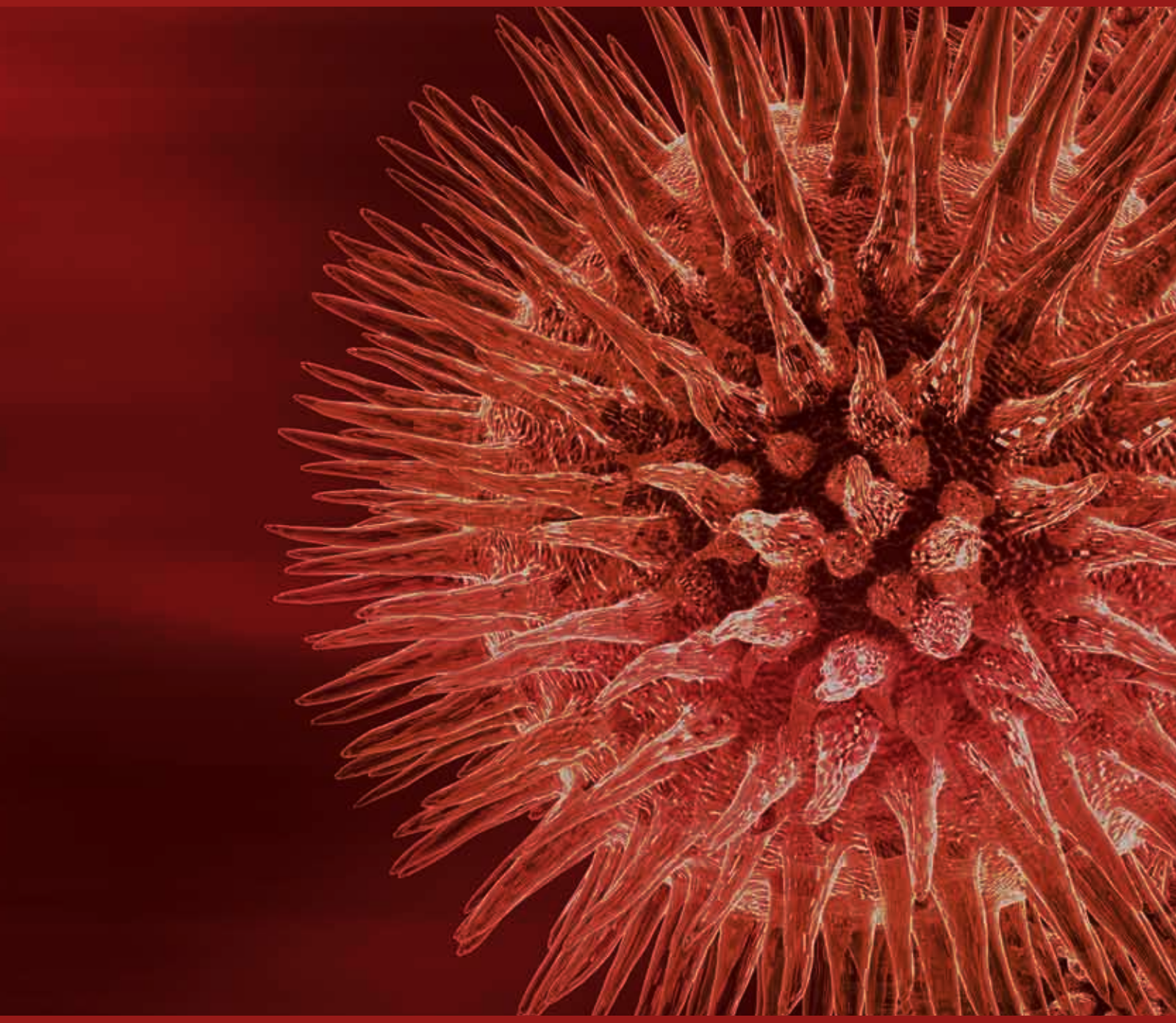
Journal of Biomedicine and Biotechnology

Regulatory RNomics and Gene Expression

Guest Editors: Zhumur Ghosh, Bibekanand Mallick, Daniel Gautheret, Pawan Malhotra, and Ravi Sachidanandam



Regulatory RNomics and Gene Expression

Journal of Biomedicine and Biotechnology

Regulatory RNomics and Gene Expression

Guest Editors: Zhumur Ghosh, Bibekanand Mallick,
Daniel Gautheret, Pawan Malhotra,
and Ravi Sachidanandam

Editorial Board

The editorial board of the journal is organized into sections that correspond to the subject areas covered by the journal.

Agricultural Biotechnology

Guihua Bai, USA
Christopher P. Chanway, Canada
Ravindra N. Chibbar, Canada
Silvia R. Cianzio, USA
Ian Godwin, Australia
Hari B. Krishnan, USA
Carol A. Mallory-Smith, USA
Dennis P. Murr, Canada
Henry T. Nguyen, USA
Rodomiro Ortiz, Mexico
Badal C. Saha, USA
Mariam B. Sticklen, USA
Chiu-Chung Young, Taiwan

Animal Biotechnology

Ernest S. Chang, USA
Hans H. Cheng, USA
Bhanu P. Chowdhary, USA
Noelle E. Cockett, USA
Peter Dovc, Slovenia
Scott C. Fahrenkrug, USA
Dorian J. Garrick, USA
Thomas A. Hoagland, USA
Tosso Leeb, Switzerland
James D. Murray, USA
Anita M. Oberbauer, USA
Jorge A. Piedrahita, USA
Daniel Pomp, USA
Kent M. Reed, USA
Lawrence Reynolds, USA
Sheila M. Schmutz, Canada
Lawrence B. Schook, USA
Mari A. Smits, The Netherlands
Leon Spicer, USA
John P. Verstegen, USA
Matthew B. Wheeler, USA
Kenneth L. White, USA

Biochemistry

Robert Blumenthal, USA
David R. Brown, UK
Saulius Butenas, USA
Vittorio Calabrese, Italy
Francis J. Castellino, USA
Roberta Chiaraluce, Italy
David M. Clarke, Canada
Francesca Cutruzzolà, Italy
Paul W. Doetsch, USA
Hicham Fenniri, Canada
Nick Grishin, USA
J. Guy Guillemette, Canada
Yusuf A. Hannun, USA
Paul W. Huber, USA
Chen-Hsiung Hung, Taiwan
Michael Kalafatis, USA
Bruce Kemp, Australia
Phillip E. Klebba, USA
Wen-Hwa Lee, USA
Richard D. Ludescher, USA
George Makhatadze, USA
Leonid Medved, USA
Susan A. Rotenberg, USA
Jason Shearer, USA
Mark A. Smith, USA
John B. Vincent, USA
Yujun George Zheng, USA

Bioinformatics

Tatsuya Akutsu, Japan
Miguel A. Andrade, Germany
Mark Borodovsky, USA
Rita Casadio, Italy
Artem Cherkasov, Canada
David Wolfe Corne, UK
Sorin Draghici, USA
Stavros J. Hamodrakas, Greece
Paul Harrison, Canada
George Karypis, USA
Jack Leunissen, The Netherlands
Guohui Lin, Canada
Satoru Miyano, Japan
Zoran Obradovic, USA
Florencio Pazos, Spain
Zhirong Sun, China
Ying Xu, USA
Alexander Zelikovsky, USA
Albert Zomaya, Australia

Biophysics

Miguel Castanho, Portugal
P. Bryant Chase, USA
Kuo-Chen Chou, USA
Rizwan Khan, India
Ali A. Khraibi, Saudi Arabia
Rumiana Koynova, USA
Serdar Kuyucak, Australia
Jianjie Ma, USA
S. B. Petersen, Denmark
Peter Schuck, USA
Claudio M. Soares, Portugal

Cell Biology

Ricardo Benavente, Germany
Omar Benzakour, France
Sanford I. Bernstein, USA
Phillip I. Bird, Australia
Eric Bouhassira, USA
Mohamed Boutjdir, USA
Chung-Liang Chien, Taiwan
Richard Gomer, USA
Paul J. Higgins, USA
Pavel Hozak, Czech Republic
Xudong Huang, USA
Anton M. Jetten, USA
Seamus J. Martin, Ireland
Manuela Martins-Green, USA
Shoichiro Ono, USA
George Perry, USA
Mauro Piacentini, Italy
George E. Plopper, USA
Lawrence Rothblum, USA
Ulrich Scheer, Germany
Michael Sheetz, USA
James L. Sherley, USA
Claudio A. Soto, USA
Gary S. Stein, USA
Richard Tucker, USA
Thomas van Groen, USA
Andre Van Wijnen, USA
Steve Winder, UK
Chuanyue Wu, USA
Bin-Xian Zhang, USA

Genetics

Adewale Adeyinka, USA
Claude Bagnis, France
James Birchler, USA
Susan Blanton, USA
Barry J. Byrne, USA
Ranajit Chakraborty, USA
Sarah H. Elsea, USA
Celina Janion, Poland
J. Spencer Johnston, USA
M. Ilyas Kamboh, USA
Manfred Kayser, The Netherlands
Brynn Levy, USA
Xiao Jiang Li, USA
Thomas Liehr, Germany
James M. Mason, USA
Raj S. Ramesar, South Africa
Elliot D. Rosen, USA
Michael Schmid, Germany
Markus Schuelke, Germany
Wolfgang A. Schulz, Germany
Jorge Sequeiros, Portugal
Mouldy Sioud, Norway
Meena Upadhyaya, UK
Rongjia Zhou, China

Genomics

Vladimir Bajic, Saudi Arabia
Margit Burmeister, USA
Settara Chandrasekharappa, USA
Yataro Daigo, Japan
Sean Grimmond, Australia
J. Spencer Johnston, USA
Vladimir Larionov, USA
Hans Lehrach, Germany
Thomas Lufkin, Singapore
Joakim Lundeberg, Sweden
John L McGregor, France
John V. Moran, USA
Henry T. Nguyen, USA
Yasushi Okazaki, Japan
Gopi K. Podila, USA
Mariano Rocchi, Italy
Paul B. Samollow, USA
Momiao Xiong, USA

Immunology

Hassan Alizadeh, USA
Peter Bretscher, Canada
Robert E. Cone, USA
Terry Delovitch, Canada
Anthony L. DeVico, USA
Nick Di Girolamo, Australia
Don Mark Estes, USA
Soldano Ferrone, USA
Jeffrey A. Frelinger, USA
John Gordon, UK
John Robert Gordon, Canada
James D. Gorham, USA
Silvia Gregori, Italy
Thomas Griffith, USA
Young S. Hahn, USA
Stella C. Knight, UK
Dorothy E. Lewis, USA
Bradley W. McIntyre, USA
R. Lee Mosley, USA
Marija Mostarica-Stojković, Serbia
Hans Konrad Muller, Australia
Ali Ouaissi, France
Kanury V. S. Rao, India
Yair Reisner, Israel
Harry W. Schroeder, USA
Wilhelm Schwaeble, UK
Nilabh Shastri, USA
Yufang Shi, China
Piet Stinissen, Belgium
Hannes Stockinger, Austria
Jan Willem Tervaert, The Netherlands
Vincent K. Tuohy, USA
Graham R. Wallace, UK

Microbial Biotechnology

Jozef Anné, Belgium
Yoav Bashan, Mexico
Marco Bazzicalupo, Italy
Nico Boon, Belgium
Luca Simone Cocolin, Italy
Peter Coloe, Australia
Daniele Daffonchio, Italy
Han de Winde, The Netherlands
Yanhe Ma, China
Bernd Rehm, New Zealand
Angela Sessitsch, Austria
Effie Tsakalidou, Greece
Juergen Wiegel, USA

Microbiology

David Beighton, UK
Steven R. Blanke, USA
Stanley Brul, The Netherlands
H. J. Busscher, The Netherlands
Isaac K. O. Cann, USA
John E. Degener, The Netherlands
Peter Dimroth, Switzerland
Stephen K. Farrand, USA
Alain Filloux, UK
Gad Frankel, UK
Nancy Freitag, USA
Roy Gross, Germany
Hans-Peter Klenk, Germany
Tanya Parish, UK
Gopi K. Podila, USA
Frederick D. Quinn, USA
Didier Raoult, France
Isabel Sá-Correia, Portugal
Pamela L. C. Small, USA
Lori Snyder, UK
Vanessa Sperandio, USA
Michael Thomm, Germany
Henny van der Mei, The Netherlands
Schwan William, USA

Molecular Biology

Rudi Beyaert, Belgium
Michael Bustin, USA
Douglas Cyr, USA
Kostas Iatrou, Greece
Lokesh Joshi, Ireland
David W. Litchfield, Canada
Noel F. Lowndes, Ireland
Wuyuan Lu, USA
Patrick Matthias, Switzerland
John L McGregor, France
Sherry Mowbray, Sweden
Elena Orlova, UK
Yeon-Kyun Shin, USA
William S. Trimble, Canada
Lisa Wiesmuller, Germany
Masamitsu Yamaguchi, Japan

Oncology

Ronald E. Cannon, USA
Colin Cooper, UK
F. M. J. Debruyne, The Netherlands
Michael Eccles, New Zealand
Nathan Ames Ellis, USA
Dominic Fan, USA
Mauro Ferrari, USA
Gary E. Gallick, USA
Daila S. Gridley, USA
Xin-yuan Guan, Hong Kong
Anne Hamburger, USA
Manoor Prakash Hande, Singapore
Beric Henderson, Australia
Steve B. Jiang, USA
Daehee Kang, South Korea
Abdul R. Khokhar, USA
Rakesh Kumar, USA
Macus Tien Kuo, USA
Eric W Lam, UK
Sue-Hwa Lin, USA
Kapil Mehta, USA
Orhan Nalcioglu, USA
Vincent C. O. Njar, USA
Peter J. Oefner, Germany
Allal Ouhtit, USA
Frank Pajonk, USA
Waldemar Priebe, USA
Annie J. Sasco, France
Fernando Carlos Schmitt, Portugal
Sonshin Takao, Japan
Ana M. Tari, USA
Henk G. Van Der Poel, The Netherlands
Haodong Xu, USA
David J. Yang, USA

Pharmacology

Abdel A. Abdel-Rahman, USA
Krishna C. Agrawal, USA
Rene Anand, USA
Mostafa Z. Badr, USA
Stelvio M. Bandiera, Canada
Ronald E. Baynes, USA
R. Keith Campbell, USA
Hak-Kim Chan, Australia
Michael D. Coleman, UK
Jacques Descotes, France
Dobromir Dobrev, Germany
Ayman El-Kadi, Canada
Zuleica Bruno Fortes, Brazil
Jeffrey Hughes, USA
Kazim Husain, USA
Farhad Kamali, UK
Michael Kassiou, Australia
Joseph J. McArdle, USA
Mark McKeage, New Zealand
Daniel T. Monaghan, USA
Toshio Narahashi, USA
Kennerly S. Patrick, USA
Vickram Ramkumar, USA
Michael J. Spinella, USA
Quadiri Timour, France
Todd W. Vanderah, USA
Val J. Watts, USA
David J. Waxman, USA

Plant Biotechnology

Prem L. Bhalla, Australia
Jose Botella, Australia
Elvira Gonzalez De Mejia, USA
H. M. Häggman, Finland
Liwen Jiang, Hong Kong
Pulugurtha B. Kirti, India
Yong Pyo Lim, South Korea
Gopi K. Podila, USA
Ralf Reski, Germany
Sudhir Kumar Sopory, India
Neal Stewart, USA

Toxicology

Michael Aschner, USA
Douglas Bristol, USA
Michael L. Cunningham, USA
Laurence D. Fechter, USA
Hartmut Jaeschke, USA
Youmin James Kang, USA
M. Firoze Khan, USA
Pascal Kintz, France
Ronald Tjeerdema, USA
Kenneth Turteltaub, USA
Brad Upham, USA

Virology

Contents

TIS11 Family Proteins and Their Roles in Posttranscriptional Gene Regulation, Maria Baou, Andrew Jewell, and John J. Murphy
Volume 2009, Article ID 634520, 11 pages

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 691286, 2 pages
doi:10.1155/2009/691286

Editorial

Regulatory RNomics and Gene Expression

Zhumur Ghosh,[1] Bibekanand Mallick,[2] Daniel Gautheret,[3] Pawan Malhotra,[4] and Ravi Sachidanandam[5]

[1] *Department of Medicine, School of Medicine, Stanford University, Grant Building S140, Stanford, CA 94305, USA*
[2] *Wadsworth Center, New York State Department of Health, New York, NY 12208, USA*
[3] *CNRS Institut de Génétique et Microbiologie, Université Paris Sud, Orsay, France*
[4] *International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067, India*
[5] *Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York University, NY 10029, USA*

Correspondence should be addressed to Zhumur Ghosh, ghosh.jhumur@gmail.com

Received 11 October 2009; Accepted 11 October 2009

Regulatory RNA research has marked a new paradigm of RNA-directed gene expression regulation and the most promising voice of the post-genomic era in developing new drugs and therapeutics for various diseases. It helps to determine what genes are accessible to each type of cell, a crucial choice for multicellular animals that require, for example, a liver cell to read off one set of genes and a brain cell to be governed by a different set. And it coordinates suites of genes that may be under different control systems but need to act together in response to sudden stresses. The generation of a huge amount of sequences from a number of key organisms and complementary development of computational bio-tools for regulatory RNA annotation and analysis, have accelerated this field of research. The small RNAs have been a boon to scientists bringing reverse genetics to assess the function of a gene. The new regulatory role of RNA began to emerge in the last 10 years as researchers discovered a class of short RNA molecules known as silencing RNAs and a second class called micro-RNAs. And these probably constitute the tip of the iceberg. The opportunity to harness the tantalizing field of regulatory RNA mediated RNA interference (RNAi) pathway in silencing disease-causing genes hold great promise for the development of therapeutics directed against targets that are not addressable with current medicines. Bioinformatics as well plays a major role and aids in analyzing, interpreting and screening of such RNA sequences and their targets.

MicroRNAs are only the best studied members of the expanding family of regulatory non-coding RNAs that populates all domains of life. MicroRNAs tell us many stories like stories of evolution, with their quickly evolving and adaptable structures that exploded in animal and plant genomes through duplication of existing microRNAs and exaptation of elements that served other functions such as snoRNAs. They tell us stories of development, with their intricate role in the differentiation of so many tissues, including animal brain, immune cells and muscle, as well as plant organs. They are also closely involved in human disease. An astounding 1700 papers have been published about RNA and cancer since the first report of downregulated miRNAs in cancer cells in 2002.

With the revelation that most intergenic and junk DNA in eukaryotic genomes was actually transcribed, a whole new understanding of the non-messenger transcriptome is emerging, where transcripts produced during RNA polymerase initiation or as the result of cleavage by a variety of Dicer-like enzyme are acting in regulating gene expression at many different levels. Regulatory RNAs are also gaining importance in the prokaryotic world in the form of trans-acting small RNAs and cis-acting riboswitches which, just as their eukaryotic counterparts, are able to switch genes on and off with exquisite accuracy in response to various environmental challenges, triggering deep changes in cellular functions that are reminiscent of the developmental events triggered by miRNAs in multi-cellular organisms. There is a long way ahead in introducing this huge diversity of RNA molecules into our global picture of cell regulatory networks. As our understanding of the functions of small RNAs and

their mode of regulation continues to increase, we will be able to translate these regulatory RNAs from *lab-to-clinic* for therapeutic applications.

This area of research has attracted some of the most innovative research groups in the molecular biology and bioinformatics field and as Editors, we are truly privileged that many of these investigators have contributed to this issue. We express our sincere gratitude to the contributing authors as well as to the vision of the Founder Editor, Dr Abdelali Haoudi, for this opportunity provided by the Journal of Biomedicine and Biotechnology.

Zhumur Ghosh
Bibekanand Mallick
Daniel Gautheret
Pawan Malhotra
Ravi Sachidanandam

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 594678, 6 pages
doi:10.1155/2009/594678

Review Article

MicroRNA-Biogenesis and Pre-mRNA Splicing Crosstalk

Noam Shomron[1] and Carmit Levy[2]

[1] *Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel Aviv University, 69978 Tel Aviv, Israel*
[2] *Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, USA*

Correspondence should be addressed to Noam Shomron, nshomron@post.tau.ac.il

Received 27 March 2009; Accepted 18 May 2009

Recommended by Zhumur Ghosh

MicroRNAs (miRNAs) are often hosted in introns of protein-coding genes. Given that the same transcriptional unit can potentially give rise to both miRNA and mRNA transcripts raises the intriguing question of the level of interaction between these processes. Recent studies from transcription, pre-mRNA splicing, and miRNA-processing perspectives have investigated these relationships and yielded interesting, yet somewhat controversial findings. Here we discuss major studies in the field.

1. Transcription, Pre-mRNA Splicing, and miRNA Biogenesis

The gene expression pathway initiates at nuclear transcription generating a pre-mRNA, which very often undergoes splicing, post-transcriptional regulation, and then translation into a protein. During the pre-mRNA splicing process, introns are removed and exons are joined in order to generate the mature mRNA [1–3]. The highly coordinated splicing event takes place in a large complex called the Spliceosome. The formation of this functional megacomplex is an orchestrated assembly of proteins and RNA that requires identification of exon-intron boundaries [4]. Exons are regularly alternatively spliced, meaning that they are either included or excluded from the final mature mRNA transcript. A recent comprehensive sequencing study observed that more than 90% of the genes undergo alternative splicing [5]. This vastly increases the transcriptome repertoire, and emphasizes both the significance of splicing and the requirement for its accurate execution.

In addition to protein coding genes, noncoding genes are transcribed. microRNAs (miRNA), the most comprehensive noncoding group, are a class of ∼22 nt noncoding RNAs that inhibit gene expression through binding to the 3′ UnTranslated Region (UTR) of target mRNA transcripts [6, 7]. There are hundreds of unique miRNAs in a given species [8], each predicted to regulate a plethora of target genes [9–13]. In fact, computational predictions indicate that miRNAs may regulate 60% of all human protein coding genes [14]. Therefore, it came as no surprise that miRNAs were linked to many cellular processes such as differentiation, growth, and apoptosis [15], while miRNA perturbations were associated with numerous diseases, including cancer [16, 17]. In the past few years, the pivotal role played by miRNAs in gene regulation has been recognized [18–20].

miRNAs are processed through a series of post-transcriptional biogenesis steps. The canonical maturation pathway, similar to protein-coding genes, initiates at transcription (mostly by RNA polymerase II) generating a primary (pri-) miRNA. The pri-miRNA is characterized by a hairpin RNA structure recognized by the nuclear RNAse-III enzyme Drosha, and its cofactor DGCR8 [21]. These proteins work in a complex of several proteins, known as the Microprocessor. The Microprocessor cleaves the pri-miRNA to generate a shorter hairpin of about 70 nt length—the pre-miRNA. This intermediate miRNA is exported from the nucleus to the cytoplasm via Exportin-5 where the RNase III endonuclease Dicer generates the final mature miRNA. This short RNA loses one of its strands (the complementary miRNA* strand) while the other is loaded onto an Argonaute-containing RNA-induced silencing complex (RISC) which mediates gene silencing. Once the miRNA binds to its target gene, regulation takes place mainly through mRNA degradation or translation inhibition [22, 23] (see Figure 1). For simplicity, the widely used term "miRNA

biogenesis" hereafter refers to the initial step of miRNA excision from its RNA transcript.

miRNAs can be located inter- or intragenically. When intergenic, their expression is coordinated with other miRNAs as a cluster [25, 26]. When intragenic, namely, positioned within a protein-coding gene (almost exclusively in introns), they are often expressed from the same strand as their host-gene [27–30] and at correlated levels [31]. Given that both coding mRNAs and miRNAs are generated from the same transcriptional unit, and that they cooccur in close cellular proximity, it would be puzzling if these events exhibited total independence. Recent studies, from transcription, miRNA-processing, and splicing oriented perspectives, have investigated these fascinating interactions and yielded interesting, yet somewhat controversial findings.

2. Intronic miRNA Biogenesis in Light of Pre-mRNA Splicing

Relationships between intronic miRNAs and the processing events of their host mRNA, namely, transcription and splicing have been addressed. Here we outline the major studies in the field.

Expressed Sequence Tag (EST) libraries of expressed mRNAs are derived from various cells and tissues. The ESTs represent a snapshot of cellular transcripts at a particular time point and thus display the given mRNA plethora and its variety at a particular cellular state. Analysis of this data revealed several chimeric transcripts containing miRNA and part of the adjacent mRNA sequences [32]. At an early stage of miRNA research this indicated the existence of a shared RNA transcript. Notably, at a later stage, some of these EST fragments were shown to be partially spliced, with either 5′ or 3′ ends matching putative Drosha cleavage sites [30]. These results strengthened the possibility that miRNAs and mRNAs are processed from the same RNA substrate. In addition, the correlated expression pattern of host-gene transcripts and their miRNAs [31] suggested that miRNAs have coevolved to use the same promoter for transcription [26]. Along with this work, by comparing miRNA processing in a construct containing only the intronic sequence versus one that also includes the flanking exons, Pawlicki and Steitz [33] found that the levels of pri-miRNA transcribed from introns are increased in the presence of flanking exons, due to prolonged retention at the site of transcription. This supported the notion that flanking exons may facilitate miRNA processing by increasing the time pri-miRNAs spend tethered to the DNA template [33, 34]. Altogether, the data indicates that intronic miRNA processing is enhanced by physical proximity to the site of transcription, and possibly also by splicing of the host gene. Several groups have isolated and identified various proteins associated with the human Microprocessor complex [35–37]. In these studies, numerous Microprocessor-associated proteins were identified as splicing factors (e.g., hnRNPH1; [37]) or involved in pre-mRNA processing (e.g., DHX15; [38]).

Taken together, based on miRNA-mRNA transcriptional (EST) evidence; shared promoters; facilitated biogenesis when flanked by exons; and overlapping proteins between the functional complexes, the data suggests that the Microprocessor is potentially enhanced and present during transcription and most likely also during splicing. If the same RNA substrate is subjected to both host-gene and intronic miRNA maturation, the intriguing question raised is how do all these processes—transcription, pre-mRNA splicing, and miRNA processing—crosstalk?

The complexity of the miRNA-host-gene interaction model has increased recently when studies from the Proudfoot Laboratory demonstrated that pre-miRNAs are generated through cotranscriptional cleavage by Drosha. Morlando et al. [39] suggested that efficient clearance of intronic sequences following Microprocessor (Drosha) cleavage may act to enhance the splicing efficiency. This occurs both in intergenic miRNAs and intronic miRNA genes [39]. These researchers showed that the Microprocessor complex, as well as 5′-3′ and 3′-5′ RNA exonucleases, are recruited to chromatin associated with intronic miRNAs during transcription of the host primary transcript, and that Drosha cleavage occurs before host intron splicing. They found that miRNA-harboring transcripts preferentially associated with chromatin fractions, from which they concluded that both pre-miRNA cleavage and intronic splicing must occur on the same nascent transcripts. The rapid exonucleolytic removal of intronic sequences may clear the proximal vicinity of RNA processing for the purpose of efficiently completing the pre-mRNA splicing task. The enhancement of splicing by the Microprocessor does not agree with other studies [30, 40] (discussed below) and does not concur with experiments in yeast that showed enhanced processing for siRNA flanked exons in splicing mutants [41].

A crosstalk between two physically overlapping RNA transcripts is not unheard of. Dependencies are seen, for example, during the biogenesis process of small nucleolar RNA (snoRNA) [42–44]. In the process of snoRNA maturation, functional links between intronic snoRNP assembly, pre-mRNA synthesis and processing have been described [45–47]. An antithesis to this dependency is the alternative miRNA biogenesis pathway that bypasses the Microprocessor via generation of "Mirtrons" [48, 49]. This mechanistically distinct class of intronic miRNAs stem from very short introns where splicing substitutes the first step of miRNA biogenesis. In this case, splicing activities replace the requirements for a Microprocessor.

However, not all roads lead to the observed dependency between microprocessing and splicing. Ying and Lin [50] have designed an artificial intron containing a pre-miRNA secondary structure. They used this construct to show that the mature miRNA was released only from the spliced intron. This suggested that spliced introns are subsequently used by Drosha and argued against any physical link between the Microprocessor and the transcriptional unit or between the Microprocessor and the Spliceosome [50]. Dye et al. [40] showed that exons of pre-mRNA are tethered to the elongating RNA polymerase II either directly or indirectly without affecting processing, indicating that cotranscriptional cleavage of nascent intronic miRNA transcripts does not affect splicing efficiency [40, 51]. Supporting the same

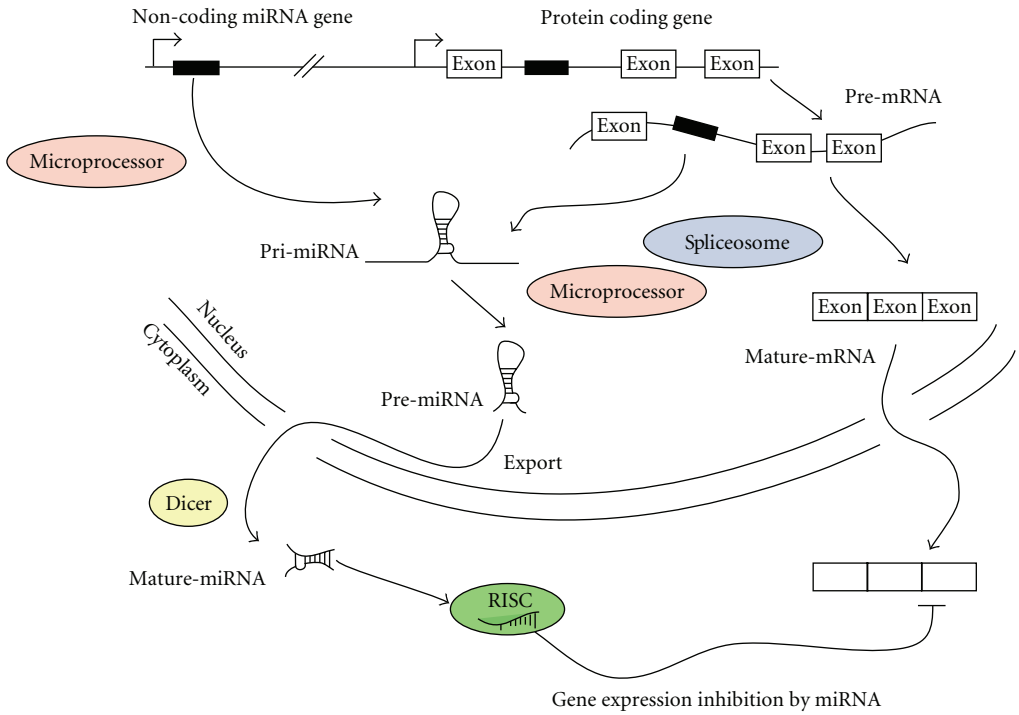


Figure 1: The canonical miRNA biogenesis pathway and its affect on gene expression. Elaborated mechanisms and exceptions to this pathway are reviewed in [24].

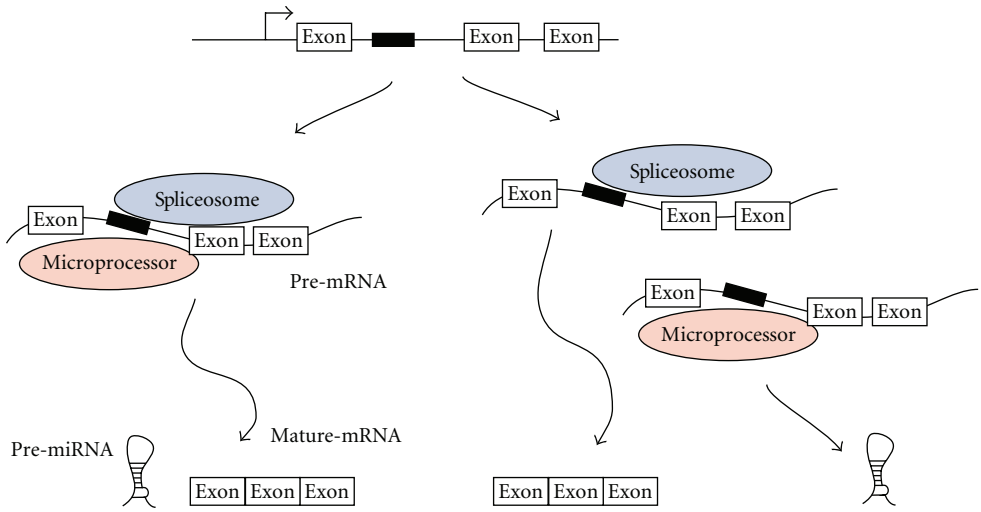


Figure 2: Possible models for intronic miRNA biogenesis. Either both miRNA and mRNA are generated from one RNA transcript (left scheme), or each is generated from an individual transcript (right scheme).

view, Kim and Kim [30] addressed miRNA biogenesis in light of the splicing mechanism. They demonstrated that cleavage of an intronic miRNA did not significantly affect the production of mature mRNA and, conversely, the production of mature miRNA was not significantly affected by splicing. In their experiments, knockdown of Drosha, or mutations in the miRNA hairpin, eliminated miRNA generation without dramatically affecting mRNA splicing. This suggested that miRNA biogenesis and splicing are coordinated but not functionally linked or interdependent. Taking a closer look, however, they also mention that Drosha knockdown led to a modest increase in spliced mRNA production and so did mutations in the miRNA hairpin. Their work showed that the adjacent introns were spliced more rapidly than miRNA-encoding introns, suggesting that binding of the Microprocessor may eventually interfere with the splicing to some extent. Taken together, Kim and Kim's data [30] imply mostly independent activities but cannot exclude the possibility that the Microprocessor interferes, to some level, with splicing.

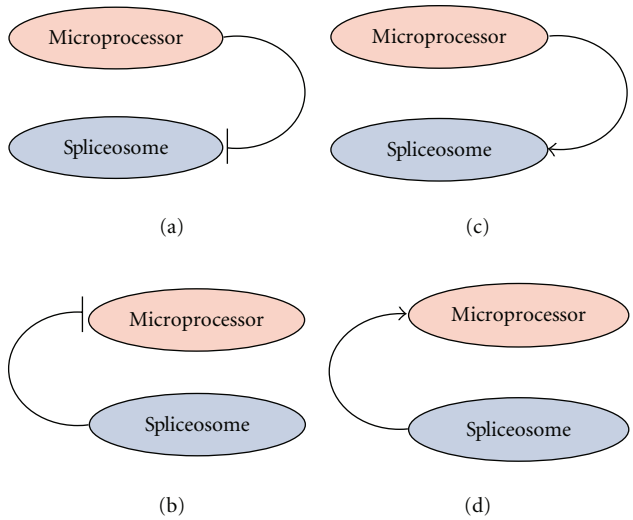


Figure 3: Possible crosstalk models between the miRNA biogenesis process, carried out by the Microprocessor, and the pre-mRNA splicing reaction, carried out by the Spliceosome.

Attempting to explain how both miRNA and mRNA are generated from the same DNA locus, reconciling with the studies described here, we come up with two distinct models. The first envisions a single miRNA and mRNA arising from the same RNA precursor. The second predicts generation of an miRNA and mRNA products arising from two independent RNA transcripts (see Figure 2). If the latter scenario was true, microprocessing and splicing would be independent of each other. The outcome would be either no functional hindrance between the Microprocessor and Spliceosome activities or a competition for available pre-mRNA substrates. In the event that both RNA products originate from the same precursor, it is conceivable that the two processes happen consecutively. This would imply that execution of one process would be a prerequisite for the other to occur. Alternatively, miRNA processing and mRNA splicing may be coordinated so that the Microprocessor and Spliceosome interact with each other. This interaction may be minimal, without affecting the amounts of miRNA and mRNA produced—as was suggested recently [30], or it may constitute a level of regulation. We note that these events should always be looked at in the spatial-temporal context meaning that the Microprocessor might act at an independent rate prior to the Spliceosome assembly, and thus their direct interaction would be prevented. Due to accumulating evidence, both in favor and against dependencies between splicing and microprocessing, further investigation is necessary in order to uncover the full complexity of these processes.

3. Coordinated Crosstalk

Cellular regulatory events are commonly embedded in complex networks of interactions. The potential coordinated activity between the Microprocessor and the Spliceosome can be stratified into four possible relationships. The Microprocessor can inhibit or activate the Spliceosome, and the Spliceosome can inhibit or activate the Microprocessor (Figure 3). These relationships are not mutually exclusive. For example, the Microprocessor could activate the Spliceosome by recruiting splicing factors to intronic miRNAs, while at the same time the Spliceosome could inhibit microprocessing. We cannot rule out, however, that these relationships occur in one large complex depending on the presence of particular RNA processing proteins within the Microprocessor [37]. Many complex regulatory loops, both positive and negative, were seen in other cellular systems (e.g., see [52, 53]).

The Spliceosome is a mega complex of hundreds of proteins and snRNPs [54]. Its assembly and dynamic structure have been extensively studied [55, 56]. The Microprocessor, on the other hand, is composed of a handful of proteins [21, 36], minimally described as a two-protein complex [24, 35] (alternatively, see [37]). It is hard to visualize these two very differently-sized complexes aligned at the same position, competing for the same substrate. Thus, a coordinated processing and crosstalk seems necessary for these complexes—the Microprocessor and the Spliceosome—to be able to process the same transcript with intricate accuracy.

During the mRNA splicing process, the rate at which transcription takes place may affect the transcripts' pattern of splicing [57]. Thus, kinetics of intron removal and exon ligation may play a role in selecting particular spliced isoforms. Given that some introns undergo miRNA excision, unless the removal is extremely rapid, one can imagine a possible effect on splicing outcome. Thus, from an evolutionary perspective, an intronic miRNA might evolve to participate in determining splicing kinetics. Consequently, an evolutionary driving force may direct miRNA positioning within the intron to prevent disruption of relevant splice signals (also see [39]).

In summary, growing evidence indicates a complex crosstalk between transcription and splicing. It is not surprising then that microprocessing is also linked to these events. Many questions still remained unanswered. Are these processes coordinated by *cis* regulatory sequences or trans acting factors? Does one process dominate over the other? How widespread is the mechanism and does it govern all intronic miRNAs? To date, not all Microprocessor and Spliceosome crosstalk scenarios (as described in Figure 3) have been identified. Yet, given the complexity of cellular pathways, it is probably only a matter of time before their elucidation.

Acknowledgments

The authors would like to thank Kathleen Robinson, Adrienne Yanez, Steffen Schubert, Eyal Mor, Shira Modai, Naama Hazan, Yakir Vaknin, and Carl Novina for commenting on the manuscript.

References

[1] T. Maniatis and R. Reed, "An extensive network of coupling among gene expression machines," *Nature*, vol. 416, no. 6880, pp. 499–506, 2002.

[2] T. W. Nilsen, "The spliceosome: the most complex macromolecular machine in the cell?" *BioEssays*, vol. 25, no. 12, pp. 1147–1149, 2003.

[3] M. J. Moore and N. J. Proudfoot, "Pre-mRNA processing reaches back to transcription and ahead to translation," *Cell*, vol. 136, no. 4, pp. 688–700, 2009.

[4] E. Kim, A. Goren, and G. Ast, "Alternative splicing: current perspectives," *BioEssays*, vol. 30, no. 1, pp. 38–47, 2008.

[5] E. T. Wang, R. Sandberg, S. Luo, et al., "Alternative isoform regulation in human tissue transcriptomes," *Nature*, vol. 456, no. 7221, pp. 470–476, 2008.

[6] E. C. Lai, "Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation," *Nature Genetics*, vol. 30, no. 4, pp. 363–364, 2002.

[7] D. P. Bartel, "MicroRNAs: target recognition and regulatory functions," *Cell*, vol. 136, no. 2, pp. 215–233, 2009.

[8] S. Griffiths-Jones, H. K. Saini, S. van Dongen, and A. J. Enright, "miRBase: tools for microRNA genomics," *Nucleic Acids Research*, vol. 36, database issue, pp. D154–D158, 2008.

[9] B. P. Lewis, I.-H. Shih, M. W. Jones-Rhoades, D. P. Bartel, and C. B. Burge, "Prediction of mammalian microRNA targets," *Cell*, vol. 115, no. 7, pp. 787–798, 2003.

[10] A. Krek, D. Grun, M. N. Poy, et al., "Combinatorial microRNA target predictions," *Nature Genetics*, vol. 37, no. 5, pp. 495–500, 2005.

[11] C. B. Nielsen, N. Shomron, R. Sandberg, E. Hornstein, J. Kitzman, and C. B. Burge, "Determinants of targeting by endogenous and exogenous microRNAs and siRNAs," *RNA*, vol. 13, no. 11, pp. 1894–1910, 2007.

[12] A. Grimson, K. K.-H. Farh, W. K. Johnston, P. Garrett-Engele, L. P. Lim, and D. P. Bartel, "MicroRNA targeting specificity in mammals: determinants beyond seed pairing," *Molecular Cell*, vol. 27, no. 1, pp. 91–105, 2007.

[13] J. Brennecke, A. Stark, R. B. Russell, and S. M. Cohen, "Principles of microRNA-target recognition," *PLoS Biology*, vol. 3, no. 3, p. e85, 2005.

[14] R. C. Friedman, K. K.-H. Farh, C. B. Burge, and D. P. Bartel, "Most mammalian mRNAs are conserved targets of microRNAs," *Genome Research*, vol. 19, no. 1, pp. 92–105, 2009.

[15] R. W. Carthew, "Gene regulation by microRNAs," *Current Opinion in Genetics and Development*, vol. 16, no. 2, pp. 203–208, 2006.

[16] R. Garzon, M. Fabbri, A. Cimmino, G. A. Calin, and C. M. Croce, "MicroRNA expression and function in cancer," *Trends in Molecular Medicine*, vol. 12, no. 12, pp. 580–587, 2006.

[17] R. Schickel, B. Boyerinas, S.-M. Park, and M. E. Peter, "MicroRNAs: key players in the immune system, differentiation, tumorigenesis and cell death," *Oncogene*, vol. 27, no. 45, pp. 5959–5974, 2008.

[18] V. Ambros, "The functions of animal microRNAs," *Nature*, vol. 431, no. 7006, pp. 350–355, 2004.

[19] L. P. Lim, N. C. Lau, P. Garrett-Engele, et al., "Microarray analysis shows that some microRNAs downregulate large numbers of-target mRNAs," *Nature*, vol. 433, no. 7027, pp. 769–773, 2005.

[20] W. Filipowicz, S. N. Bhattacharyya, and N. Sonenberg, "Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?" *Nature Reviews Genetics*, vol. 9, no. 2, pp. 102–114, 2008.

[21] H. Seitz and P. D. Zamore, "Rethinking the microprocessor," *Cell*, vol. 125, no. 5, pp. 827–829, 2006.

[22] N. Bushati and S. M. Cohen, "MicroRNA functions," *Annual Review of Cell and Developmental Biology*, vol. 23, pp. 175–205, 2007.

[23] N. Shomron, D. Golan, and E. Horstein, "An evolutionary perspective of animal microRNAs and their targets," *Journal of Biomedicine and Biotechnology*. In press.

[24] J. Winter, S. Jung, S. Keller, R. I. Gregory, and S. Diederichs, "Many roads to maturity: microRNA biogenesis pathways and their regulation," *Nature Cell Biology*, vol. 11, no. 3, pp. 228–234, 2009.

[25] Y. Altuvia, P. Landgraf, G. Lithwick, et al., "Clustering and conservation patterns of human microRNAs," *Nucleic Acids Research*, vol. 33, no. 8, pp. 2697–2706, 2005.

[26] F. Ozsolak, L. L. Poling, Z. Wang, et al., "Chromatin structure analyses identify miRNA promoters," *Genes and Development*, vol. 22, no. 22, pp. 3172–3183, 2008.

[27] A. Rodriguez, S. Griffiths-Jones, J. L. Ashurst, and A. Bradley, "Identification of mammalian microRNA host genes and transcription units," *Genome Research*, vol. 14, no. 10A, pp. 1902–1910, 2004.

[28] N. Liu, K. Okamura, D. M. Tyler, M. D. Phillips, W.-J. Chung, and E. C. Lai, "The evolution and functional diversification of animal microRNA genes," *Cell Research*, vol. 18, no. 10, pp. 985–996, 2008.

[29] K. Okamura, W.-J. Chung, and E. C. Lai, "The long and short of inverted repeat genes in animals: microRNAs, mirtrons and hairpin RNAs," *Cell Cycle*, vol. 7, no. 18, pp. 2840–2845, 2008.

[30] Y.-K. Kim and V. N. Kim, "Processing of intronic microRNAs," *EMBO Journal*, vol. 26, no. 3, pp. 775–783, 2007.

[31] S. Baskerville and D. P. Bartel, "Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes," *RNA*, vol. 11, no. 3, pp. 241–247, 2005.

[32] N. R. Smalheiser, "EST analyses predict the existence of a population of chimeric microRNA precursor-mRNA transcripts expressed in normal human and mouse tissues," *Genome Biology*, vol. 4, no. 7, article 403, 2003.

[33] J. M. Pawlicki and J. A. Steitz, "Primary microRNA transcript retention at sites of transcription leads to enhanced microRNA production," *Journal of Cell Biology*, vol. 182, no. 1, pp. 61–76, 2008.

[34] J. M. Pawlicki and J. A. Steitz, "Subnuclear compartmentalization of transiently expressed polyadenylated pri-microRNAs: processing at transcription sites or accumulation in SC35 foci," *Cell Cycle*, vol. 8, no. 3, pp. 345–356, 2009.

[35] R. I. Gregory, K.-P. Yan, G. Amuthan, et al., "The Microprocessor complex mediates the genesis of microRNAs," *Nature*, vol. 432, no. 7014, pp. 235–240, 2004.

[36] R. I. Gregory, T. P. Chendrimada, and R. Shiekhattar, "MicroRNA biogenesis: isolation and characterization of the microprocessor complex," *Methods in Molecular Biology*, vol. 342, pp. 33–47, 2006.

[37] A. Shiohama, T. Sasaki, S. Noda, S. Minoshima, and N. Shimizu, "Nucleolar localization of DGCR8 and identification of eleven DGCR8-associated proteins," *Experimental Cell Research*, vol. 313, no. 20, pp. 4196–4207, 2007.

[38] X. Wen, S. Tannukit, and M. L. Paine, "TFIP11 interacts with mDEAH9, an RNA helicase involved in spliceosome

disassembly," *International Journal of Molecular Sciences*, vol. 9, no. 11, pp. 2105–2113, 2008.

[39] M. Morlando, M. Ballarino, N. Gromak, F. Pagano, I. Bozzoni, and N. J. Proudfoot, "Primary microRNA transcripts are processed co-transcriptionally," *Nature Structural and Molecular Biology*, vol. 15, no. 9, pp. 902–909, 2008.

[40] M. J. Dye, N. Gromak, and N. J. Proudfoot, "Exon tethering in transcription by RNA polymerase II," *Molecular Cell*, vol. 21, no. 6, pp. 849–859, 2006.

[41] E. H. Bayne, M. Portoso, A. Kagansky, et al., "Splicing factors facilitate RNAi-directed silencing in fission yeast," *Science*, vol. 322, no. 5901, pp. 602–606, 2008.

[42] L.-H. Qu, Y. Henry, M. Nicoloso, et al., "U24, a novel intron-encoded small nucleolar RNA with two 12 nt long, phylogenetically conserved complementarities to 28S rRNA," *Nucleic Acids Research*, vol. 23, no. 14, pp. 2669–2676, 1995.

[43] W. Filipowicz and V. Pogacic, "Biogenesis of small nucleolar ribonucleoproteins," *Current Opinion in Cell Biology*, vol. 14, no. 3, pp. 319–327, 2002.

[44] V. De Turris, G. Di Leva, S. Caldarola, F. Loreni, F. Amaldi, and I. Bozzoni, "TOP promoter elements control the relative ratio of intron-encoded snoRNA versus spliced mRNA biosynthesis," *Journal of Molecular Biology*, vol. 344, no. 2, pp. 383–394, 2004.

[45] T. Hirose, M.-D. Shu, and J. A. Steitz, "Splicing-dependent and -independent modes of assembly for intron-encoded box C/D snoRNPs in mammalian cells," *Molecular Cell*, vol. 12, no. 1, pp. 113–123, 2003.

[46] P. K. Yang, C. Hoareau, C. Froment, B. Monsarrat, Y. Henry, and G. Chanfreau, "Cotranscriptional recruitment of the pseudouridylsynthetase Cbf5p and of the RNA binding protein Naf1p during H/ACA snoRNP assembly," *Molecular and Cellular Biology*, vol. 25, no. 8, pp. 3295–3304, 2005.

[47] P. Richard, A. M. Kiss, X. Darzacq, and T. Kiss, "Cotranscriptional recognition of human intronic box H/ACA snoRNAs occurs in a splicing-independent manner," *Molecular and Cellular Biology*, vol. 26, no. 7, pp. 2540–2549, 2006.

[48] E. Berezikov, W.-J. Chung, J. Willis, E. Cuppen, and E. C. Lai, "Mammalian mirtron genes," *Molecular Cell*, vol. 28, no. 2, pp. 328–336, 2007.

[49] J. G. Ruby, C. H. Jan, and D. P. Bartel, "Intronic microRNA precursors that bypass Drosha processing," *Nature*, vol. 448, no. 7149, pp. 83–86, 2007.

[50] S.-Y. Ying and S.-L. Lin, "Current perspectives in intronic micro RNAs (miRNAs)," *Journal of Biomedical Science*, vol. 13, no. 1, pp. 5–15, 2006.

[51] N. Gromak, G. Talotti, N. J. Proudfoot, and F. Pagani, "Modulating alternative splicing by cotranscriptional cleavage of nascent intronic RNA," *RNA*, vol. 14, no. 2, pp. 359–366, 2008.

[52] J. Y. Leung, F. T. Kolligs, R. Wu, et al., "Activation of AXIN2 expression by beta-catenin-T cell factor: a feedback repressor pathway regulating Wnt signaling," *Journal of Biological Chemistry*, vol. 277, no. 24, pp. 21657–21665, 2002.

[53] O. Brandman and T. Meyer, "Feedback loops shape cellular signals in space and time," *Science*, vol. 322, no. 5900, pp. 390–395, 2008.

[54] Z. Zhou, L. J. Licklider, S. P. Gygi, and R. Reed, "Comprehensive proteomic analysis of the human spliceosome," *Nature*, vol. 419, no. 6903, pp. 182–185, 2002.

[55] J. Sperling, M. Azubel, and R. Sperling, "Structure and function of the pre-mRNA splicing machine," *Structure*, vol. 16, no. 11, pp. 1605–1615, 2008.

[56] M. C. Wahl, C. L. Will, and R. Luhrmann, "The spliceosome: design principles of a dynamic RNP machine," *Cell*, vol. 136, no. 4, pp. 701–718, 2009.

[57] M. de la Mata, C. R. Alonso, S. Kadener, et al., "A slow RNA polymerase II affects alternative splicing in vivo," *Molecular Cell*, vol. 12, no. 2, pp. 525–532, 2003.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 803069, 9 pages
doi:10.1155/2009/803069

Review Article

Computational Challenges in miRNA Target Predictions: To Be or Not to Be a True Target?

Christian Barbato,[1] Ivan Arisi,[2] Marcos E. Frizzo,[3] Rossella Brandi,[2] Letizia Da Sacco,[4] and Andrea Masotti[4]

[1] *European Brain Research Institute-Fondazione EBRI-Rita Levi-Montalcini, Via del Fosso di Fiorano, 64/65, 00143 Roma, Italy*
[2] *Neurogenomics Facility, European Brain Research Institute-Fondazione EBRI-Rita Levi-Montalcini, Via del Fosso di Fiorano, 64/65, 00143 Roma, Italy*
[3] *Departamento de Ciências Morfológicas, ICBS, UFRGS, Rua Sarmento Leite 500, Porto Alegre, RS, CEP 90050-170, Brazil*
[4] *Gene Expression - Microarrays Laboratory, Bambino Gesù Children's Hospital, P.za S.Onofrio 4, 00165 Roma, Italy*

Correspondence should be addressed to Andrea Masotti, andrea.masotti@opbg.net

Received 2 January 2009; Accepted 20 March 2009

Recommended by Zhumur Ghosh

All microRNA (miRNA) target—finder algorithms return lists of candidate target genes. How valid is that output in a biological setting? Transcriptome analysis has proven to be a useful approach to determine mRNA targets. Time course mRNA microarray experiments may reliably identify downregulated genes in response to overexpression of specific miRNA. The approach may miss some miRNA targets that are principally downregulated at the protein level. However, the high-throughput capacity of the assay makes it an effective tool to rapidly identify a large number of promising miRNA targets. Finally, loss and gain of function miRNA genetics have the clear potential of being critical in evaluating the biological relevance of thousands of target genes predicted by bioinformatic studies and to test the degree to which miRNA-mediated regulation of any "validated" target functionally matters to the animal or plant.

1. Introduction

The microRNA- (miRNA-) guided "RNA" silencing pathway is a recently discovered process that is able to regulate gene expression by acting on messenger RNA (mRNA) at posttranscriptional level. miRNA biogenesis is mediated by Dicer which catalyzes the processing of double-stranded RNAs (dsRNAs) into ≈22 nt-long small miRNAs. The initial transcript, or "primary miRNA" (pri-miRNA), can be hundreds to thousands nucleotides long and, like any other Pol II transcript, undergoes capping and polyadenylation. The mature miRNA is part of a 60 to 80-nucleotide stem-loop structure contained within the pri-miRNA. The first step in miRNA biogenesis occurs in the nucleus and requires the excision of this hairpin structure. The excised hairpin, called pre-miRNA, is exported to the cytoplasm, and the pre-miRNA is then processed by another RNase III enzyme called Dicer. This endonuclease removes the loop region of the hairpin, releasing the mature miRNA:miRNA* duplex. During the assembly of the RNA-induced silencing complex (RISC) with the miRNA, only one strand of the duplex is loaded, whereas the complementary miRNA* strand is removed and degraded. The mature miRNA is now ready to direct its activity on a target mRNA by binding miRNA responsive elements usually located in the 3'untranslated region (3'UTR) of the transcript. This association may result in either cleavage or translational repression of the target mRNA, depending on the degree of base-pairing between the miRNA and the responsive element. Perfect complementarity generally results in cleavage, whereas imperfect base-pairing leads to translational repression. These alternative effects might also reflect differences in the biochemical composition of the RISC complex associated to each specific miRNA:mRNA duplex. The proteins in the Argonaute (AGO) family are very tightly bound to small single-stranded RNAs within RISC, as the RNA-protein interaction persists

even under high-salt conditions. The PAZ domain of Ago has been implicated in RNA binding, and the PIWI domain seems to furnish RISC with effector-nuclease function [1]. The wide range of molecular weights reported for RISC complex (between 140 and 500 kDa) represents several different versions of the complex that contain other factors in addition to AGO. Because the other components of RISC are not required for slicing, they may have a role in other aspects of RISC activity, for example, substrate turnover and/or RISC subcellular localization. This variation may also represent species differences or may reflect developmental- or tissue-specific variations in RISC composition. The exact composition of the RISC complex is currently unknown [2].

miRNA genes represent about 1%-2% of the known eukaryotic genomes and constitute an important class of fine-tuning regulators that are involved in several physiological or disease-associated cellular processes. miRNAs are conserved throughout the evolution, and their expression may be constitutive or spatially and temporally regulated. Even in viral infections these small noncoding RNAs can contribute to the repertoire of host-pathogen interactions. The resources needed to study in details such interactions or to investigate their therapeutic implications have been recently reviewed [3]. Increasing efforts have been made to identify the specific targets of miRNAs, leading to speculation that miRNAs may regulate at least 30% of human genes. Computational predictions suggest that each miRNA can target more than 200 transcripts and that a single mRNA may be regulated by multiple miRNAs [4]. This entails that miRNAs and their targets are part of complex regulatory network and outline the widespread impact of miRNAs on both the expression and evolution of protein-coding genes [5].

The mechanism of miRNA-mediated gene regulation remains controversial. However, artificial tethering of AGO proteins to the 3'UTR of a reporter mRNA is sufficient to induce its translational repression. This evidence suggests that miRNAs may act to guide the deposition of the RISC complex onto a specific site of the target mRNA [6].

To date, the computational identification of miRNA targets and the validation of miRNA-target interactions represent fundamental steps in disclosing the contribution of miRNAs toward cell functions. The prediction of miRNA targets by computational approaches is based mainly on miRNAs complementarity to their target mRNAs, and several web-based or stand-alone computer softwares are used to predict miRNA targets [4]. Among them, *TargetScanS, PicTar*, and *miRanda* are the most common target prediction programs while *miRBase, Argonaute, miRNAMap,* and *miRGen* are databases combining the compilation of miRNAs with target prediction modules.

Here, we summarize and discuss the most recent *in silico* and biological approaches aimed to unravelling the functional interactions between miRNAs and their targets with a special emphasis to combined methods for more accurate miRNA target gene prediction.

2. Combining mRNA and miRNA Expression Profiles for an Accurate Target Prediction

It is now well established that the formation of a double-stranded RNA duplex through the binding of miRNA to mRNA in the RNA-induced silencing complex (RISC) triggers either the degradation of the mRNA transcript or the inhibition of protein translation. However, experimental identification of miRNA targets is not straightforward, and in the last few years, many computational methods and algorithms have been developed to predict miRNA targets [7]. Even though target prediction criteria may vary widely, most often they include: (1) strong Watson-Crick basepairing of the 5′ seed (i.e., positions 2–8) of the miRNA to a complementary site in the 3'UTR of the mRNA, (2) conservation of the miRNA binding site, and (3) a local miRNA-mRNA interaction with a positive balance of minimum free energy (MFE). These requirements should be accompanied by a good structural accessibility of the surrounding mRNA sequence. However, it is likely that other important parameters for functional miRNA-target interactions remain to be identified.

The first step in the prediction procedure requires the identification of potential miRNA binding sites in the mRNA 3'UTR according to specific base-pairing rules. The second step involves the implementation of cross-species conservation requirements [8]. Among the most popular prediction algorithms, we recall PicTar [9], TargetScan [10], and miRanda [11]. Each algorithm has a definite rate of both false positive and false negative predictions [7]. In common practice, more than one algorithm is used to make reliable predictions about a particular gene or a specific miRNA.

Surprisingly, different algorithms provide different predictions, and the degree of overlap between different lists of predicted targets is sometimes poor or null [8].

It has been predicted that up to 30% of mammalian genes are regulated by miRNAs [11–13], and many regulatory patterns are likely to be regulated by them [14]. However, when the number of genes under study is on the order of several hundreds or thousands (like in microarray experiments), a gene-by-gene search of miRNA targets of interest becomes impractical. Furthermore, when dealing with such a number of genes that may be coregulated, the evaluation of groups of genes with common binding sites for one or specific miRNAs or families of miRNAs is surely more informative. This goal may be reached using classical enrichment statistics, testing over-representation of the miRNA target predictions within the selected set of genes (see also next paragraph): the statistical methods are similar to those used for the Gene Ontology annotation (http://www.geneontology.org/GO.tools.html).

However, few prediction algorithms able to clarify miRNA function or integrate data coming from different experimental high-throughput techniques are currently available. Therefore, there is the need to develop accurate computational methods for the identification of functional miRNA-target interactions. Undoubtedly, a computational method able to efficiently combine gene expression studies

Table 1: Common softwares for "–omics" data analysis allowing in-depth analysis of high-throughput data.

Method name	Reference	Brief description	Computer platform	Web interface	Availability	URL
Babelomics	Al-Shahrour et al. 2006	Web-based tools for genomic data analysis. Gene annotations include predicted microRNA	Any platform, web browser	yes	Free access	http://www.babelomics.org/
M@ia	Le Bechec et al. 2008	Modular tools for genomic data analysis. Gene annotations include predicted microRNA	Linux, MacOs, Windows. PHP language, Apache web server and MySQL database required	no	Open-source	http://maia.genouest.org/
TIGR Multiexperiment Viewer (MeV)		Integrated environment for -omics data analysis. Gene annotations include predicted microRNA	Windows, MacOs; Java required.	no	Free executable	http://www.tm4.org/mev.html
BRB-ArrayTools		Tools for -omics data analysis. The working environment is Microsoft Excel, an R engine is providing to Excel through and add-in module. Gene annotations include predicted microRNA	Windows. Java, Excel and R language required	no	Free executable	http://linus.nci.nih.gov/~brb/download.html
GeneSpring GX		Integrated environment for -omics data analysis. Gene annotations include predicted microRNA	Windows, Java required	no	Commercial from Agilent Technologies, free trial	http://www.silicongenetics.com/
Ingenuity Pathway Analysis		Integrated environment for -omics data analysis. Gene annotations include predicted microRNA. Functional annotation and analysis of biological networks.	Windows, Java required	no	Commercial from Ingenuity Systems Inc., free trial	http://www.ingenuity.com/index.html
R Bionconductor		A common open source environment for -omics data analysis and statistics. It includes tools for microRNA analysis and annotation.	Linux, MacOs, Windows.	no	Open Source	http://www.bioconductor.org/

(mRNA profiles) with miRNAs expression profiles for a reliable prediction of miRNA target is essential. In fact, using the results of both miRNA and gene expression profiling, the prediction of miRNA-mRNA associations through the identification of anticorrelated pairs should be refined; based on the well-established knowledge of miRNA function, an upregulation of a specific miRNA will lead to lower expression of its mRNA targets, and a downregulation of a specific miRNA will lead to higher levels of its target genes. This effect is more clearly visible from in vitro studies where the system is perturbed either by the over-expression or by the silencing of a specific miRNA [15, 16]. Therefore, a ranking of downregulated (or upregulated) genes coupled to several mRNA predictions should allow the researcher to obtain a more reliable estimate of the "real" miRNA targets and finally their function [12, 13].

Unfortunately, so far this approach led to few examples, and the available software and algorithms will be briefly commented here. In contrast, a biological approach has led to the development of several techniques that appear to be efficient alternatives to computational methods. These applications, briefly reviewed in this paper, are able to solve, at least in part, the problem of high-throughput validation of miRNA targets in vivo.

2.1. Gene Expression Analysis. Several software for the analysis of "-omics" data are commercially available or free for nonprofit organizations (Table 1). These systems are usually general purpose environments in which small databases of experimental samples can be built; the data can be filtered and normalized and also analyzed in depth using a number of statistical techniques such as analysis of variance (ANOVA), hierarchical clustering, Principal Component Analysis (PCA), among others. The same systems also offer annotation instruments such as enrichment statistics for a set of reference databases, including lists of miRNAs targeting all the known genes. The predictions come usually from

Table 2: Algorithms and software tools specifically developed for functional interpretation of miRNA expression data, inference of miRNA gene regulation from mRNA trascriptomic profiles, combination of parallel mRNA and miRNA expression data.

Method name	Reference	Brief description	Computer platform	Web interface	Availability	URL
miRGator	Nam et al. [17]	A web-based system to analyze microRNA expression data and to integrate parallel microRNA, mRNA, and protein profiles	Any platform, web browser	yes	Free access	http://genome.ewha.ac.kr/miRGator/
SigTerms	Creighton et al. [18]	Series of Microsoft Excel macros that compute an enrichment statistic for over-representation of predicted microRNA targets within the analyzed gene set. The software supports PicTar, TargetScan, and miRanda prediction algorithms.	Windows, Excel required	no	free source code	http://sigterms.sourceforge.net/
TopKCEMC	Lin and Ding [19]	Integration of different analysis results of the same data, each represented by a ranked list of entities. The algorithm finds the optimal list combining all the input ones. This system can be applied to the output lists of different microRNA target predictors as well as to different differentially expressed gene lists.	Linux, MacOs, Windows. R language	no	Open Source	http://www.stat.osu.edu/~statgen/SOFTWARE/TopKCEMC/
GenMIR++	Huang et al. [20]	Using a Bayesian learning network, the algorithm accounts for patterns of mRNA gene expression using miRNA expression data and a set of predicted miRNA targets. A smaller set of high-confidence functional miRNA targets then obtained from the data using the algorithm.	Any platform, Matlab language	no	Free source code	http://www.psi.toronto.edu/genmir/
MIR	Cheng and Li [21]	This method infers the level of microRNA expression starting from the gene expression profile and a gene target prediction. It is similar to GSEA for the analysis of gene expression. Every microRNA has an enrichment score based on the differential expression of its targets, weighted by a binding energy matrix.	Windows, Linux	no	Free executable	http://leili-lab.cmb.usc.edu/yeastaging/projects/microrna

the most popular computational predictors (TargetScan, PicTar, Miranda) and are not validated by databases of experimental miRNA-mRNA interactions. Given any mRNA expression profile and a selected gene list, this approach allows a first investigation of the miRNAs likely to directly modulate, at least partially, the mRNA degradation rate or indirectly modulate the mRNA transcription and translation rates. These techniques are not specifically tailored to the problem of integrating parallel miRNA and mRNA gene profiles obtained within the same experiment but are useful in combining data within the same analytical environment.

Of these tools, only Babelomics is available via web. Algorithms for functional annotation, such as FatiGO, have been integrated into a single and user friendly interface. The software GeneSpring is a commercial package that offers, together with a wide range of standard and advanced statistical analysis methods, other enrichment statistics for functional annotations. This last feature is further developed in the Ingenuity Pathway Analysis system, specifically designed for functional and pathway analysis. Other analysis software such as the popular Bioconductor package and the MeV from the TIGR institute, are open source projects that undergo constant updates. Bionconductor works within the R language environment, which enables it to be directly integrated with several other R libraries such as the TopKCEMC reported in Table 2.

2.2. Integration and Analysis of mRNA and miRNA Data. The usefulness of bioinformatic integration of mRNA and miRNA expression data into an interaction database (Transcriptome Interaction Database) [22] was emphasized by Chen et al. [23]. However, the functional significance of

many miRNAs is still largely unknown due to the difficulty in identifying target genes and the lack of genome wide expression data combining miRNA results.

In Table 2 there is a list of some recent algorithms or tools developed to investigate the effect of miRNAs on mRNA expression profiles, to better predict miRNA targets and to integrate different data sources.

SigTerms is a novel software package (a set of Microsoft Excel macros) that has been recently developed: for a given target prediction database, it retrieves all miRNA-mRNA functional pairs represented by an input set of genes [18]. For each miRNA, the software computes an enrichment statistic for over-representation of predicted targets within the gene set. This could help to define roles of specific miRNAs and miRNA-regulated genes in the system under study. In the hands of researchers, SigTerms is a powerful tool that allows rates of false positive and false negative responses to be minimized. One method to decrease the incidence of false positive predictions and to narrow down the list of putative miRNA targets is to compare the *in silico* target predictions to the genes that are differentially expressed in the biological system of interest. SigTerms can support this type of analytical approach allowing the user to manipulate, filter, and extract different output from miRNA-mRNA sets.

Another recently reported application is miRGator [17] that integrates target predictions, functional analyses, gene expression data and genome annotations. Since the function of miRNA is mostly unknown, diverse experimental and computational approaches have been applied to elucidate their role [24, 25]. In this context, miRGator provides a utility for statistical enrichment tests of target genes, performed for gene ontology (GO) function, GenMAPP and KEGG pathways, and for various diseases. Expression correlation between miRNA and target mRNA/proteins is evaluated, and their expression patterns can be readily compared with a user friendly interface. At present, miRGator supports only human and mouse genomes.

Another major task facing researchers studying complex biological systems is the integration of data from high-throughput "-omics" platforms such as DNA variations, transcriptome profiles, and RNAomics. Recently, some miRNA-bioinformatic aspects like the biological and therapeutic repertoire of miRNAs, the *in silico* prediction of miRNA genes and their targets, and the bioinformatic challenges lying ahead have been reviewed [26]. Combined modeling of multiple raw datasets can be extremely challenging due to their enormous differences, while rankings from each dataset might provide a common base for integration. Aggregation of miRNA targets, predicted from different computational algorithms is one of these problems. Another challenging issue is the integration of results from multiple mRNA studies based on different platforms. However, one of the methods recently proposed in the literature makes use of a global optimization technique, the so-called Cross Entropy Monte Carlo (CEMC) [19]. This algorithm, called TopKCEMC, searches iteratively for the optimal list that minimizes the sum of weighted distances between the candidate (aggregate) list and each of the input-ranked lists. The distance between two ranked lists is measured using both the modified Kendall's tau measure and the Spearman's footrule [27]. The application of this technique in the field of miRNA seems appropriate when the diverse predicted targets from different computational algorithms are combined together to give an aggregate list that is more informative for downstream experiments [12, 13]. This algorithm is a clear example of what we think may be well suited for combining mRNA and miRNA data to furnish a list of more reliable miRNA targets. In fact, the comparison should be made combining the "classical" list of miRNA targets (obtained from different prediction softwares) and a list of ranked downregulated (or upregulated) mRNAs.

Another proposed method of inferring the effective regulatory activities of miRNAs requires integrating microarray expression data with miRNA target predictions. As previously mentioned, the method is based on the idea that regulatory activity changes of miRNAs could be reflected by the expression changes of their target transcripts (measured by microarray techniques) [21]. To verify the hypothesis, this method has been applied to selected microarray data sets measuring gene expression changes in cell lines after transfection or inhibition of specific miRNAs. Results indicate that this method can detect activity enhancement of the transfected miRNAs as well as activity reduction of the inhibited miRNAs with high sensitivity and specificity. Furthermore, this inference is robust with respect to false positive predictions (i.e., nonspecific interactions when silencing a miRNA or when the gene downregulation is erroneously associated to a direct miRNA targeting) [15]. This method is a generalization of the gene set enrichment analysis (GSEA), which was proposed to identify gene sets associated with expression change profiles [28].

The first example of a direct correlation between mRNA expression levels and the 3'UTR motif composition has been recently reported [29]. This algorithm, a novel application of REDUCE [30], has also led to the hypothesis that the number of vertebrate miRNA could be larger than previously estimated. The algorithm's rationale is based on the assumption that motifs within 3'UTRs make a linear contribution to enhancing or inhibiting mRNA levels. The significant motifs are chosen by iteratively looking at the individual contribution that brings the greatest reduction in the difference between the model and the expression data. Motifs with a P-value lower than a defined threshold are retained and listed. This method was ultimately demonstrated to be more sensitive than the current target prediction algorithms not relying on cross-species comparisons.

The same approach has been followed in another recent paper [31]. Here, the authors demonstrated that the effect of a miRNA on its target mRNA levels can be measured within a single gene expression profile. This method, however, used a known public dataset of expression both for miRNA and mRNA, limiting the usefulness of the conclusions. However, the success of this approach has revealed the vast potential for extracting information about miRNA function from other gene expression profiles.

A novel Bayesian model and learning algorithm, GenMiR++ (Generative model for miRNA regulation), has also been proposed. GenMiR++ accounts for patterns of

Table 3: Other computational and experimental approaches capable of performing more reliable analysis by combining miRNA and mRNA expression data.

Reference	Brief description	Computer platform
Kort et al. [32]	Two signatures of differentially expressed mRNAs and microRNAs are used to cluster the data. Qualitative combination of mRNA and microRNA expression data.	Any platform, web browser, R language
Lanza et al. [33]	One signature of differentially expressed mRNAs and microRNAs in combination is used to correctly cluster the data. Qualitative combination of mRNA and microRNA expression data.	Any platform, GeneSpring software
Salter et al. [34]	Qualitative combining of mRNA profiling and microRNA expression, by clustering separately the data and analyzing differentially modulated pathways.	Any platform, GeneSpring software, R Language, GenePattern software
Nicolas et al. [15]	Experimental identification of real microRNA targets by overexpression or silencing of miR-140.	Any platform, web browser
Sood et al. [29]	A computational tool to directly correlate 3'UTR motifs with changes in mRNA levels upon miRNA overexpression or knockdown.	Linux, Cygwin (Windows), Mac OS X, SunOS platform. A web version is also available

gene expression using miRNA expression data and a set of candidate miRNA targets [20]. A set of high-confidence functional miRNA targets is obtained from the data using a Bayesian learning algorithm. With this model, the expression of a targeted mRNA transcript can be explained through the regulatory action of multiple miRNAs. GenMiR++ allows accurate identification of miRNA targets from both sequence and expression data and allows the recovery of a significant number of experimentally verified targets, many of which provide insight into miRNA regulation.

In Table 3 we summarize some research articles where the authors have combined expression data for miRNA and mRNA, using standard analytical techniques but without the use of specifically designed algorithms.

In a recent approach aimed at identifying miRNA targets, an experimental and analysis workflow was used to find a set of genes whose expression is modulated by miR-140 [15]. This method is based on the manipulation of a miRNA activity in mouse cell lines, where miR-140 is expressed at a moderate level, thus making it easier both to repress or enhance its activity. Expression of mRNAs repressed or enhanced upon miRNA overexpression and silencing, respectively, was profiled. Within the set obtained by the intersection of the up- and down regulated mRNAs measured by microarrays, the authors searched for complementary seed sequences in the 3'UTR section of transcripts: 21 out of 49 mRNAs were identified as candidate direct targets, while the others as potential indirect ones. Interestingly, none of the 21 identified candidates were computed by popular predictors such as TargetScan, MiRBase, and PiCTar, though one of these targets, Cxcl12, was validated by Northern Blot and Luciferase assay. This method suggests that the use of more cell lines would certainly increase the set of experimentally identified targets. In fact, since some of them were already found to have escaped the analysis, they were unaffected by the type of cell manipulation chosen in this approach. This method appears to be conservative and tends to find false negative targets especially if they are not affected at the mRNA level.

A different type of combined analysis of mRNA and miRNA profiles is often used in the field of tumors: cancers may be classified into various subclasses or may respond differently to various chemotherapeutic procedures. To correctly distinguish two subtypes of carcinomas (i.e., the colorectal cancer that can be characterized by microsatellite pathway either stability or instability), the authors have identified two different gene signatures from the mRNA and miRNA expression profiles [33]. The two signatures were extracted by standard statistical techniques such as correct T-test, PAM (Prediction Analysis of Microarray) and SVM (support vector machine, provided by Gene Spring software, see Table 1). Then, their ability to classify the samples was tested through a hierarchical clustering, both separately and together. Results showed that the better performance was obtained when the two signatures were combined together in a single clustering tree, proving once more the well-assessed crucial role played by miRNAs in the genesis of cancers. Both mRNA and miRNA gene profiles coupled to hierarchical clustering techniques were recently used in obtaining a deeper understanding of the cancer biology of the Wilm's tumor [32].

A serious problem that affects the results of antineoplastic treatments is, together with a correct diagnosis and classification, the choice of the right chemotherapeutic agent [34]. Again, both mRNA and miRNA expression signatures of sensitive and resistant cell lines were used to predict patient response to a panel of commonly used chemotherapy agents. The signatures were first used to cluster analyze samples from real breast cancer patients, then also as predictors to separate patients into nonresponders/responders to each treatment. The miRNA profiles were also finally analyzed to investigate the biological mechanisms underlying the resistance/response to the agents used in the study, making use of the prior knowledge about the experimentally validated targets of the selected miRNAs.

3. Novel Biochemical Approaches for miRNA Target Characterization.

Finally, we would like to report a few examples that show how a biochemical approach may overcome all the difficulties encountered with the computational approach.

So far, the small number of available validated miRNA targets has hindered the evaluation of the accuracy of miRNA-target prediction software. Recently, the "mirWIP" method has been proposed for the capture of all known conserved miRNA-mRNA target relationships in *Caenorhabditis elegans*, with a lower false positive rate than other standard methods [35]. This quantitative miRNA target prediction method allows an accurate weighting of some immunoprecipitation-enriched parameters, finally optimizing sensitivity to verified miRNA-target interactions and specificity.

As indicative examples, two recent studies on *C. elegans* used immunoprecipitation of miRNA-containing ribonucleoprotein complexes and evaluated that only 30%–45% of miRNAs associated with these complexes contain perfectly matched, conserved seed elements in their 3'UTRs [36, 37]. Although these datasets have provided important insights into parameters associated with functional interactions, this approach is limited to the detection of miRNA-target interactions that result in transcript destabilization and does not identify stable, translationally repressed target mRNAs. Recently, immunoprecipitation of the RISC has been used to identify mRNAs that stably associate with the endogenous RISC [38]. This study recovered 3404 mRNA transcripts that specifically coprecipitate with the miRNA-induced silencing complex (miRISC) proteins AIN-1 and AIN-2. This "AIN-IP" set of mRNA transcripts provided a biologically derived estimate of how many genes are targeted by miRNAs: in this case, at least one-sixth of *C. elegans* genes. The authors used these features to develop the prediction algorithm mirWIP, which scores miRNA target sites by weighting site characteristics in proportion to their enrichment in the experimental AIN-IP set. MirWIP has improved overall performance compared to previous algorithms, in both recovery of the AIN-IP transcripts and correct identification of genetically verified miRNA-target relationships without a requirement for alignment of target sequences. MirWIP in its current form is supported by immunoprecipitation experiments that identify transcripts by their probable association with miRNAs, even if these experiments do not directly provide information about what particular miRNA (or set of miRNAs) is responsible for miRISC association.

Finally, because the miRISC immunoprecipitation approach may be biased toward the identification of stable miRNA-target complexes, miRNA-induced target destabilization can be screened using complementary datasets, such as microarray assays to identify mRNA transcripts that change in response to miRNA activity.

To overcome the above mentioned difficulties and since the identification of the downstream targets of miRNAs is essential to understand cellular regulatory networks, a direct biochemical method for miRNA target discovery has been proposed that combines RISC purification with microarray analysis of bound mRNAs [39]. A biochemical method of identifying miRNA targets holds the promise of deepening the understanding of the determinants of miRNA-mediated regulation, particularly by revealing targets that are repressed without changes in mRNA levels. Identification of this class of targets will provide an opportunity to study sequences or structural features determining miRNAs regulatory fate. As a model, miR-124a has been used because its targets are well known and studied. This method consisted in the Ago2 co-immunoprecipitation of mRNA targets followed by microarray profiling of mRNAs. As a result, it has been proven that not only most of the immunoprecipitated mRNAs analyzed were direct miR-124a targets but also a significant subset was downregulated.

4. Conclusions

A novel sequencing era is going to dramatically change our view of studying gene expression, posttranscriptional modifications, DNA copy number variations, and SNPs. Novel high-throughput sequencing techniques are emerging at an impressive speed on the market and on the scientific community. In the near future, these novel approaches will surely help to elucidate the function of miRNAs and their role as fine regulators. One of the most important recently reported work is based on this approach [40]. Whereas conventional methods rely on computational prediction and subsequent experimental validation of target RNAs, the proposed method consists in the direct sequencing of more than 28 000 000 signatures from the 5′ ends of polyadenylated products of miRNA-mediated mRNA decay. Briefly, by matching millions of 5′ end sequences of RNA cleavage products back to their corresponding sequences in the genome, additional sequences flanking the potential cleavage sites were identified. These were used to identify matches to known or new potential miRNAs that could direct their cleavage. Even though this study was conducted on *Arabidopsis thaliana*, we expect that the proposed method will also be rapidly applied to other genomes for the understanding of the role and functions of miRNAs.

In summary, we have addressed the issue of combining mRNA and miRNA expression data from different points of view. While biological validation of a predicted target is critical, failure to biologically validate the expression of a certain miRNA does not necessarily imply that the bioinformatic approach is incorrect. It is possible that the miRNA is not expressed in the examined tissues, the miRNA is expressed only in specific phase of cell cycle, or that the miRNA is expressed in low abundance, which escapes detection by the technique used. This latter cause is especially problematic for miRNA that shares a high degree of sequence homology with another miRNA. Expression of an abundant miRNA may therefore mask the expression of a rare one that is very similar in sequence, especially when using polymerase chain reaction amplification. While several methods already exist to predict miRNA targets, albeit with a heterogeneous and wide range of results, there are few

tools and algorithms or even only analysis workflow capable of elucidating the functional role of miRNAs. The wider availability of experimentally validated miRNA targets and their action mechanisms will certainly permit in the near future more reliable computational predictions.

Acknowledgment

The authors thank editors and reviewers for their useful suggestions and comments to improve the manuscript. This work was supported by grants of the Italian Ministry of Health (to A.M and L.D.S), partially funded by "Project EBRI-IIT" of the Italian Institute of Technology (IIT) and supported by "Fondazione Alazio Award 2007" (http://www.fondazionealazio.org/ - Via Torquato Tasso 22, 90144 Palermo) (to C.B).

References

[1] J.-J. Song, S. K. Smith, G. J. Hannon, and L. Joshua-Tor, "Crystal structure of argonaute and its implications for RISC slicer activity," *Science*, vol. 305, no. 5689, pp. 1434–1437, 2004.

[2] V. N. Kim, J. Han, and M. C. Siomi, "Biogenesis of small RNAs in animals," *Nature Reviews Molecular Cell Biology*, vol. 10, no. 2, pp. 126–139, 2009.

[3] Z. Ghosh, B. Mallick, and J. Chakrabarti, "Cellular versus viral microRNAs in host-virus interaction," *Nucleic Acids Research*, vol. 37, no. 4, pp. 1035–1048, 2009.

[4] M. Lindow and J. Gorodkin, "Principles and limitations of computational microRNA gene and target finding," *DNA and Cell Biology*, vol. 26, no. 5, pp. 339–351, 2007.

[5] D. P. Bartel, "MicroRNAs: target recognition and regulatory functions," *Cell*, vol. 136, no. 2, pp. 215–233, 2009.

[6] R. S. Pillai, C. G. Artus, and W. Filipowicz, "Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis," *RNA*, vol. 10, no. 10, pp. 1518–1525, 2004.

[7] N. Rajewsky, "microRNA target predictions in animals," *Nature Genetics*, vol. 38, supplement 1, pp. S8–S13, 2006.

[8] P. Sethupathy, M. Megraw, and A. G. Hatzigeorgiou, "A guide through present computational approaches for the identification of mammalian microRNA targets," *Nature Methods*, vol. 3, no. 11, pp. 881–886, 2006.

[9] A. Krek, D. Grün, M. N. Poy, et al., "Combinatorial microRNA target predictions," *Nature Genetics*, vol. 37, no. 5, pp. 495–500, 2005.

[10] B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell*, vol. 120, no. 1, pp. 15–20, 2005.

[11] B. John, A. J. Enright, A. Aravin, T. Tuschl, C. Sander, and D. S. Marks, "Human microRNA targets," *PLoS Biology*, vol. 2, no. 11, article e363, pp. 1–18, 2004.

[12] B. P. Lewis, I.-H. Shih, M. W. Jones-Rhoades, D. P. Bartel, and C. B. Burge, "Prediction of mammalian microRNA targets," *Cell*, vol. 115, no. 7, pp. 787–798, 2003.

[13] X. Xie, J. Lu, E. J. Kulbokas, et al., "Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals," *Nature*, vol. 434, no. 7031, pp. 338–345, 2005.

[14] J. Li, G. Musso, and Z. Zhang, "Preferential regulation of duplicated genes by microRNAs in mammals," *Genome Biology*, vol. 9, no. 8, article R132, pp. 1–10, 2008.

[15] F. E. Nicolas, H. Pais, F. Schwach, et al., "Experimental identification of microRNA-140 targets by silencing and overexpressing miR-140," *RNA*, vol. 14, no. 12, pp. 2513–2520, 2008.

[16] S. S. Li, S. L. Yu, L. P. Kao, et al., "Target identification of microRNAs expressed highly in human embryonic stem cells," *Journal of Cellular Biochemistry*, vol. 106, no. 6, pp. 1020–1030, 2009.

[17] S. Nam, B. Kim, S. Shin, and S. Lee, "miRGator: an integrated system for functional annotation of microRNAs," *Nucleic Acids Research*, vol. 36, database issue, pp. D159–D164, 2008.

[18] C. J. Creighton, A. K. Nagaraja, S. M. Hanash, M. M. Matzuk, and P. H. Gunaratne, "A bioinformatics tool for linking gene expression profiling results with public databases of microRNA target predictions," *RNA*, vol. 14, no. 11, pp. 2290–2296, 2008.

[19] S. Lin and J. Ding, "Integration of ranked lists via cross entropy Monte Carlo with applications to mRNA and microRNA studies," *Biometrics*, vol. 65, no. 1, pp. 9–18, 2009.

[20] J. C. Huang, Q. D. Morris, and B. J. Frey, "Bayesian inference of microRNA targets from sequence and expression data," *Journal of Computational Biology*, vol. 14, no. 5, pp. 550–563, 2007.

[21] C. Cheng and L. M. Li, "Inferring microRNA activities by combining gene expression with microRNA target prediction," *PLoS ONE*, vol. 3, no. 4, article e1989, pp. 1–9, 2008.

[22] R. W. Georgantas III, R. Hildreth, S. Morisot, et al., "$CD34^+$ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 8, pp. 2750–2755, 2007.

[23] A. Chen, M. Luo, G. Yuan, et al., "Complementary analysis of microRNA and mRNA expression during phorbol 12-myristate 13-acetate (TPA)-induced differentiation of HL-60 cells," *Biotechnology Letters*, vol. 30, no. 12, pp. 2045–2052, 2008.

[24] T. W. Nilsen, "Mechanisms of microRNA-mediated gene regulation in animal cells," *Trends in Genetics*, vol. 23, no. 5, pp. 243–249, 2007.

[25] A. Rodriguez, E. Vigorito, S. Clare, et al., "Requirement of *bic/microRNA-155* for normal immune function," *Science*, vol. 316, no. 5824, pp. 608–611, 2007.

[26] Z. Ghosh, J. Chakrabarti, and B. Mallick, "miRNomics—the bioinformatics of microRNA genes," *Biochemical and Biophysical Research Communications*, vol. 363, no. 1, pp. 6–11, 2007.

[27] R. Fagin, R. Kumar, and D. Sivakumar, "Comparing top k lists," *SIAM Journal on Discrete Mathematics*, vol. 17, no. 1, pp. 134–160, 2003.

[28] A. Subramanian, P. Tamayo, V. K. Mootha, et al., "Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 43, pp. 15545–15550, 2005.

[29] P. Sood, A. Krek, M. Zavolan, G. Macino, and N. Rajewsky, "Cell-type-specific signatures of microRNAs on target mRNA expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 8, pp. 2746–2751, 2006.

[30] H. J. Bussemaker, H. Li, and E. D. Siggia, "Regulatory element detection using correlation with expression," *Nature Genetics*, vol. 27, no. 2, pp. 167–171, 2001.

[31] A. Arora and D. A. C. Simpson, "Individual mRNA expression profiles reveal the effects of specific microRNAs," *Genome Biology*, vol. 9, no. 5, article R82, pp. 1–16, 2008.

[32] E. J. Kort, L. Farber, M. Tretiakova, et al., "The E2F3-oncomir-1 axis is activated in Wilms' tumor," *Cancer Research*, vol. 68, no. 11, pp. 4034–4038, 2008.

[33] G. Lanza, M. Ferracin, R. Gafà, et al., "mRNA/microRNA gene expression profile in microsatellite unstable colorectal cancer," *Molecular Cancer*, vol. 6, article 54, pp. 1–11, 2007.

[34] K. H. Salter, C. R. Acharya, K. S. Walters, et al., "An integrated approach to the prediction of chemotherapeutic response in patients with breast cancer," *PLoS ONE*, vol. 3, no. 4, article e1908, pp. 1–8, 2008.

[35] M. Hammell, D. Long, L. Zhang, et al., "mirWIP: microRNA target prediction based on microRNA-containing ribonucleoprotein-enriched transcripts," *Nature Methods*, vol. 5, no. 9, pp. 813–819, 2008.

[36] G. Easow, A. A. Teleman, and S. M. Cohen, "Isolation of microRNA targets by miRNP immunopurification," *RNA*, vol. 13, no. 8, pp. 1198–1204, 2007.

[37] M. Beitzinger, L. Peters, J. Y. Zhu, E. Kremmer, and G. Meister, "Identification of human microRNA targets from isolated argonaute protein complexes," *RNA Biology*, vol. 4, no. 2, pp. 76–84, 2007.

[38] L. Zhang, L. Ding, T. H. Cheung, et al., "Systematic identification of *C. elegans* miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2," *Molecular Cell*, vol. 28, no. 4, pp. 598–613, 2007.

[39] F. V. Karginov, C. Conaco, Z. Xuan, et al., "A biochemical approach to identifying microRNA targets," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 49, pp. 19291–19296, 2007.

[40] M. A. German, M. Pillay, D.-H. Jeong, et al., "Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends," *Nature Biotechnology*, vol. 26, no. 8, pp. 941–946, 2008.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 952304, 9 pages
doi:10.1155/2009/952304

Research Article

Novel miR390-Dependent Transacting siRNA Precursors in Plants Revealed by a PCR-Based Experimental Approach and Database Analysis

M. S. Krasnikova,[1] I. A. Milyutina,[2] V. K. Bobrova,[2] L. V. Ozerova,[3] A. V. Troitsky,[2] A. G. Solovyev,[1,2] and S. Y. Morozov[2]

[1] *Institute of Agricultural Biotechnology, Russian Academy of Agricultural Sciences, Timiryazevskaya 42, 127550 Moscow, Russia*
[2] *A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119992 Moscow, Russia*
[3] *Main Botanic Garden Russian Academy of Sciences, Botanicheskaya 4, 127276 Moscow, Russia*

Correspondence should be addressed to S. Y. Morozov, morozov@genebee.msu.su

Received 28 February 2009; Accepted 31 July 2009

Recommended by Zhumur Ghosh

TAS loci in plant genomes encode transacting small interfering RNAs (ta-siRNAs) that regulate expression of a number of genes. The function of TAS3 precursor in *Arabidopsis thaliana* is controlled by two miR390 target sites flanking two ta-siARF sequences targeting mRNAs of ARF transcription factors. Cleavage of the 3′-miR390-site initiates ta-siRNAs biogenesis. Here we describe the new method for identification of plant ta-siRNA precursors based on PCR with oligodeoxyribonucleotide primers mimicking miR390. The method was found to be efficient for dicotiledonous plants, cycads, and mosses. Based on sequences of amplified loci and a database analysis, a novel type of miR390-dependent TAS sequences was identified in dicots. These TAS loci are characterized by a smaller distance between miR390 sites compared to TAS3, a single copy of ta-siARF, and a sequence conservation pattern pointing to the possibility that processing of novel TAS-like locus is initiated by cleavage of the 5′-terminal miR390 target site.

1. Introduction

Small RNA-mediated gene silencing plays important roles in many cellular processes, including development, genome maintenance and integrity, and adaptive responses to biotic and abiotic stress in most eukaryotes. Small RNAs, usually 20–25 nucleotides (nt) in length, guide heterochromatin formation, mRNA degradation, translational inhibition, and DNA elimination. In plants, small RNAs are highly diverse and significant progress has been achieved in unraveling the components and mechanisms involved in their biogenesis and function [1–8]. MicroRNAs (miRNAs) are a class of small noncoding RNAs (21-22-nt long) transcribed from the genomes of all multicellular organisms and some viruses [5, 9–13]. Plant miRNA biogenesis starts with the transcription of miRNA precursors by RNA polymerase II. These precursors contain the mature miRNA sequence within the stem of a long imperfect RNA hairpin which is processed in the nucleus by RNaseIII-like protein DCL1 to give an imperfect RNA duplex with 2 nt 3′ overhangs on each strand [5, 14, 15]. In total, several dozens of plant miRNA families (hundreds of individual miRNA species) are currently identified and found to target mainly protein-coding mRNAs [7, 12, 13, 15, 16]. However, some microRNAs guide the cleavage of the non-protein-coding primary transcripts of TAS genes directing the formation of trans-acting siRNAs (ta-siRNAs). In this case, miRNAs represent a cleavage guider for ARGONAUTE (AGO) proteins with RNaseH-like activity that cleaves TAS single-stranded RNA transcript in the region complementary to small RNA [4]. TAS cleavage product(s) is converted by cooperative action of RNA-binding protein SGS3 and RNA-polymerase RDR6 to double-stranded form and subsequently processed by DCL4 to produce a cluster of ta-siRNAs that are phased in 21 nt increment relative to the original cleavage site on both strands [4, 5, 13, 17, 18]. The generated 21 nt ta-siRNAs further work as components

of RISC complex to guide AGO-dependent cleavage of their target mRNAs. Recent studies have reported that some ta-siRNAs also work as cleavage guiders to cut other TAS RNA precursors similarly to microRNA and thus generate secondary ta-siRNAs with new specificity [19]. Most TAS RNA precursors have only single miRNA target motif (e.g., miR173) positioned 5′ to ta-siRNA-producing site, and the target motif is cleaved by AGO1 guided by the respective miRNA. However, two miR390 target sites (5′ and 3′ to ta-siRNA site) were shown to be necessary for TAS3 precursor RNA cleavage that appeared to be dependent on specific interaction between AGO7 and miR390 [4, 18, 20].

In this study, we analyzed TAS3-related sequences encoded by genomes of different land plants. Using multiple alignments of TAS3-like RNAs in dicotyledonous plants [17, 18], we synthesized oligonucleotide primers complementary to miR390 target sites positioned 5′ and 3′ to ta-siRNA site. In a control PCR reaction with *Arabidopsis thaliana* chromosomal DNA, we obtained a PCR product of expected size. However, in *Nicotiana benthamiana* and tobacco, in addition to the PCR products corresponding to TAS3-like RNAs, we found smaller PCR fragments. Sequencing of these fragments and further database analysis revealed that they represent a new, previously undescribed, type of miR390-mediated TAS genes producing potential precursors of ta-siARF RNA in different dicotyledonous plants.

2. Materials and Methods

2.1. Plant Material. Plants were taken from collections of Main Botanic Garden of the Russian Academy of Sciences and Department of Virology, Moscow State University. Solanaceous plants were germinated in standard growth chamber conditions for several weeks until stage of 6–8 leaves.

2.2. Analysis of Nucleic Acids. Genomic plant DNA was isolated from 200 mg of plant material by DNA extraction kit (Macherey-Nagel) according to the protocol of the manufacturer. Total RNA was isolated from young tobacco leaves with the Trizol reagent according to the manufacturer's instructions (Invitrogen). Digestion of any contaminating DNA was achieved by treatment of samples with RQI RNase-free DNase (Promega). Reverse transcription was performed with 1 μg of total RNA and oligo (dT)-primer using the RT system (Invitrogen) according to the protocol of the manufacturer. Primers for dicotiledonous plants and *Cycas revoluta* were: forward primer: TAS-P 5′-GGT-GCTATCCTATCTGAGCTT-3′ and reverse primers TAS-Mcaa 5′-AGCTCAGGAGGGATAGCAA-3′ and TAS-Maca 5′-AGCTCAGGAGGGATAGACA-3′.

The primers for mosses (Physcomitrella-specific) were: forward primers: Bryo TAS-P1 5′-GGCGCTATCCCTCCT-GAGCT-3′ and Bryo TAS-P3 5′-GACGCTACCCTTCCT-GAGCT-3′, reverse primer: Bryo TAS-M 5′-TAGCTCAGG-AGTGATA(G/T)A(C/A)AA-3′. For PCR, 25–35 cycles were used for amplification with a melting temperature of 95°C, an annealing temperature of 58°C (for seed plants) or 60°C (for mosses), and an extending temperature of 72°C, each for 30 seconds, followed by a final extension at 72°C for 3 minutes. PCR products were visualized by electrophoresis of samples in 1% agarose gel. For cloning, the PCR-amplified DNA bands were isolated from gel and ligated into pGEM-T (Promega). In each case, 7–18 independent clones were sequenced. Sequences of 20–50% of clones depending on particular plant species showed no presence of tasi-ARF RNA sequences and these clones were regarded as false ones. In some cases (less than 10% of clones), nucleotide sequences of tasi-ARF precursor genes have the ends (primer sites) which are different by single nucleotide substitutions or deletions from the primer sequences. We propose that this discrepancy may result from rare mutations after cloning of PCR products in *Escherichia coli*.

2.3. GeneBank Accession Numbers. DNA sequences were deposited at NCBI databank under the following accession numbers: *Nicotiana benthamiana*—FJ804742; *Nicotiana tabacum*—FJ804743 ("short" TAS) and FJ804751 (classical TAS); *Datura stramonium*—FJ804744; *Solanum demissum*—FJ804745; *Physalis longifolia*—FJ804746; *Brachythecium latifolium* (clone 50 Br)—FJ804747; *Brachythecium latifolium* (clone 47 Br)—FJ804748; *Hookeria lucens*—FJ804749; *Cycas revolute*—FJ804750.

3. Results

To design primers for PCR and cloning of TAS3-like genes from *Nicotiana benthamiana* and *Nicotiana tabacum*, we used a multiple alignment of TAS3-like RNA precursors from flowering plants published by Axtell and others [18]. PCR analysis was performed with two pairs of primers: P-Tas3 corresponded to the 5′ miR390 target site and either M-Tas3/caa or M-Tas3/aca, both complementary to the 3′ miR390 target/cleavage site [18, 20]. Control PCR reaction using chromosomal *Arabidopsis thaliana* DNA as template and both paires of primers resulted in efficient synthesis of a single PCR-fragments with expected size of 260 bp (Figure 1(a)) that was in agreement with calculated distance between 5′ and 3′ miR390 target sites in *Arabidopsis* ta-siARF precursor RNAs [17, 18]. Sequencing of these PCR-amplified and cloned DNA fragments confirmed specific amplification of at least two of three known *A. thaliana* TAS3 chromosomal loci (data not shown) [17, 21].

PCR amplification of chromosomal DNA from *Nicotiana benthamiana* and *Nicotiana tabacum* (cv. Samsun) also resulted in synthesis of one major band of 250 bp and 280 bp, respectively (Figure 1(a) and data not shown). Cloning and sequencing of the obtained DNA bands revealed that the amplified sequences contained putative ta-siARF site composed of two tandem copies of ARF-specific ta-siRNAs and located between miR390 target sites corresponding to PCR primers. Moreover, amplified sequences showed obvious similarity to TAS3-like genes from other dicotyledonous plants (Figure 2) [17, 18]. Bioinformatic analysis of the putative TAS3-like sequences from *Nicotiana benthamiana* and *Nicotiana tabacum* using NCBI Blast revealed closely

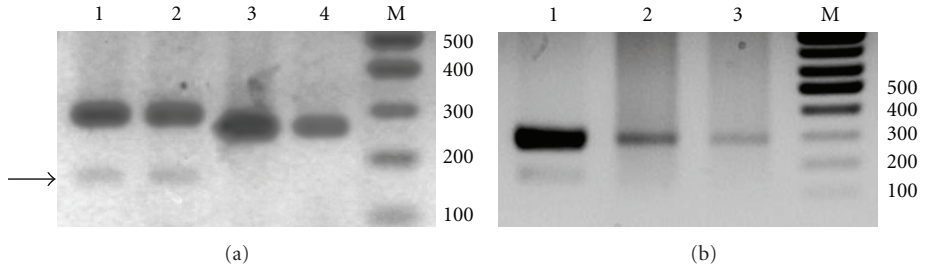


Figure 1: Agarose gel analysis of PCR products. (a) Amplification of genomic DNA sequences flanked by miR390 sites. PCR products were obtained on *Nicotiana tabacum* genomic DNA with primers P-Tas3 and M-Tas3/caa (Lane 1), on *N. tabacum* genomic DNA with primers P-Tas3 and M-Tas3/aca (Lane 2), on *Arabidopsis thaliana* genomic DNA with primers P-Tas3 and M-Tas3/caa (Lane 3), and on *A. thaliana* genomic DNA with primers P-Tas3 and M-Tas3/aca (Lane 4). Arrow points to the 170-nt PCR product. M is DNA size markers. Marker fragment sizes in base pairs are indicated on the left. (b) Amplification of *N. tabacum* genomic DNA and cDNA preparation. PCR products were obtained on *N. tabacum* genomic DNA with primers P-Tas3 and M-Tas3/caa (Lane 1), on *N. tabacum* cDNA with primers P-Tas3 and M-Tas3/caa (Lane 2), and on *N. tabacum* cDNA with primers P-Tas3 and M-Tas3/aca (Lane 3). M is DNA size markers. Marker fragment sizes in base pairs are indicated on the left.

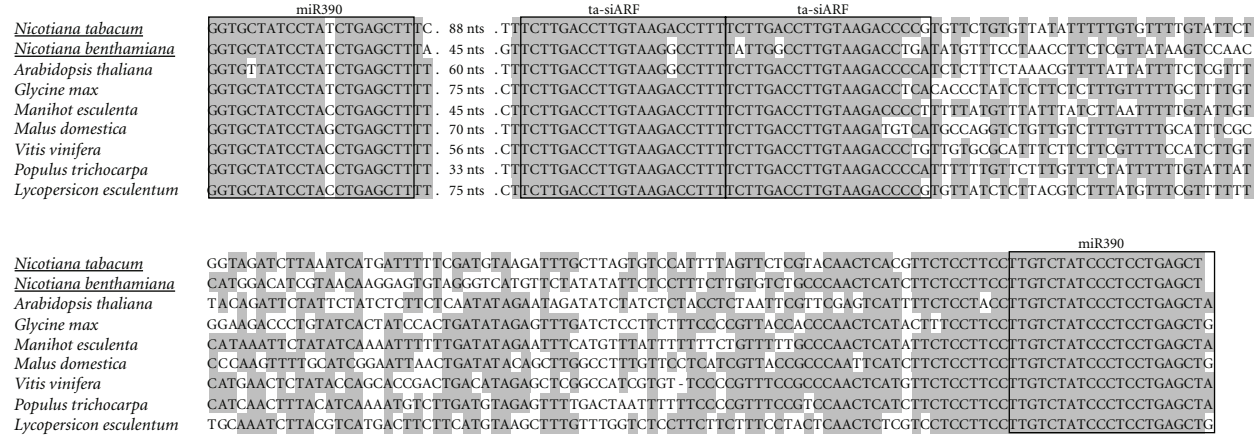


Figure 2: Nucleotide sequence alignment of "280-nt-long" ta-siARF loci of dicot plants. Sequences of *Nicotiana tabacum* and *Nicotiana benthamiana* (undelined) were determined in this study, the others were from GeneBank. Boxes indicate miR390 complementary sites and ta-siARF-encoding sequence regions. The length of nonconserved sequence between the 5′-miR390 complementary site and ta-siARF sequence not included in the alignment is indicated. Shading shows residues identical for the given position in most sequences. Sequences of *N. tabacum* and *N. benthamiana* miR390 complementary sites originate from primers used for the amplification of ta-siARF loci. GeneBank accession numbers of the sequences included in the alignment are as follows: *Nicotiana tabacum*, FJ804751; *Nicotiana benthamiana*, FJ804742; *Arabidopsis thaliana*, BX838290; *Glycine max*, BE330988; *Manihot esculenta*, CK652751; *Malus domestica*, CN490861; *Vitis vinifera*, DT025007; *Populus trichocarpa*, DT498974; *Lycopersicon esculentum*, DV105041.

related sequences in representatives of the family *Solanaceae*. For example, *Nicotiana tabacum* (cv. Samsun) TAS3-like sequence showed almost 100% identity to the *Nicotiana tabacum* (cv. Hicks Broadleaf) genomic sequence obtained in the frame of Tobacco Genome Initiative (GenBank accession no. FH434354) and 78% identity to the *Solanum lycopersicum* (cv. Heinz 1706) chromosome 11 BAC clone (GenBank accession no. DU917444) (Figure 2).

Unlike *Arabidopsis thaliana* DNA showing only one visible DNA band after PCR amplification, *Nicotiana tabacum* DNA consistently produced a second minor PCR product of about 170 bp in length (Figure 1(a)). Since no indications on existence of miR390-dependent TAS3-like genes with so closely arranged miRNA target sites were found in the literature and sequence databases, cloning and sequencing of the minor PCR fragment was carried out. The 170 bp DNA sequence was found to contain the putative ta-siARF site composed of one copy of ARF-specific ta-siRNAs (Figure 3). To rule out the possibility of PCR/cloning artifact we compared this sequence to the available nucleotide sequence databases. As it was found for the 280-nucleotide-long *Nicotiana tabacum* (cv. Samsun) PCR product, 170 bp fragment contained sequence closely related to some yet uncharacterized database entries (Figure 3). Particularly, it showed 96% identity to *Nicotiana tabacum* (cv. Hicks Broadleaf) genomic survey sequences (GSS) obtained in the frame of Tobacco Genome Initiative (GenBank accessions nos. FH734100, FH203124 and FH011695), 91% identity to EST sequence of *Nicotiana tabacum* (cv. SNN) (GenBank accession nos. AM791738), 79% identity to the *Solanum tuberosum* EST

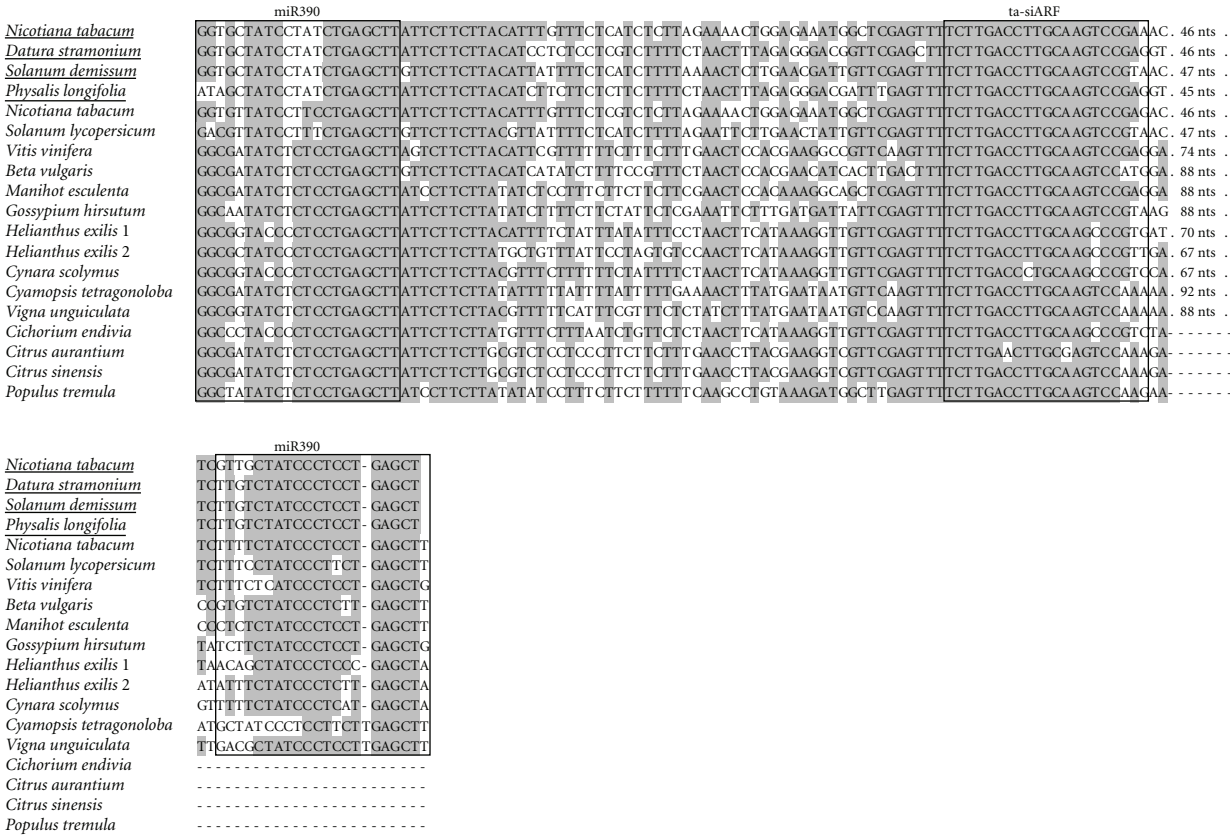


Figure 3: Nucleotide sequence alignment of "170-nt-long" ta-siARF loci of dicot plants. Sequences of *Nicotiana tabacum, Datura stramonium*, *Solanum demissum,* and *Physalis longifolia* (undelined) were determined in this study the others were from GeneBank. Boxes indicate miR390 complementary sites and ta-siARF-encoding sequence regions. The length of nonconserved sequence between the ta-siARF sequence and 3′-miR390 complementary site not included in the alignment is indicated. Dashes in the sequences of *Cichorium endivia*, *Citrus aurantium*, *Citrus sinensis,* and *Populus tremula* indicate the sequence region lacking the 3′-miR390 complementary site that could not be aligned with other sequences. Shading shows residues identical for the given position in most sequences. Sequences of *N. tabacum, D. stramonium*, *S. demissum,* and *P. longifolia* miR390 complementary sites originate from primers used for the amplification of ta-siARF loci. GeneBank accession numbers of the sequences included in the alignment are as follows: *Nicotiana tabacum*, FJ804743; *Datura stramonium*, FJ804744; *Solanum demissum*, FJ804745; *Physalis longifolia*, FJ804746; *Nicotiana tabacum*, AM791738; *Solanum lycopersicum*, BE459870; *Vitis vinifera*, CU775162; *Beta vulgaris*, CV301446; *Manihot esculenta*, DV447689; *Gossypium hirsutum*, DW502659; *Helianthus exilis* 1, EE630512; *Helianthus exilis* 2, EE657417; *Cyamopsis tetragonoloba*, EG977206; *Vigna unguiculata*, FG922881; *Cynara scolymus*, GE588140; *Cichorium endivia*, EL348656; *Citrus aurantium,* EY848024; *Citrus sinensis*, CK935773; *Populus tremula*, DN500355.

sequence (GenBank accession no. FG548921), 78% identity to the *Solanum lycopersicum* chromosome 12 clone LE_HBa-26C13 (GenBank accession nos. AC209585) and the *Solanum lycopersicum* EST sequence (GenBank accession nos. BE459870). Somewhat lesser identity (73-74%) was also revealed for EST sequences from *Solanum phureja* (GenBank accession nos. FG647537) and *Solanum tuberosum* (GenBank accessions nos. BQ514736 and BI431636) (Figure 3 and data not shown). These data indicated that 170 bp-long PCR-amplified fragments corresponded to the genuine *Nicotiana tabacum* (cv. Samsun) genome fragment potentially encoding a new type of ta-siARF RNA precursors. Sequence analysis revealed that, except their length, the 170-nt-long ta-siARF RNA differ from well-characterized 280-nt-long precursors in the structure of ta-siARF-encoding region: while the longer precursors contain two tandem copies of ta-siARF arranged as the conserved 42-nt region, the smaller precursors described in this paper have only one monomeric 21-nt ta-siARF copy (Figures 2 and 3).

We further experimentally analyzed the occurrence of such putative TAS precursor RNAs in other *Solanaceae* representatives. Genomic DNAs of *Datura stramonium*, *Solanum demissum* and *Physalis longifolia* were taken as templates for PCR with primers P-Tas3 and either M-Tas3/caa or M-Tas3/aca. In all cases, major 280-nucleotide-long and minor 170 bp amplified fragments were easily detected similarly to *Nicotiana tabacum* (data not shown). Sequencing of the smaller DNA band confirmed the occurrence of novel putative monomeric ta-siARF precursor sequences in different *Solanaceae* representatives (order *Solanales*) (Figure 3).

The TAS3 loci and miR390 are known to be well conserved among diverse flowering plants [18, 21, 22]. To explore the possibility that the novel monomeric ta-siARF precursor sequences might have emerged in plant evolution

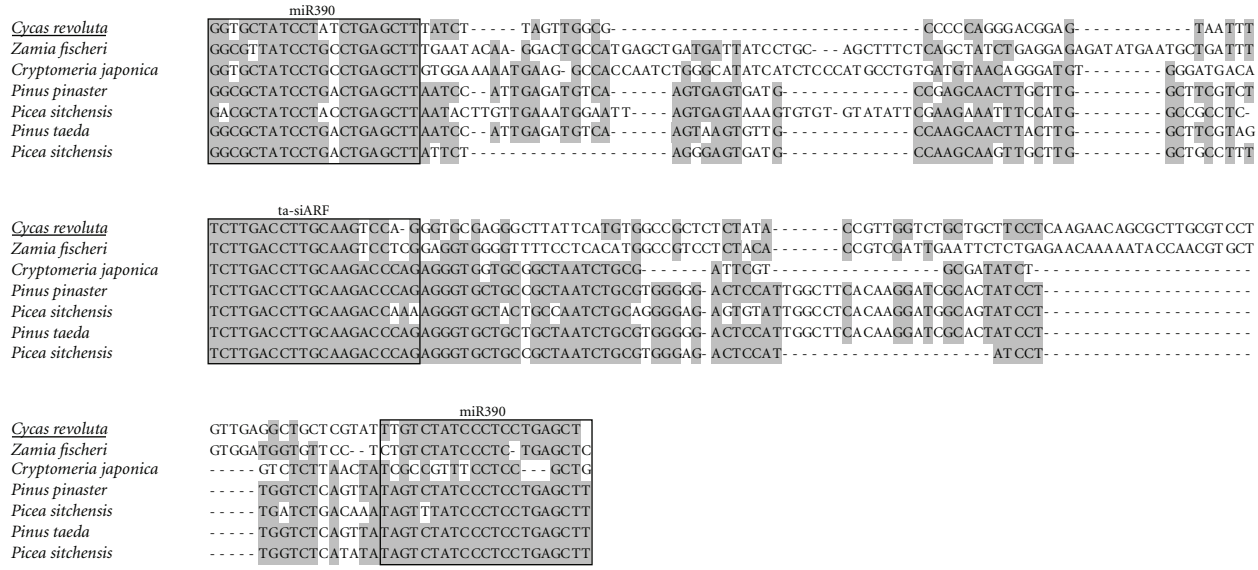


Figure 4: Nucleotide sequence alignment of "short" ta-siARF loci of cycad and conifer plants. The sequence of *Cycas revoluta* (undelined) was determined in this study, the others were taken from GeneBank. Boxes indicate miR390 complementary sites and ta-siARF sequence regions. Shading shows residues identical for the given position in most sequences. Sequences of *C. revoluta* miR390 complementary sites originate from primers used for the amplification of ta-siARF loci. GeneBank accession numbers of the sequences included in the alignment are as follows: *Cycas revoluta*, FJ804750; *Picea sitchensis*, EF086492; *Pinus pinaster*, BX682439; *Pinus taeda*, CV034496; *Picea sitchensis*, CO220369; *Zamia fischeri*, DY034932; *Cryptomeria japonica*, DC432705.

earlier than the appearance of family *Solanaceae*, we searched EST database at NCBI. We found that many dicot plants code for putative monomeric ta-siARF loci surrounded in most cases by complementary sites for miR390 positioned 160–190 bp from each other, thus confirming that targeting of ta-siRNA precursors is an evolutionarily conserved function of miR390. These plants include representatives of eurosids—orders *Malpighiales*, *Fabales*, *Malvales,* and *Sapindales*, asterids—orders *Asterales* and *Solanales*, as well as orders *Caryophyllales* and *Vitales* (Figure 3). Analysis of all identified monomeric ta-siARF precursor sequences revealed, in addition to the only copy of ta-siARF sequence, one more characteristic feature. The region between the 5′-miR390 target site and the ta-siARF sequence appeared to be well conserved in length and, to some extent, in sequence, whereas the region between the ta-siARF sequence and the 3′-miR390 target site was found to be variable both in length and sequence (Figure 3). This arrangement of the novel 170 bp-TAS loci differed considerably from that of the well characterized 280 bp-TAS loci, where the sequence and distance conservation was observed for the region between the tandem ta-siARF sequence and the 3′-miR390 target site and not for the sequence between the 5′-miR390 target site and the ta-siARF sequence (Figure 2).

To verify expression of novel putative monomeric ta-siARF precursor RNAs in plant leaves, total RNA was isolated from tobacco plants at 6-8-leaf stage and used as a template for cDNA synthesis with oligo(dT)-primer. It was found that PCR with primers P-Tas3, and either M-Tas3/caa or M-Tas3/aca carried out on this cDNA preparation gave rise only to 280-nucleotide-long DNA fragment but not detectable 170 bp-fragment (Figure 1(b)).

Conifers (*Pinus taeda*) TAS-like locus, like that of higher plants, was shown to contain the 42-nt ta-siARF region flanked by two miR390 complementary sites. However, unlike TAS3 of flowering plants, only the 5′ miR390-target site is cleaved in conifer TAS3-like RNA precursor [18]. To reveal the possible conservation of TAS3-like loci in lower seed plants, we performed PCR amplification of total DNA from Cycads (*Cycas revoluta*) using dicot-specific primers P-Tas3, M-Tas3/caa and M-Tas3/aca. As it was found for tobacco, PCR on *Cycas revoluta* DNA gave rise to one major DNA fragment of 250 bp and, in addition, several larger and smaller minor bands including those of 170–190 bp in length (data not shown). Sequencing and BLAST analysis of the latter sort of bands revealed that they corresponded to the monomeric ta-siARF precursors of flowering plants (Figure 4). Analysis of databases revealed conifers-encoded sequences showing similarity to the sequenced *Cycas revoluta* putative ta-siARF precursor (Figure 4), showing therefore that this type of regulatory element might have emerged early in seed plant evolution.

Previously, four ta-siRNA loci targeted by miR390 were found in moss *Physcomitrella patens* and referred to as PpTAS3a-d. All four loci contain 5′ and 3′ miR390-target sites, and ta-siRNAs derived from these moss loci regulate, similarly to those encoded by the angiosperm TAS3 loci, ARF genes and, additionally, AP2 transcription factor genes [18, 23, 24]. To confirm applicability of the approach based on PCR amplification of TAS3-like loci to lower plants, we designed new primers BryoTAS-P1,

Figure 5: Nucleotide sequence alignment of ta-siARF loci of mosses. (a) Alignment of *Brachythecium latifolium* clone 50 Br sequence and *Physcomitrella patens* TAS3d. (b) Alignment of *B. latifolium* clone 47 Br sequence and *Hookeria lucens* with *P. patens* TAS3b and TAS3c. Sequences of *B. latifolium* and *H. lucens* (undelined) were determined in this study, the others were from GeneBank. Boxes indicate miR390 complementary sites, ta-siARF sequence regions and ta-siAP2 regions. Shading shows residues identical for the given position in most sequences. Sequences of *B. latifolium* and *H. lucens* miR390 complementary sites originate from primers used for the amplification of ta-siARF loci. GeneBank accession numbers of the sequences included in the alignment are as follows: *Brachythecium latifolium* clone 50 Br, FJ804747; *Brachythecium latifolium* clone 47 Br, FJ804748; *Hookeria lucens*, FJ804749; *Physcomitrella patens* TAS3d, BK005828; *P. patens* TAS3b, BK005826; *P. patens* TAS3c, BK005827.

Bryo TAS-P3, and Bryo TAS-M complementary to miR390-target sites of PpTAS3a-d. These primers were used for PCR on total DNA isolated from *Brachythecium latifolium* and *Hookeria lucens*. Sequencing of major amplified DNA bands showed that *Brachythecium latifolium* encodes at least two types of miR390-dependent AP2/ARF-targeting TAS loci demonstrating sequence similarity to different elements of this kind characterized for *Physcomitrella patens,* namely, PpTAS3d (Figure 5(a)) and PpTAS3b/3c (Figure 5(b)), whereas *Hookeria lucens*, despite its distant taxonomic relation to *Physcomitrella patens,* code for a rather close homolog of PpTAS3b/3c (Figure 5(b)).

4. Discussion

MicroRNAs and ta-siRNAs are small RNAs around 21 nt in length and have widespread roles as posttranscriptional regulators of plant physiology and development [25]. They can affect target genes through a variety of mechanisms, including transcript cleavage and translational repression. The mature miRNAs and ta-siRNAs are derived from larger precursor transcripts that are a few hundred base pairs in size. These precursor RNAs are capped and polyadenylated and can be spliced, in agreement with them being transcribed by RNA polymerase II [12, 26, 27]. Many plant miRNAs and ta-siRNAs are encoded by gene families. For example, four ta-siRNA loci targeted by miR390 were found in *P. patens* and three miR390-dependent TAS loci were identified in *A. thaliana* [17, 18, 21, 23]. Given their similar complementarity to miR390, it was reasoned that multiple TAS3-like loci might be paralogs derived from duplication and divergence of an ancestral locus, and perhaps the areas producing biologically relevant ta-siRNAs may have been preferentially conserved [18, 23]. However, despite obvious progress in studies of miRNA precursor evolution in plants [12, 28, 29], little is known about TAS gene molecular evolution.

Comparing sequences of ta-siRNA precursors within plant genera and between distantly related species should help to determine patterns of molecular evolution that is subject to different evolutionary constraints in comparison with patterns of protein-coding gene evolution. However, the discovery of ta-siRNA genes in plants is still an ongoing process. Although much effort has been directed toward ta-siRNA identification in *Arabidopsis*, poplar and moss *Physcomitrella patens*, many economically or evolutionarily important species have yet to be examined, and more ta-siRNAs likely remain to be discovered even in the species that have been extensively studied. One limiting factor in the ta-siRNA discovery process is the availability of a sequenced

genome, without which a comprehensive analysis of the potential precursor structures of cloned small RNAs is not possible. In *Arabidopsis*, three major methods have been used for ta-siRNA discovery: forward genetics, direct cloning and sequencing, and bioinformatic prediction. Although forward genetic studies have only resulted in the identification of a few ta-siRNAs, this method provides hints to the functions of these miRNAs in addition to their isolation [3]. A second approach to miRNA discovery was bioinformatic prediction. While a number of published studies employed several different algorithms to predict ta-siRNAs, the features that the algorithms search for in the genomic sequences are based on our current knowledge of plant miRNAs and are largely similar among the studies [19]. A third, and perhaps the most effective, method for ta-siRNA discovery was direct cloning and sequencing. In particular, deep sequencing of cloned small RNA libraries using massively parallel signature sequencing (MPSS) or pyrosequencing allowed the identification of numerous small RNAs from *Arabidopsis* and provided a picture of the genomic landscape of small RNAs [18, 23]. One added advantage of this approach is that most small RNA species from a particular genomic loci are exhibited, which helps discern whether the locus gives rise to a single or multiple siRNAs. If multiple small RNAs mapping to both strands of a locus are present, these small RNAs are most likely siRNAs.

Here, we describe a new method for identification of plant miR390-flanked ta-siRNAs precursors based on PCR on the template of genomic DNA or cDNA using oligodeoxyribonucleotide primers mimicking in their specificity miR390. General idea of this experimental approach was suggested by the recently published paper on cloning of animal miRNA-regulated target genes by reverse transcription with direct use of miRNA as a primer [30]. Importantly, the recognition of ta-siRNA precursor by miR390 occurs in most cases by forming imperfect base pairs with 5′ and 3′ target sites [18, 23]. Assuming that imperfect base-pairing is tolerated by Taq polymerase, there is a high chance that designed miR390-related oligodeoxyribonucleotides will recognize both target sites in phylogenetically distant species. To investigate functional constraints on sequence evolution of ta-siARF precursor genes, we have studied sequence variation in the putative precursors of miR390-dependent TAS-like genetic elements between species from the *Solanaceae*. The application of the novel PCR-based method to *Nicotiana tabacum* showed obvious difference from *A. thaliana*: *N. tabacum* DNA directed a major 280-nucleotide-long PCR product and the second minor PCR product of about 170 bp in length (Figure 1). When the method was also applied to *Datura stramonium*, *Solanum demissum,* and *Physalis longifolia,* the minor PCR short product was again identified. Sequencing of 280-nucleotide-long PCR product in the *Solanaceae* and *Arabidopsis* as a control showed that we amplified and cloned the chromosomal region closely related to typical TAS3-related precursor sequences found in diverse dicot plants [17, 18]. This region included 42-nt region from AtTAS3 tasiRNAs that target ARF3 and ARF4 (tasiARFs) and 5′- and 3′- terminal 21-nt regions corresponding to the miR390 complementary sites (PCR primer sites) (Figure 2). These results demonstrate that the PCR-based method is a useful way to clone and identify targets on the basis of base pairing with individual miRNAs. Nucleotide sequence of the minor 170-nucleotide-long product of four selected solanaceous plants revealed the putative 21-nt ta-siARF site composed of one copy of ARF-specific ta-siRNAs and positioned between the miR390 complementary sites (PCR primer sites) (Figure 3). We searched publicly available nucleotide databases of genome survey sequences (GSSs), high-throughput genomics sequences (HTGSs), expressed sequenced tags (ESTs), and nonredundant (NR) nucleotides and identified 16 putative "short" miR390-dependent TAS sequences in 15 diverse plant species (Figure 3 and data not shown).

Using this PCR approach, ta-siARF precursors characterized by the monomeric ta-siARF were found in the cycad *Cycas revolute.* Database search confirmed this experimental finding and revealed novel miR390-dependent TAS sequences in one more cycad (*Zamia fischeri*) and four conifer species (Figure 4). The experimental approach used in this paper was further verified by the analysis of mosses with primers based on *Physcomitrella patens* miR390 complementary; the PCR products obtained on *Brachythecium latifolium* and *Hookeria lucens* DNA represented ta-siRNA precursor sequences that were not known before for these species and related to the previously characterized *P. patens* ta-siRNA loci (Figures 5(a) and 5(b)).

To analyze expression of the novel putative ta-siARF precursor RNAs in plants, total RNA was isolated from tobacco plants at 6-8-leaf stage and used as template for cDNA synthesis with oligo(dT)-primer. PCR analysis of leaf cDNA revealed only 280-nucleotide-long DNA fragment which sequence corresponded to classical tobacco TAS3-like element (Figure 1(b)). On the other hand, RNA template for the EST sequence of *Nicotiana tabacum* (cv. SNN) (GenBank accession no. AM791738) with 91% identity to our "short" TAS sequence from *Nicotiana tabacum* (cv. Samsun) (Figure 3) was isolated from 5-day seedlings. These data suggest that the novel type of miR390-dependent TAS elements might be expressed in tobacco only at early stages of ontogenesis.

Importantly, alignment of the putative novel TAS elements revealed that the length of a region between ta-siARF site and 5′ miR390-target site is highly preserved in contrast to classical TAS3-like genes in which perfect size conservation was revealed between ta-siARF site and 3′ miR390-target site (Figures 2 and 3). In addition, "core consensus" in classical ta-siARF site was found to represent sequence: TCTTGACCTTGTAAGACCPy, whereas consensus in "short" TAS is different: TCTTGACCTTGCAAGPyCCPu. Significance of this variation remains obscure.

Strikingly, in four cases we failed to reveal the 3′ miR390-target site in "short" miR390-dependent TAS sequences (Figure 3). This type of organization of ta-siRNA precursors is common for the other, except TAS3, TAS genes in *Arabidopsis,* and other plants [4, 12] in which miRNA-dependent cleavage occurs 5′ to ta-siRNA sites. Therefore, we speculate that in "short" miR390-dependent TAS

sequences miRNA-dependent cleavage also can occur 5′ to ta-siRNA sites. This hypothesis requires experimental verification. In addition to identification of the precursor cleavage site, the role of AGO1 and AGO7 in the "short" miR390-dependent ta-siARF precursor processing should be investigated in comparison to classical TAS3-like precursors [4, 20].

Recently a novel silencing method utilizing artificial ta-siRNAs was proved to be efficient for the chimeric *Arabidopsis thaliana* TAS1 and TAS3 genes [31, 32]. This method provides an excellent tool not only for silencing of genes-of-interest but also for investigating functionality of TAS genes and their specific sequence modifications. Application of this method to "short" TAS3-like genes identified in this paper may result in a identification of their possible functions and mapping of cleavage sites in RNA precursors.

Acknowledgments

The authors thank A. C. Timonin for stimulating discussions. This work was supported by Grants 08-04-00846 and 09-04-00707 from the Russian Foundation for Basic Research.

References

[1] D. Baulcombe, "RNA silencing in plants," *Nature*, vol. 431, no. 7006, pp. 356–363, 2004.

[2] F. Vazquez, "Arabidopsis endogenous small RNAs: highways and byways," *Trends in Plant Science*, vol. 11, no. 9, pp. 460–468, 2006.

[3] H. Vaucheret, "Post-transcriptional small RNA pathways in plants: mechanisms and regulations," *Genes & Development*, vol. 20, no. 7, pp. 759–771, 2006.

[4] H. Vaucheret, "Plant ARGONAUTES," *Trends in Plant Science*, vol. 13, no. 7, pp. 350–358, 2008.

[5] E. Bonnet, Y. van de Peer, and P. Rouzé, "The small RNA world of plants," *New Phytologist*, vol. 171, no. 3, pp. 451–468, 2006.

[6] P. Laporte, F. Merchan, B. B. Amor, et al., "Riboregulators in plant development," *Biochemical Society Transactions*, vol. 35, no. 6, pp. 1638–1642, 2007.

[7] C. Y. Chu and T. M. Rana, "Small RNAs: regulators and guardians of the genome," *Journal of Cellular Physiology*, vol. 213, no. 2, pp. 412–419, 2007.

[8] B. Liu, Z. Chen, X. Song, et al., "*Oryza sativa Dicer-like4* reveals a key role for small interfering RNA silencing in plant development," *The Plant Cell*, vol. 19, no. 9, pp. 2705–2718, 2007.

[9] X. Chen, "MicroRNA metabolism in plants," *Current Topics in Microbiology and Immunology*, vol. 320, pp. 117–136, 2008.

[10] S. Griffiths-Jones, H. K. Saini, S. van Dongen, and A. J. Enright, "miRBase: tools for microRNA genomics," *Nucleic Acids Research*, vol. 36, pp. D154–D158, 2008.

[11] E. Gottwein and B. R. Cullen, "Viral and cellular microRNAs as determinants of viral pathogenesis and immunity," *Cell Host and Microbe*, vol. 3, no. 6, pp. 375–387, 2008.

[12] M. J. Axtell and J. L. Bowman, "Evolution of plant microRNAs and their targets," *Trends in Plant Science*, vol. 13, no. 7, pp. 343–349, 2008.

[13] A. C. Mallory and N. Bouché, "MicroRNA-directed regulation: to cleave or not to cleave," *Trends in Plant Science*, vol. 13, no. 7, pp. 359–367, 2008.

[14] V. Ambros and X. Chen, "The regulation of genes and genomes by small RNAs," *Development*, vol. 134, no. 9, pp. 1635–1641, 2007.

[15] B. Liu, P. Li, X. Li, et al., "Loss of function of OsDCL1 affects microRNA accumulation and causes developmental defects in rice," *Plant Physiology*, vol. 139, no. 1, pp. 296–305, 2005.

[16] A. Takeda, S. Iwasaki, T. Watanabe, et al., "The mechanism selecting the guide strand from small RNA duplexes is different among *Argonaute* proteins," *Plant & Cell Physiology*, vol. 49, no. 4, pp. 493–500, 2008.

[17] E. Allen, Z. Xie, A. M. Gustafson, and J. C. Carrington, "microRNA-directed phasing during trans-acting siRNA biogenesis in plants," *Cell*, vol. 121, no. 2, pp. 207–221, 2005.

[18] M. J. Axtell, C. Jan, R. Rajagopalan, and D. P. Bartel, "A two-hit trigger for siRNA biogenesis in plants," *Cell*, vol. 127, no. 3, pp. 565–577, 2006.

[19] H. M. Chen, Y. H. Li, and S. H. Wu, "Bioinformatic prediction and experimental validation of a microRNA-directed tandem trans-acting siRNA cascade in Arabidopsis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 9, pp. 3318–3323, 2007.

[20] T. A. Montgomery, M. D. Howell, J. T. Cuperus, et al., "Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation," *Cell*, vol. 133, no. 1, pp. 128–141, 2008.

[21] L. Williams, C. C. Carles, K. S. Osmont, and J. C. Fletcher, "A database analysis method identifies an endogenous trans-acting short-interfering RNA that targets the Arabidopsis ARF2, ARF3, and ARF4 genes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 27, pp. 9703–9708, 2005.

[22] M. J. Axtell and D. P. Bartel, "Antiquity of microRNAs and their targets in land plants," *Plant Cell*, vol. 17, no. 6, pp. 1658–1673, 2005.

[23] M. J. Axtell, J. A. Snyder, and D. P. Bartel, "Common functions for diverse small RNAs of land plants," *Plant Cell*, vol. 19, no. 6, pp. 1750–1769, 2007.

[24] M. D. Howell, N. Fahlgren, E. J. Chapman, J. S. Cumbie, et al., "Genome-wide analysis of the RNA-DEPENDENT RNA POLYMERASE6/DICER-LIKE4 pathway in Arabidopsis reveals dependency on miRNA- and tasiRNA-directed targeting," *Plant Cell*, vol. 19, no. 3, pp. 926–942, 2007.

[25] G. Chuck, H. Candela, and S. Hake, "Big impacts by small RNAs in plant development," *Current Opinion in Plant Biology*, vol. 12, no. 1, pp. 81–86, 2009.

[26] M. Ha, M. Pang, V. Agarwal, and Z. J. Chen, "Interspecies regulation of microRNAs and their targets," *Biochimica et Biophysica Acta*, vol. 1779, no. 11, pp. 735–742, 2008.

[27] M. W. Jones-Rhoades, D. P. Bartel, and B. Bartel, "MicroRNAs and their regulatory roles in plants," *Annual Review of Plant Biology*, vol. 57, pp. 19–53, 2006.

[28] A. Li and L. Mao, "Evolution of plant microRNA gene families," *Cell Research*, vol. 17, no. 3, pp. 212–218, 2007.

[29] N. Warthmann, S. Das, C. Lanz, and D. Weigel, "Comparative analysis of the MIR319a microRNA locus in Arabidopsis and related Brassicaceae," *Molecular Biology and Evolution*, vol. 25, no. 5, pp. 892–902, 2008.

[30] Y. Andachi, "A novel biochemical method to identify target genes of individual microRNAs: identification of a new Caenorhabditis elegans let-7 target," *RNA*, vol. 14, no. 11, pp. 2440–2451, 2008.

[31] T. A. Montgomery, M. D. Howell, J. T. Cuperus, et al., "Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation," *Cell*, vol. 133, no. 1, pp. 128–141, 2008.

[32] M. de la Luz Gutiérrez-Nava, M. J. Aukerman, H. Sakai, S. V. Tingey, and R. W. Williams, "Artificial trans-acting siRNAs confer consistent and effective gene silencing," *Plant Physiology*, vol. 147, no. 2, pp. 543–551, 2008.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 410260, 7 pages
doi:10.1155/2009/410260

Methodology Report

Efficient siRNA Delivery by the Cationic Liposome DOTAP in Human Hematopoietic Stem Cells Differentiating into Dendritic Cells

Sabata Martino, Ilaria di Girolamo, Roberto Tiribuzi, Francesco D'Angelo, Alessandro Datti, and Aldo Orlacchio

Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Sezione di Biochimica e Biologia Molecolare, Università di Perugia, Via del Giochetto, 06100 Perugia, Italy

Correspondence should be addressed to Aldo Orlacchio, orly@unipg.it

Received 11 December 2008; Accepted 20 March 2009

Recommended by Bibekanand Mallick

RNA interference technology is an ideal strategy to elucidate the mechanisms associated with human $CD34^+$ hematopoietic stem cell differentiation into dendritic cells. Simple manipulations in vitro can unequivocally yield alloreactive or tolerogenic populations, suggesting key implications of biochemical players that might emerge as therapeutic targets for cancer or graft-versus-host disease. To knockdown proteins typically involved in the biology of dendritic cells, we employed an siRNA delivery system based on the cationic liposome DOTAP as the carrier. Freshly-isolated $CD34^+$ cells were transfected with siRNA for cathepsin S with negligible cytotoxicity and transfection rates (>60%) comparable to the efficiency shown by lentiviral vectors. Further, cathepsin S knockdown was performed during both cell commitment and through the entire 14-day differentiation process with repeated transfection rounds that had no effect per se on cell development. Tested in parallel, other commercially-available chemical reagents failed to meet acceptable standards. In addition to safe and practical handling, a direct advantage of DOTAP over viral-mediated techniques is that transient silencing effects can be dynamically appraised through the recovery of targeted proteins. Thus, our findings identify DOTAP as an excellent reagent for gene silencing in resting and differentiating $CD34^+$ cells, suggesting a potential for applications in related preclinical models.

1. Introduction

The discovery of siRNAs and subsequent advances in RNA interference (RNAi) technology has contributed to a major methodological shift in functional genomics [1]. Gene silencing, that can be successfully extended to high-throughput screening formats [2, 3], is a procedure that directly accelerates biological and biomedical research through the identification of molecular players involved in development, homeostasis, and disease. In the new millennium, the explosive growth of this field has also raised the prospect of RNAi as a therapeutic to treat a wide range of pathologies, in particular viral infections, cancer, and neurodegenerative disorders [4–7].

Synthetic siRNAs have emerged as a widespread means for gene silencing in mammalian cells [8], notwithstanding limitations associated with a nonrenewable supply, temporary silencing effect, and occurrence of off-target activity [9, 10]. Next-generation synthetic products were recently introduced to ensure the highest silencing power under a minimal occurrence of false positives (e.g., ON-TARGET*plus* [Dharmacon] and *Silencer* Select validated siRNAs [Ambion]), and provide the opportunity to transfect virtually any cell type in the absence of a carrier (Accell siRNAs [Dharmacon]). Further, shRNA expression vectors coupled to lentiviral transduction systems were developed to obtain unlimited resources, achieve stable integration into the genome in a widest variety of both dividing and nondividing cells, produce sustained transgene expression, and comply with *in vivo* applications (e.g., MISSION [Sigma-Aldrich], BLOCK-iT [Invitrogen], SMARTvector [Dharmacon], GeneNet [System Biosciences], and Lenti-X

[Clontech]) [11–13]. Finally, access to mimic and inhibitor miRNA libraries for use in either gain- or loss-of-function analyses, respectively, have enhanced scope and flexibility of studies intended to unravel the involvement of miRNAs (e.g., PremiR [Ambion], miRIDIAN [Dharmacon], and miScript [Qiagen]) in cytoplasmic posttranscriptional gene silencing [14], translational activation [15] and transcriptional control of gene expression in the nucleus [16].

However, the extraordinary benefits of these breakthroughs in RNAi technology may imply significant research investments and special requirements, such as biocontainment facilities dedicated to viral work. Chemical agents, instead, are relatively inexpensive, easy to handle and generally already optimized by the vendor for use with specific cell lines. Nonetheless, chemical transfections in finite cultures or freshly-isolated primary cells usually raise major challenges due to a combination of cytotoxic effects and poor transfection efficiencies. These problems become more obvious in dynamic systems such as stem cells subjected to differentiation.

Lentiviral vectors can successfully transduce hematopoietic stem cells (HSCs) [12, 17], but integrate stably in the genome and preclude the recovery of knocked-down proteins. Within nonviral transfection methods for HSCs, electroporation raises major survival issues due to apoptotic cell death [18], while nucleofactor technology was shown to deliver mRNA and plasmid DNA with inconsistent transfection and cell survival rates [19].

Lipofection has typically been considered inefficient for use with HSCs [20]. Nonetheless, based on the large number of products currently available from vendors and the advantages offered by a transient gene knockdown, we screened a variety of agents with different chemical formulations and found that the liposomal version of the monocationic lipid DOTAP is an excellent vector for the treatment of freshly isolated HSCs. Further, we observed that DOTAP is an efficient vehicle for gene knockdown in HSCs induced to differentiate into immunogenic dendritic cells *in vitro* through a conventional treatment based on Flt3L, GM-CSF, IL-4 and TNF-α [21–23].

2. Materials and Methods

2.1. Isolation of Human HSCs. Blood samples were collected from healthy volunteers who provided informed consent. Human peripheral blood mononuclear cells were isolated by density gradient centrifugation over Ficoll-Paque PLUS (GE Healthcare), after which $CD34^+$cells were purified by immunomagnetic selection using the minimagnetic-activated cell sorter (MACS) system (Miltenyi Biotec). Mean purity of CD34-enriched cells, determined by flow cytometry using a FACScan (BD Biosciences), was 94.8%, with a median value of 92 throughout an 86–98.5 range.

2.2. Differentiation of HSCs into Dendritic Cells. To generate immunogenic dendritic cells, CD34-enriched cells were transferred to 25 cm^2 flasks at a density of 10^5 cells/mL and cultured for 14 days in RPMI-1640 medium (Euroclone) containing 10% fetal calf serum (FCS) (Euroclone) and a cytokine cocktail composed of human recombinant, Flt3L (50 ng/mL), GM-CSF (50 ng/mL), IL-4 (10 ng/mL), and TNF-α (2.5 ng/mL) (PeproTech EC). Every third day, half of the culture medium was replaced by fresh medium supplemented with the same cocktail. Cells were analyzed at day 3, 7, and 14 to evaluate the expression of specific markers of differentiation [23].

2.3. Evaluation of Different Reagents for siRNA Transfection of Untreated $CD34^+$ Stem Cells. The following, commercially available reagents were tested to determine transfection rates and cytotoxicity effects: TransPass R1 and TransPass R2 (New England Biolabs), Lipofectamine 2000 and Lipofectamine LTX (Invitrogen), siPORT NeoFX and siPORT Amine (Ambion), and DOTAP (Roche). Tests were performed using a 96-well plate format. Freshly prepared $CD34^+$ cells were seeded at a density of 2×10^4/well. Fluorescein-labeled siRNA control (New England Biolabs), in a final concentration of 15 nM, was used to monitor the transfection procedures. Fluorescein-labeled siRNA was regularly diluted with RPMI-1640 without serum, except for the experiment performed with DOTAP, in which the diluent was HBS (Hepes-buffered saline: 20 mM HEPES, pH 7.4, containing 150 mM NaCl).

The transfection reagents were essentially employed as per each vendor's recommendations. TransPass R1 and TransPass R2 (0.2 μL) were diluted directly in the fluorescein-labeled siRNA solution (10 μL), incubated 30 minutes at room temperature and then added to the cultures (90 μL). Lipofectamine 2000 and Lipofectamine LTX (0.25 μL) were first diluted in 12.5 μL RPMI-1640 without serum and, after 5 minutes incubation, mixed with 12.5 μL of fluorescein-labeled siRNA. After 30 minutes incubation, the transfection complexes were added to the cells (75 μL).

siPORT NeoFX and siPORT Amine were first diluted (0.5 and 0.3 μL, resp.) in 10 μL RPMI-1640 without serum. After 10 minutes at room temperature, each carrier was mixed with 10 μL of fluorescein-labeled siRNA. The mixtures (20 μL) were incubated 10 minutes, then spotted in the assay wells. Within 5 minutes, cells were dispensed in a volume of 80 μL.

DOTAP (1.4 μL) was diluted in 7 μL HBS and then mixed, very slowly, with the same volume of fluorescein-labeled siRNA. After 30 minutes at room temperature, the mixture was added to cells (186 μL) resuspended in RPMI-1640 containing 20% FCS.

Except for the experiment performed with DOTAP, fresh medium was added to the assay wells 4 hours after each transfection process in order to achieve a final volume and a concentration of FCS of 200 μL and 10%, respectively.

In all instances, transfection and cell survival were evaluated 24 hours after the transfection method.

Transfection rates were expressed as the ratio between transfected (fluorescein-labeled) and total number of viable cells measured via DAPI nuclear staining and trypan blue exclusion test. Signals were captured by a Nikon Eclipse TE2000-S fluorescence microscope equipped with a Cell-F video camera (Olympus) and processed via the Cell-F software.

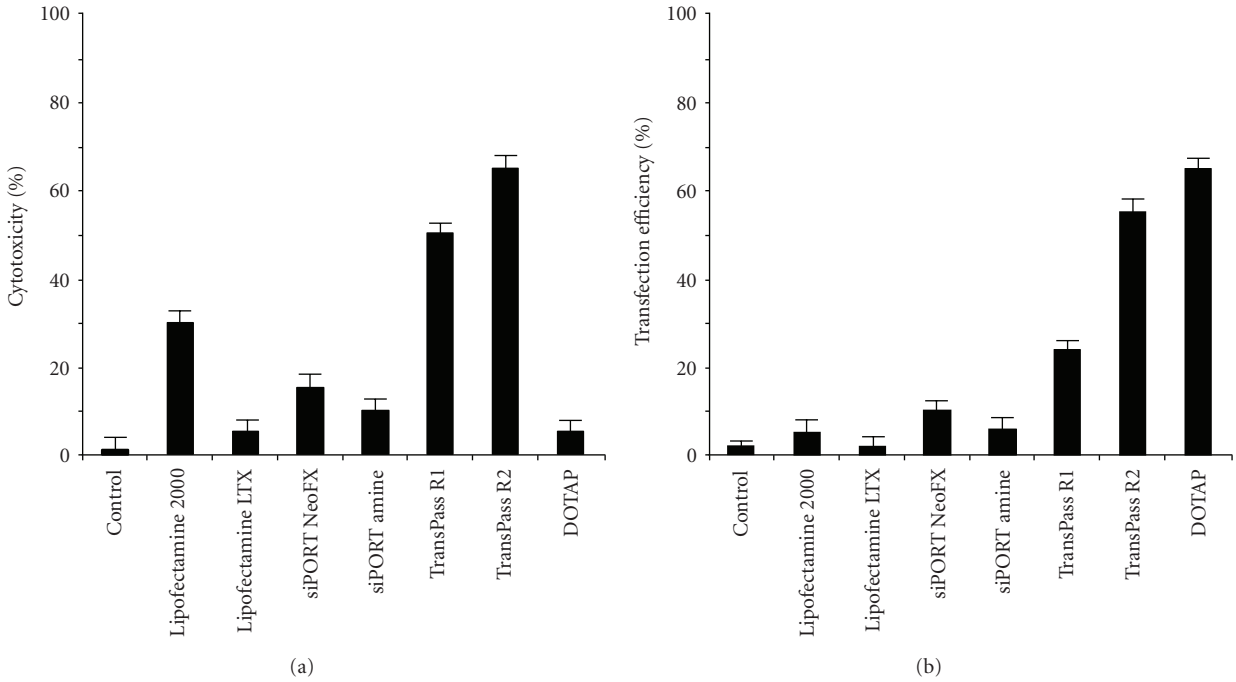


Figure 1: *Effects of siRNA delivery agents on cytotoxicity and transfection rates*. Freshly isolated HSCs were subjected to agent-specific transfection protocols using a fluorescein-labeled, nontargeting siRNA control as described in the Materials and Methods. After 24 hours incubation, cytotoxicity was assessed through the trypan blue dye exclusion test (a), while transfection rates were calculated from the ratio between fluorescein-labeled and total number of DAPI-stained cells (b). Data shown are representative of triplicate determinations.

2.4. Silencing of Cathepsin S Gene via the DOTAP Reagent in HSCs Subjected to Differentiation. Freshly isolated $CD34^+$ cells were resuspended in RPMI-1640 supplemented with the agents used to generate immunogenic dendritic cells. Immediately after treatment, cells were subjected to transfection using a predesigned siRNA targeting the CatS gene (CTSS: ID-113084-113085, Ambion).

Cells subjected to mock- and scrambled-siRNA transfection (ID-46183G, Ambion) under the same experimental conditions were employed for control purposes. The procedure, run in a 24-well plate format via triplicate tests, was implemented using a 25 nM final siRNA concentration. Each well-contained 2×10^5 cells in a volume of 500 μL. The siRNAs were combined with DOTAP and maintained for 30 minutes at room temperature to form complexes. The mixture (50 μL) was then overlaid dropwise on the cell cultures. Following 4-hour incubation, 1.2 mL of RPMI-1640 containing 10% FCS and differentiating agents was added to each well. To ensure silencing of genes for the entire 14-day differentiation process, cells were centrifuged, resuspended in 500 μL of cytokine-enriched culture medium and exposed to a second (day 3) and third (day 9) transfection round under the same experimental conditions.

2.5. Western Blotting. Preparation of cell extracts, electrophoresis (12% gel) under reducing conditions and Western blotting procedures were performed as previously described [24].

Precursors and mature forms of cathepsin S were analyzed using polyclonal antibodies from Santa Cruz Biotechnology as the primary antibodies. Immunodetection was carried out by employing the enhanced, chemiluminescent Amersham ECL Plus kit. For each blot, several time exposures were performed to confirm that the results were within the linear response range of the film. Densitometric scans via an MCID system (InterFocus Imaging) were used to demonstrate that the intensity of the bands was proportional to protein content.

3. Results and Discussion

Several transfection reagents were employed to evaluate cytotoxic effects and the capacity to deliver a fluorescein-labeled siRNA control in freshly isolated, untreated HSCs. Commercially-available agents were chosen to explore a variety of structural diversities and chemical formulations as per each vendor's product description. The screen included a cationic lipid (TransPass R1), a nonlipid cationic polymer (TransPass R2), a lipid-based formulation designed for reverse transfection protocols (siPORT NeoFX), a polyamine mixture (siPORT Amine), and different versions of cationic liposomes (Lipofectamine 2000, Lipofectamine LTX), an animal origin-free product, and DOTAP, obtained from N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate.

Four chemicals (i.e., Lipofectamine LTX, siPORT NeoFX, siPORT Amine, and DOTAP) were relatively well tolerated by HSCs (≥85% viability) (Figure 1(a)), however only DOTAP was found to combine a lack of cell cytotoxicity with efficient (60%) siRNA transfer (Figure 1(b)). TransPass R2 showed

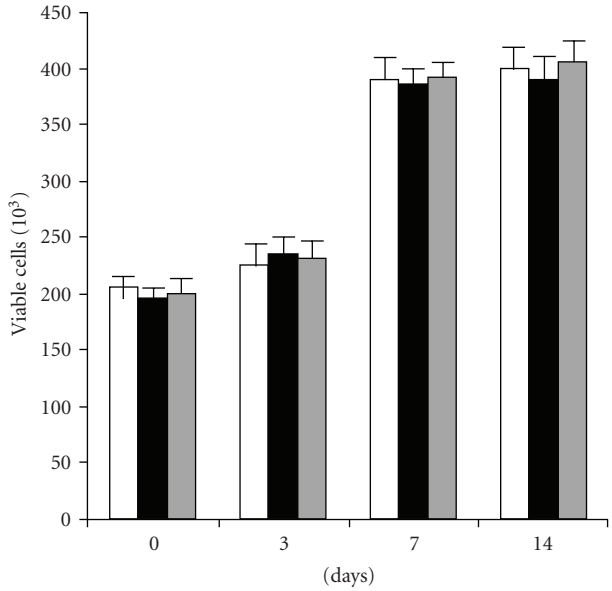


Figure 2: *Cell viability during repeated, DOTAP-mediated transfection rounds in differentiating HSCs.* Freshly isolated HSCs were induced to differentiate into immunogenic dendritic cells as described in the Materials and Methods. Immediately after treatment, cultures were split into two cell populations, of which one, used as the control, was grown in absence of manipulations (*white bars*), while the other was subjected to two and three DOTAP-based, scrambled siRNA transfection rounds performed at day 0 and day 3 (*black bars*), and day 0, day 3 and day 9 (*grey bars*). Cell viability was monitored by trypan blue staining at different time points as indicated.

a transfection capacity similar to DOTAP, but significant cytotoxicity. Thus, DOTAP emerged as the chemical with suitable attributes for use in gene knockdown studies of differentiating HSCs.

We have previously shown that changes in cathepsin S expression correlate with the differentiation of HSCs into immunogenic dendritic cells *in vitro*. In this regard, cathepsin S shows a precursor form of 66 KDa in freshly isolated HSCs, while the conventional, mature protein of 26 KDa appears following a 7-day treatment with a cytokine cocktail [22]. Based on the physiological role of cathepsin S in the biology of dendritic cells [25, 26], we employed a gene knockdown strategy to investigate the involvement of the enzyme during the entire 14-day differentiation process.

In light of the transient nature of siRNA-mediated silencing, we implemented the DOTAP protocol for three rounds of transfection performed in untreated HSCs (day 0) and, following cocktail treatment, in cells subjected to differentiation for 3 and 9 days. Trypan blue exclusion tests performed at different time points showed that multiple knockdowns did not affect cell viability (Figure 2). Furthermore, overall metabolic activity and cell proliferation were also unchanged, measured through reduction rates of the tetrazolium salt XTT (not shown) [27]. We also observed that repeated transfections of a scrambled siRNA had no impact on the temporal expression of antigens typically modulated during the differentiation of HSCs into phenotypically and functionally mature dendritic cells, namely CD34, CD1a, CD80, CD86, CD83, and CD11c (Figure 3). Similarly, the expression of additional immunophenotypic markers such as CD40 (10.04% ± 0.5), CD4 (59.02% ± 0.3), CD123 (46.08% ± 0.6) HLA-ABC (94.10% ± 0.7) in fully differentiated dendritic cells was found to compare well with previous data [23], suggesting that the differentiation process advanced normally within expected temporal timeframes. Further, mixed lymphocyte reaction tests (not shown) did not reveal any discrepancy in the functional properties of alloreactive dendritic cells [23]. On day 14, the reduction of cathepsin S expression exceeded 95% in the differentiation model, as indicated by Western blotting analysis (Figure 4(a)). Instead, gene silencing performed only twice at day 0 and, again, at day 3 caused more than 80% enzyme reduction during the cell commitment stage (day 7), followed by increasing cathepsin levels that resulted in a fully restored expression after two weeks (Figure 4(b)). Interestingly, no differences on the CD34, CD1a, CD80, CD86, CD83, and CD11c markers were measured during HSCs differentiation subjected to two and three transfection rounds compared to untrasfected cells (Figure 3).

These results were found to be consistent throughout the screen of >20 different populations of freshly isolated HSCs and therefore demonstrate a reliable and flexible experimental approach. This system was therefore explored to study the biochemical implication of cathepsin S in the molecular mechanisms associated with HSC differentiation into dendritic cells along the immunogenic or immunosuppressive pathway (Martino et al., submitted manuscript).

Together, our findings point to DOTAP as a chemical suitable for siRNA delivery into HSCs, in light of a transfection efficiency (~60%) comparable to the transduction rate recently achieved in the same human cells through lentiviral vectors [17], and the excellent tolerance to repeated exposures. The ability to dramatically enhance antisense oligodeoxyribonucleotide uptake had, in previous studies, been taken as evidence for DOTAP's biocompatibility with HSCs [28]. However, absence of any disruption during HSC differentiation further suggests that the DOTAP protocol herein described may be successfully extended to other HSC-related systems, including models aimed at addressing existing controversial perspectives on HSC transdifferentiation and overall plasticity [29, 30].

It is possible that other chemical agents, not included in our screen, may exhibit a performance comparable, if not better, to that produced by DOTAP. In this regard, the cationic lipophosphoramide KLN-5 was shown to be a nontoxic, highly efficient vehicle for transgene delivery into HSCs [31]; however, this chemical has not yet been tested in repeated siRNA transfection procedures and, to our knowledge, is not widely available through vendors. It must be noted that while a large number of lipid formulations could be designed to introduce nucleic acids in cells, a complex combination of chemical-physical and biological factors may severely limit the selection of chemicals capable of transfecting synthetic siRNAs with high transfection scores in HSCs. Indeed, cationic liposomes such as DOTAP, Lipofectamine 2000 and Lipofectamine LTX

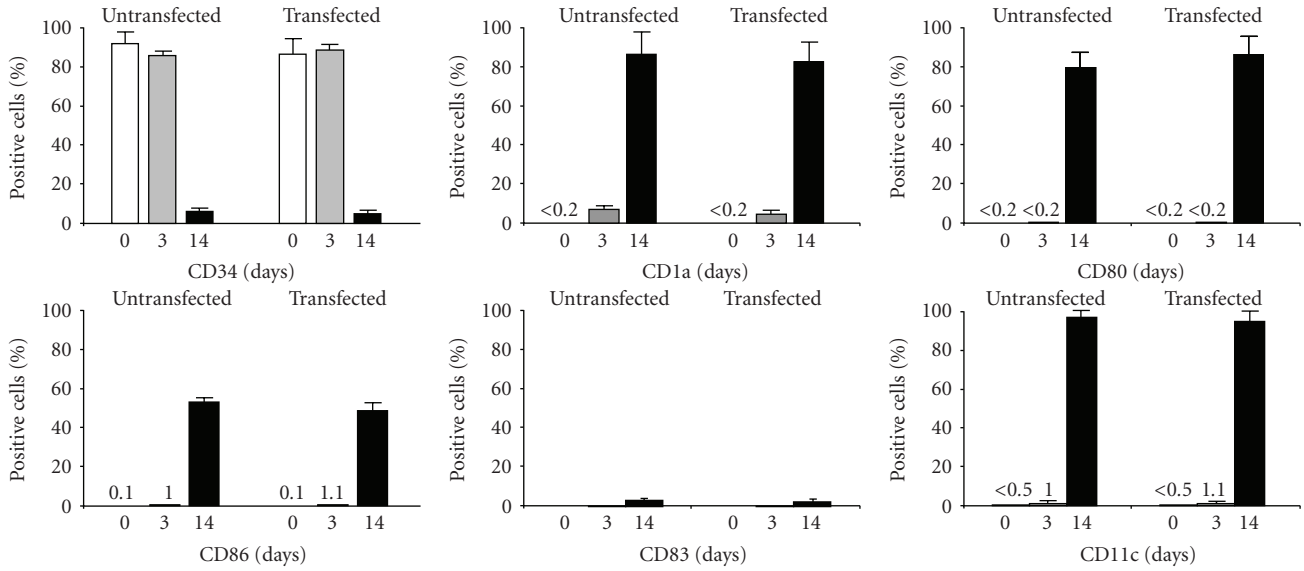


Figure 3: *Expression of phenotypic markers throughout HSC differentiation into immunogenic dendritic cells in presence of multiple, DOTAP-mediated transfection rounds.* HSCs were subjected to differentiation agents, and then immediately split into two parallel cultures, one of which was maintained in the absence of manipulations, while the other was subjected to DOTAP-based, scrambled siRNA transfection rounds. Phenotypic antigens typically modulated during HSC differentiation into immunogenic dendritic cells were analyzed in HSCs at day 0 (*white bars*), day 3 (*grey bars*), and day 14 (*black bars*). In all instances, analyses were performed through a FACScan flow cytometer using monoclonal antibodies labeled with fluorescein isothiocyanate or phycoerythrin. Data shown are representative of triplicate determinations.

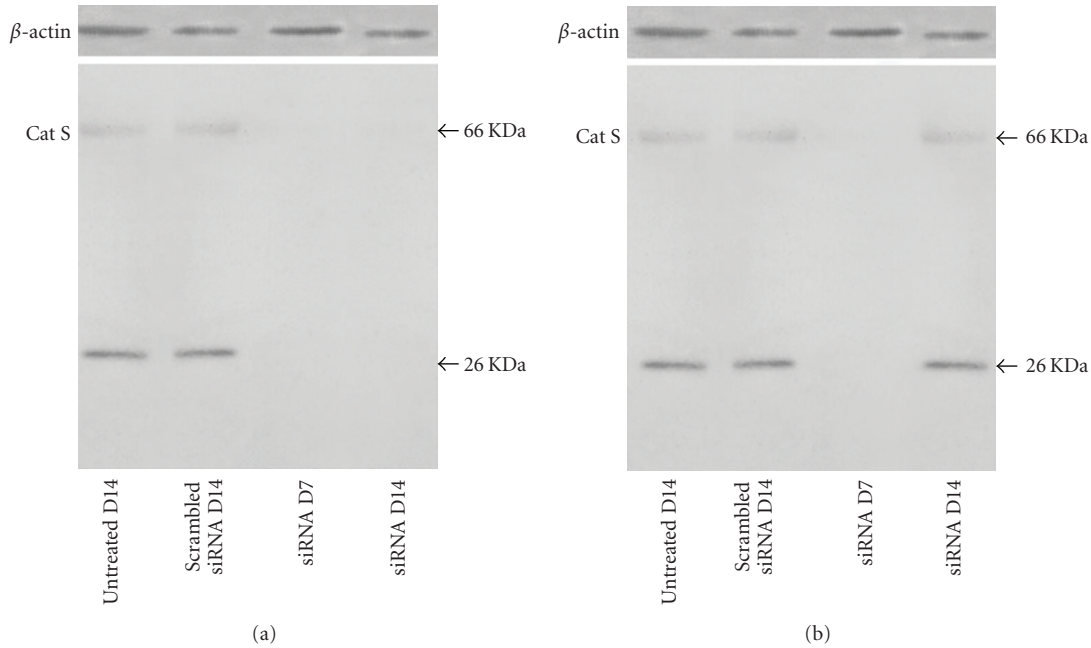


Figure 4: *Silencing of cathepsin S during HSC differentiation.* (a) Levels of cathepsin S were assessed by Western blotting in dendritic cells generated from the differentiation *in vitro*of HSCs. During the 14-day differentiation process, cells were subjected to DOTAP-based transfection rounds on day 0, 3, and 9 using a CTSS-targeting siRNA as described in the Materials and Methods. Untreated and scrambled siRNA-transfected cells were employed for control purposes. Levels of precursor (66 KDa) and mature (26 KDa) forms of cathepsin S at different time points (i.e., days 7 and 14) are indicated. β-actin was used to normalize the signals. (b) Shown is the same analysis as in panel A, except for the number of transfections, that were performed on day 0 and 3 only. Graphs are representative of experiments consistently reproduced using at least 20 different populations of HSCs.

display a positively-charged headgroup that engages electrostatic interactions with the sugar-phosphate backbone, a spacer that may or may not be designed to facilitate such interactions, and 1–3 hydrocarbon chains that may vary in length, extent of saturation and distribution of *cis* and *trans* configurations [32]. Thus, cationic liposomes can be subject to a high degree of structural permutations, likely correlated with a capacity to transfer nucleic acids in specific cell types. Despite structural similarities and a shared clathrin-mediated endocytosis process for DNA delivery [33, 34], these reagents exhibited a very wide performance range when tested in the HSC system. Marked differences in vector efficiencies may also arise from different mechanisms of internalization linked to subsequent, intracellular trafficking routes associated with macromolecular fates [33].

4. Conclusions

Our findings clearly point to DOTAP as an ideal vehicle for *in vitro* studies that require gene expression modulation in HSCs. It will, therefore, be intriguing to investigate whether this reagent can also be adapted to *in vivo* applications, for example, through combination with low-molecular-weight polyethylenimines. These may indeed synergize with liposomes for DNA uptake [35] and have already been proven to be effective in preclinical models [36, 37]. This approach, if successful, would obviate the safety concerns associated with viral-mediated insertional mutagenesis.

Acknowledgments

The authors would like to thank Anna C. Berardi from IRCCS-Bambino Gesù Roma, Italy, for useful discussions and comments on the manuscript. This work was supported by Grants from MIUR (FIRB- Idea Progettuale n.RBIP06FH7J_002), Fondazione Cassa Risparmio di Perugia (Project 2007.0149.02), Ministero della Salute (Project n, RF-UMB-2006-339457), and Consorzio INNB to A.O.

References

[1] S. E. Martin and N. J. Caplen, "Applications of RNA interference in mammalian systems," *Annual Review of Genomics and Human Genetics*, vol. 8, pp. 81–108, 2007.

[2] D. E. Root, N. Hacohen, W. C. Hahn, E. S. Lander, and D. M. Sabatini, "Genome-scale loss-of-function screening with a lentiviral RNAi library," *Nature Methods*, vol. 3, no. 9, pp. 715–719, 2006.

[3] M. Boutros and J. Ahringer, "The art and design of genetic screens: RNA interference," *Nature Reviews Genetics*, vol. 9, no. 7, pp. 554–566, 2008.

[4] D. Bumcrot, M. Manoharan, V. Koteliansky, and D. W. Y. Sah, "RNAi therapeutics: a potential new class of pharmaceutical drugs," *Nature Chemical Biology*, vol. 2, no. 12, pp. 711–719, 2006.

[5] A. Orlacchio, G. Bernardi, A. Orlacchio, and S. Martino, "RNA interference as a tool for Alzheimer's disease therapy," *Mini-Reviews in Medicinal Chemistry*, vol. 7, no. 11, pp. 1166–1176, 2007.

[6] A. Orlacchio, G. Bernardi, A. Orlacchio, and S. Martino, "Patented therapeutic RNAi strategies for neurodegenerative diseases of the CNS," *Expert Opinion on Therapeutic Patents*, vol. 18, no. 10, pp. 1161–1174, 2008.

[7] D. H. Kim and J. J. Rossi, "Strategies for silencing human disease using RNA interference," *Nature Reviews Genetics*, vol. 8, no. 3, pp. 173–184, 2007.

[8] S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl, "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells," *Nature*, vol. 411, no. 6836, pp. 494–498, 2001.

[9] D.-H. Kim, M. A. Behlke, S. D. Rose, M.-S. Chang, S. Choi, and J. J. Rossi, "Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy," *Nature Biotechnology*, vol. 23, no. 2, pp. 222–226, 2005.

[10] J. Moffat, J. H. Reiling, and D. M. Sabatini, "Off-target effects associated with long dsRNAs in *Drosophila* RNAi screens," *Trends in Pharmacological Sciences*, vol. 28, no. 4, pp. 149–151, 2007.

[11] S. A. Stewart, D. M. Dykxhoorn, D. Palliser, et al., "Lentivirus-delivered stable gene silencing by RNAi in primary cells," *RNA*, vol. 9, no. 4, pp. 493–501, 2003.

[12] M. Li and J. J. Rossi, "Lentiviral vector delivery of siRNA and shRNA encoding genes into cultured and primary hematopoietic cells," *Methods in Molecular Biology*, vol. 433, pp. 287–299, 2008.

[13] O. ter Brake, K. 't Hooft, Y. P. Liu, M. Centlivre, K. J. von Eije, and B. Berkhout, "Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition," *Molecular Therapy*, vol. 16, no. 3, pp. 557–564, 2008.

[14] R. S. Pillai, S. N. Bhattacharyya, and W. Filipowicz, "Repression of protein synthesis by miRNAs: how many mechanisms?" *Trends in Cell Biology*, vol. 17, no. 3, pp. 118–126, 2007.

[15] S. Vasudevan, Y. Tong, and J. A. Steitz, "Switching from repression to activation: microRNAs can up-regulate translation," *Science*, vol. 318, no. 5858, pp. 1931–1934, 2007.

[16] D. H. Kim, P. Sætrom, O. Snøve Jr., and J. J. Rossi, "MicroRNA-directed transcriptional gene silencing in mammalian cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 42, pp. 16230–16235, 2008.

[17] F. Di Nunzio, B. Piovani, F.-L. Cosset, F. Mavilio, and A. Stornaiuolo, "Transduction of human hematopoietic stem cells by lentiviral vectors pseudotyped with the RD114-TR chimeric envelope glycoprotein," *Human Gene Therapy*, vol. 18, no. 9, pp. 811–820, 2007.

[18] L. H. Li, P. McCarthy, and S. W. Hui, "High-efficiency electrotransfection of human primary hematopoietic stem cells," *The FASEB Journal*, vol. 15, no. 3, pp. 586–588, 2001.

[19] J. M. Wiehe, P. Ponsaerts, M. T. Rojewski, et al., "mRNA-mediated gene delivery into human progenitor cells promotes highly efficient protein expression," *Journal of Cellular and Molecular Medicine*, vol. 11, no. 3, pp. 521–530, 2007.

[20] V. F. I. Van Tendeloo, H.-W. Snoeck, F. Lardon, et al., "Nonviral transfection of distinct types of human dendritic cells: high-efficiency gene transfer by electroporation into hematopoietic progenitor- but not monocyte-derived dendritic cells," *Gene Therapy*, vol. 5, no. 5, pp. 700–707, 1998.

[21] G. Ferlazzo, J. Klein, X. Paliard, W.-Z. Wei, and A. Galy, "Dendritic cells generated from $CD34^+$ progenitor cells with flt3 ligand, c-Kit ligand, GM-CSF, IL-4, and TNF-α are functional antigen-presenting cells resembling mature monocyte-derived dendritic cells," *Journal of Immunotherapy*, vol. 23, no. 1, pp. 48–58, 2000.

[22] R. Tiribuzi, S. Martino, E. Ciraci, et al., "Non-redundant roles of cathepsins L, B and S in $CD1a^+$ dendritic cells knocked-down for cathepsin S by RNA interference," *Minerva Biotecnologica*, vol. 20, no. 2, pp. 59–67, 2008.

[23] S. Della Bella, S. Nicola, I. Timofeeva, M. L. Villa, A. Santoro, and A. C. Berardi, "Are interleukin-16 and thrombopoietin new tools for the in vitro generation of dendritic cells?" *Blood*, vol. 104, no. 13, pp. 4020–4028, 2004.

[24] S. Martino, C. Emiliani, A. Orlacchio, R. Hosseini, and J. L. Stirling, "*β-N*-acetylhexosaminidases A and S have similar sub-cellular distributions in HL-60 cells," *Biochimica et Biophysica Acta*, vol. 1243, no. 3, pp. 489–495, 1995.

[25] K. Honey and A. Y. Rudensky, "Lysosomal cysteine proteases regulate antigen presentation," *Nature Reviews Immunology*, vol. 3, no. 6, pp. 472–482, 2003.

[26] L. Delamarre, M. Pack, H. Chang, I. Mellman, and E. S. Trombetta, "Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate," *Science*, vol. 307, no. 5715, pp. 1630–1634, 2005.

[27] D. A. Scudiero, R. H. Shoemaker, K. D. Paull, et al., "Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines," *Cancer Research*, vol. 48, no. 17, pp. 4827–4833, 1988.

[28] R. Kronenwett, U. Steidl, M. Kirsch, G. Sczakiel, and R. Haas, "Oligodeoxyribonucleotide uptake in primary human hematopoietic cells is enhanced by cationic lipids and depends on the hematopoietic cell subset," *Blood*, vol. 91, no. 3, pp. 852–862, 1998.

[29] D. Orlic, J. Kajstura, S. Chimenti, et al., "Bone marrow cells regenerate infarcted myocardium," *Nature*, vol. 410, no. 6829, pp. 701–705, 2001.

[30] C. E. Murry, M. H. Soonpaa, H. Reinecke, et al., "Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts," *Nature*, vol. 428, no. 6983, pp. 664–668, 2004.

[31] T. Montier, P. Delépine, K. Le Ny, et al., "KLN-5: a safe monocationic lipophosphoramide to transfect efficiently haematopoietic cell lines and human $CD34^+$ cells," *Biochimica et Biophysica Acta*, vol. 1665, no. 1-2, pp. 118–133, 2004.

[32] X. Gao and L. Huang, "Cationic liposome-mediated gene transfer," *Gene Therapy*, vol. 2, no. 10, pp. 710–722, 1995.

[33] A. W. Wong, S. J. Scales, and D. E. Reilly, "DNA internalized via caveolae requires microtubule-dependent, Rab7-independent transport to the late endocytic pathway for delivery to the nucleus," *The Journal of Biological Chemistry*, vol. 282, no. 31, pp. 22953–22963, 2007.

[34] J. Rejman, A. Bragonzi, and M. Conese, "Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes," *Molecular Therapy*, vol. 12, no. 3, pp. 468–474, 2005.

[35] P. Lampela, P. Soininen, A. Urtti, P. T. Männistö, and A. Raasmaja, "Synergism in gene delivery by small PEIs and three different nonviral vectors," *International Journal of Pharmaceutics*, vol. 270, no. 1-2, pp. 175–184, 2004.

[36] A. Bragonzi, G. Dina, A. Villa, et al., "Biodistribution and transgene expression with nonviral cationic vector/DNA complexes in the lungs," *Gene Therapy*, vol. 7, no. 20, pp. 1753–1760, 2000.

[37] H.-W. Liao and K.-W. Yau, "In vivo gene delivery in the retina using polyethylenimine," *BioTechniques*, vol. 42, no. 3, pp. 285–288, 2007.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 871313, 8 pages
doi:10.1155/2009/871313

Review Article

Searching for MIND: MicroRNAs in Neurodegenerative Diseases

Christian Barbato,[1,2] Francesca Ruberti,[2] and Carlo Cogoni[1,3]

[1] *European Brain Research Institute (EBRI), Fondazione EBRI- Rita Levi-Montalcini, Via del Fosso di Fiorano 64/65, 00143 Roma, Italy*
[2] *Istituto di Neurobiologia e Medicina Molecolare (INMM), CNR, Via del Fosso di Fiorano 64/65, 00143 Roma, Italy*
[3] *Dipartimento di Biotecnologie Cellulari ed Ematologia, Sezione di Genetica Molecolare, Università di Roma "La Sapienza", Viale Regina Elena 324, 00161 Roma, Italy*

Correspondence should be addressed to Christian Barbato, c.barbato@inmm.cnr.it

Received 9 January 2009; Accepted 12 June 2009

Recommended by Pawan Malhotra

In few years our understanding of microRNA (miRNA) biogenesis, molecular mechanisms by which miRNAs regulate gene expression, and the functional roles of miRNAs has been expanded. Interestingly, numerous miRNAs are expressed in a spatially and temporally controlled manner in the nervous system, suggesting that their posttrascriptional regulation may be particularly relevant in neural development and function. MiRNA studies in neurobiology showed their involvement in synaptic plasticity and brain diseases. In this review ,correlations between miRNA-mediated gene silencing and Alzheimer's, Parkinson's, and other neurodegenerative diseases will be discussed. Molecular and cellular neurobiological studies of the miRNAs in neurodegeneration represent the exploration of a new Frontier of miRNAs biology and the potential development of new diagnostic tests and genetic therapies for neurodegenerative diseases.

1. Introduction

Neurodegenerative diseases represent a large group of neurological disorders with heterogeneous clinical and pathological expressions, affecting specific groups of neurons, in specialized functional anatomic systems. A mixture of environmental and genetic factors seems to engender neurodegenerative diseases, and aging has been found a common risk factor. Neurodegenerative diseases result from the gradual and progressive loss of neuronal cells, leading to nervous system dysfunction. They are characterized by the formation of distinct pathological changes in the brain, including extracellular protein deposits, cellular inclusions, and remodelling of cell morphology. However, while many different forms of neurodegenerative disease are recognized, the lines that separate one from another are often unclear. For instance, symptoms such as motor impairment and memory loss may occur in many different types of neurodegenerative disease. Alzheimer's disease, Parkinson's disease, prion diseases, and polyglutamine disorders, including Huntington's disease and various spinocerebellar ataxias, are well-known neurodegenerative disorders [1]. To date, with few exceptions, no diagnostic laboratory tools exist that can clearly indicate the presence, absence, or category of a neurodegenerative disease. Diagnoses are usually based on clinical evaluation of the symptoms.

The microRNA-(miRNA-) guided RNA silencing pathway is a recently discovered process found to regulate gene expression acting on messenger RNA (mRNA). MiRNA biogenesis is mediated by Dicer which catalyzes the processing of double-stranded RNAs (dsRNAs) into ≈22 nt-long small miRNAs. These small noncoding RNA molecules operate as guides for RISC (RNA Induced Silencing Complex) to cleave a target mRNA in case of a perfect complementarity (siRNA) or to block the target mRNA translation (miRNA) when there is an imperfect pairing between miRNAs and the targets. In mammalian cells the repression of translation by miRNA is mediated by an imperfect pairing with 3′UTR of the mRNA target [2]. MiRNAs are conserved throughout the evolution, and their expression may be constitutive or spatially and temporally regulated. Increasing efforts to identify the specific targets of miRNAs lead to speculate

that miRNAs can regulate more than 90% of human genes. Specific miRNA subsets were expressed in specific brain area and in neuronal and glial cell subtypes [3]. The studies of microRNAs expression profiles in nervous system represent the first step in understanding how, where, and when miRNAs are involved in the regulation of neurodevelopment, differentiation, dendritic spine development, local protein synthesis, and synaptic plasticity [4]. Several works have shown spatially and/or temporally restricted distribution of miRNAs, suggesting that they might regulate neuronal gene expression. By comparative analysis of miRNA expression in the normal and pathologic brain, the microRNA signatures in several neurodegenerative diseases, including polyglutamine expansions, Parkinson's and Alzheimer's diseases are coming up. To date we have few pieces of information about the expression profiles, and the complex composition of the brain, containing several neuronal and glial istotypes, while representing its main biological characteristic, is also the principal obstacle for an accurate analysis. Furthermore in order to interpretate miRNA expression data from postmortem human brains affected by neurodegenerative diseases, the source of neural tissues, together with the RNA isolation techniques used, need to be carefully considered. Overall, the identification of miRNA's physiological target genes should be a primary approach to reveal the specific contribution of microRNAs to neural function. Recent studies of microRNA in nonneuronal cellular systems could drive future research in primary neuronal cells. In fact, prediction of microRNA targets utilizing different algorithms based on the general rule of the seed region has been complemented by an elegant proteomic approach which uses a mass spectrometric method called stable-isotope labelling with aminoacid in cell culture (SILAC) to measure changes in protein levels in response to miRNA induction or knockdown. SILAC approach showed that individual microRNA can reduce the production of hundred proteins [5, 6]. Many targets are repressed at both mRNA and protein level and others are predominantly regulated at protein level. Although several miRNA-induced changes in the proteome correlate with the presence of seeds in the mRNA of the affected proteins, some changes remain to be explained. They might be due both to indirect effects and/or to miRNA direct targeting mediated by still unknown rules. Remarkably an increasing level of complexity of species-specific miRNA expression during evolution emerged. However, 447 new miRNA genes expressed in human fetal and chimpanzee adult brains were identified. Many of them are not conserved beyond primates, indicating a recent evolutionary origin. Since 8% of miRNA were found to be human-specific, they might play a role in the human brain evolution. However, expression levels of miRNAs common to human and chimpanzee were not determined, because different regions were analyzed at various ages. Several features in cognitive functions of humans and chimpanzees are probably elucidated by variations in cortical structures. Therefore, the diversity of miRNA repertoire in the brain likely contributes to the dissimilarities between human and chimpanzee, arguing for a role of miRNA in brain evolution and function [7].

In this review we discuss the recent studies on the involvement of miRNA-mediated gene silencing in neurodegenerative diseases.

2. MicroRNAs in Neurodegenerative Diseases

2.1. MicroRNAs in Alzheimer's Disease. Alzheimer's Disease (AD) is the best known degenerative disease affecting the central nervous system [8]. AD is a chronic progressive disease characterized by early memory impairments followed by these cognitive deficits: aphasia (language disturbances), agnosia (failure to recognize people or objects in presence of intact sensory function), apraxia (inability to perform motor acts in presence of intact motor system). Neuropathologically the areas of brain most affected are the hippocampus followed by association cortices and subcortical structures. The neurodegeneration is characterised by synapse and cellular loss, β-amyloid plaques, and neurofibrillary lesions. The major component of plaques is the Aβ peptide which derives from the proteolytic processing of its precursor protein (APP). The neurofibrillary lesions contain aggregates of hyperphosphorylated microtubule-associated protein tau. This histopathological hallmark is used in Braak's Alzheimer's system [9] to describe postmortem AD brain samples in six stages: in the transentorhinal stage (Stages I and II), the neurofibrillary pathology is essentially confined to the transentorhinal and entorhinal cortex and slightly to the CA1/CA2 sections of the hippocampus; the limbic stage (Stages III and IV) frames a severe involvement of the entorhinal areas and a moderate engagement of the hippocampus; the hallmark of the neocortical stage (Stages V and VI) was the dismantlement of the neocortex. AD is the most common cause of dementia in aged populations. About 1% early onset familial form of the disease (onset before 60 to 65 years of age) is due to mutations in three genes, *APP*, *presenilin 1* (*PSEN1*), and *presenilin 2* (*PSEN2*) all of which cause Aβ overproduction. Aβ production is initiated by the processing of APP by the β-amyloid cleavage enzyme 1 (BACE1) which generated a C-terminal fragment of APP, labelled as C99. This fragment is further cleaved by γ-secretase complex, which includes the presenilins, and generates the more abundant Aβ40 and the less abundant, but more pathogenic, Aβ42 [8]. Aβ load in AD brain was suggested to trigger neuronal dysfunctions. For the majority of AD cases, which shows less obvious familial aggregation (hence they are also called sporadic AD), the molecular bases of the disease are matter of intensive research.

A discrete number of studies has suggested that a dysregulated microRNA expression could be aging-associated and could contribute to AD. miRNA expression profiles are changed in pathological conditions in all studies published until now. Lukiw's laboratory evaluated the expression of 12 miRNAs in hippocampal region of fetal, adult, and AD brain [10]. They found that miR-9 was upregulated in both fetal and AD hippocampus, and miR-128 was increased specifically in AD hippocampus. However, the translational changes induced by these specific miRNAs in AD hippocampus remain to be investigated. More recently

espression profiles of 328 microRNAs in anterior temporal cortex from five sporadic AD patients and five age-mathced controls showed 13 microRNAs significantly downregulated in cortex of sporadic AD [11]. Using various prediction algorithms to identify AD-related potential target genes, 7 of the 13 microRNAs had candidate binding sites in the 3′UTR of BACE (miR-15a, -29b-1, -9, and -19b) or APP (let-7, miR-101, miR-15a, and miR-106b,) or in the 3′UTR of PSEN1 (miR-9). On the other hand, 6 microRNAs do not seem to be related to obvious targets (miR-210, -181c, -22, -26b, -363, -93). In particular the relationship between BACE1 and miR-29 was deeply investigated (mentioned in what follows). The changes of miRNA profiles might be specific for sporadic AD and might cause or exacerbate the neuropathology. Furthermore, linking microRNA expression to their specific targets could suggest novel pathways of the disease.

A recent study evaluated the importance of deregulation of miRNA expression in brains and cerebrospinal fluid (CSF) of Alzheimer's patients [12]. Over 300 microRNAs were determined in the hippocampus, medial frontal gyrus, and cerebellum from early and late stage AD compared to age-matched control. Deregulated microRNAs have been associated to known and novel molecular pathways in AD pathogenesis such as neurogenesis, oxidative stress, insulin resistance, and innate immunity. For example, miR-9 and miR-132 downregulation was correlated to impaired neurogenesis and neuronal differentiation. The finding that miR-423 was upregulated in hippocampus while miR-98 was decreased in cerebellum was appealing. In fact both miRNAs modulate IDH2 (isocitrate dehydrogenase 2) expression and IDH2 reduction was described to be involved in oxidative stress in AD prefrontal cortex. These observations suggest a mechanism for the specific susceptibility of particular AD brain areas such as the hippocampus and the relative sparing of others such as the cerebellum.

Sixty miRNAs were differentially expressed in the CSF of patients between Braak stage 5 and stage 1. Among these miRNAs few were brain enriched while several were not correlated to the miRNAs changes observed in AD brain regions. Therefore, CSF microRNAs were suggested to derive from T lymphocytes present in the CSF. The altered expression of miRNA in the CSF of patients affected by Alzheimer's disease, opens a new scenario on the use of these expression profiles as putative AD biomarkers. To date, CSF analysis from AD patients produced some of the most reproducible biomarkers, such as decreased Aβ42, increased total tau (ttau), and increased phosphorylated tau (p-tau) [13]. Combinations of these CSF markers have been also proposed to diagnosticate AD. Future work might be to evaluate if the CSF miRNAs profile correlates with Aβ, total and phosphorylated tau protein presently carried out in the CSF of AD patients.

Specific molecular mechanisms involving microRNAs and expression of BACE1 and APP are emerging in the AD field. By microarray and in situ hybridization of superior-medial frontal cortex of AD, Nelson's laboratory showed that the expression of miR-107 decreased during progression of the disease in parallel to BACE1 mRNA increase. In addition cell culture experiments showed that the expression of a luciferase reporter gene fused to a 3′UTR containing BACE1 microRNA 107 binding site is modulated by miR-107 [14]. Another study, investigating changes in microRNA expression profiles of anterior temporal cortex from sporadic AD patients found that the expression of the cluster miR-29a/b-1 was significantly decreased in a subgroup of AD patients in which BACE 1 protein was abnormally upregulated while BACE 1 mRNA levels were unchanged [11]. Consistently, during mouse brain development from E17 to 1 year, BACE1 protein level decrease was correlated to miR-29a/b-1 upregulation while BACE1 mRNA level was stable. In cell culture experiments, BACE1 target validation was demonstrated by monitoring the effects of miR-29a/b-1 on the translation of a BACE1 3′UTR luciferase reporter carrying wilde-type or mutated miR29a/b-1 responsive site. Finally, upon either overexpression or downregulation of miR-29a/b-1 in human cell culture both BACE1 protein levels and APP cleavage product Aβ were, respectively, reduced and increased.

In both previous studies [11–14], the loss of microRNA in AD is not specific for a certain brain area more susceptible to the disease, that is, miR-107 is also downregulated in motor cortex of AD patients and miR-29a/b-1 expression also decreases in AD cerebellum. Therefore, altered BACE expression due to microRNA deregulation is not responsible for increased sensitivity of particular brain regions. However also in the AD familial cases, mutations of *APP* and *PSEN* are present in all cells of the brain and only specific regions are affected from AD.

Interestingly other two microRNAs, miR-298 and miR-328, regulate BACE 1 protein expression in cultured neuronal cells [15]. It is relevant that in APPswe/Psen1 transgenic mice, an AD mouse model which recapitulates some features of the disease, it was observed that BACE1 mRNA decreased and protein levels increased in the hippocampus at 19 months of age. In transgenic mice, the expression of miR-298 and miR-328 decreased in the granular neurons of the hippocampus during aging. However, while the miR-328 sequence is perfectly conserved between mouse and human, that of miR-298 is only 72% identical. Clearly, additional work will be needed to determine whether all of these microRNAs are really active in human brain and their relative contribution to BACE expression in physiological and pathological conditions and in different neuronal populations.

It has been shown that AD can be caused by increased expression of the APP gene due to either genomic duplication or regulatory sequence alterations. In C. elegans, *APP* orthologue *APL*-1 is regulated by developmentally timed microRNA [16]. In particular, apl-1 expression in seam cells is indirectly repressed by let-7 family microRNA, and apl-1 transcription is regulated by downstream targets of let-7 microRNA. This study opens new insights into the time-dependent progression of AD. The 3′UTR of APP mRNA is a potential target for several microRNAs. Recently, utilizing human HEK-293 cells, it has been demonstrated that miR106a and miR-520c negatively regulate expression of reporter genes containing their predicted target sequences present in the APP 3′UTR [17]. In addition, overexpression of miR-106a or miR-520c (which is not expressed in brain)

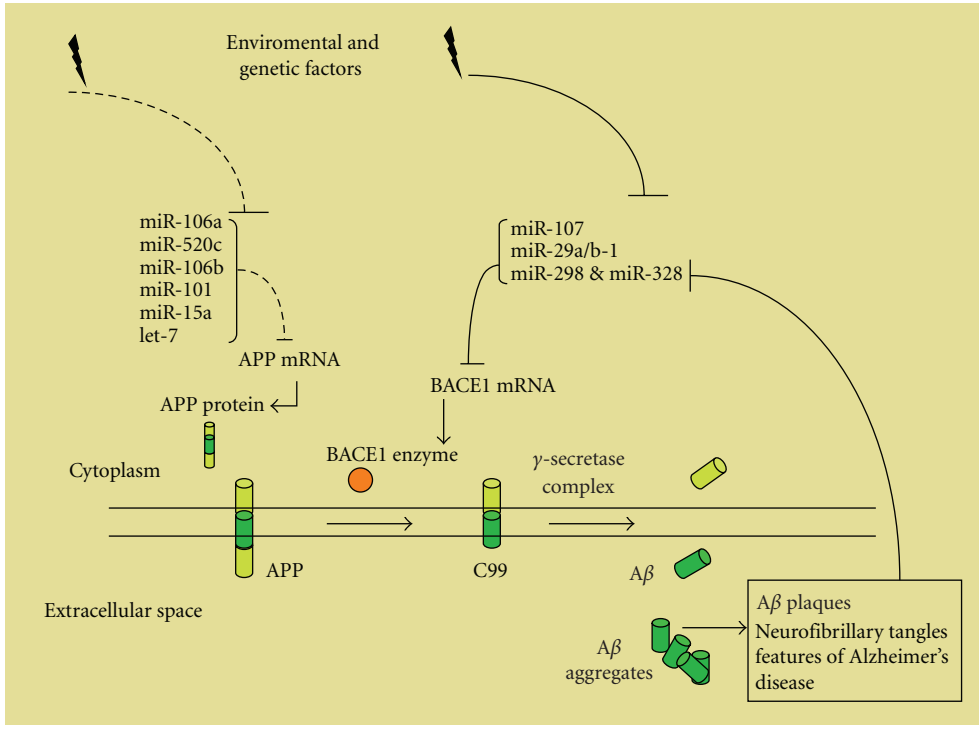


Figure 1: *APP or BACE1 upregulation might lead to Aβ overproduction in Alzheimer's Disease.* The picture shows molecular pathways modulating APP and BACE1 expression and amyloidogenic processing of APP by BACE1 and the γ-secretase complex leading to *Aβ* production. Reduction of miRNA/RISC posttranscriptional regulation of APP and/or BACE1 mRNA induces the increase of the relative proteins, which drive to *Aβ* accumulation. Changes of miRNA expression might trigger molecular events inducing AD pathology or generate a feed-forward mechanism during AD progression (as suggested for miR-298 and miR-328).

reduces APP levels by 50%. It will be important to translate these results in a cellular context relevant for AD pathology. Interestingly, miR-106b is one of the four microRNA that have been predicted to target the 3′UTR of APP (let-7, miR-101, miR-15a, and miR-106b) and that were found to be downregulated in anterior temporal cortex from five sporadic AD patients [11]. All these investigations suggest that dysregulation of miRNAs, by modulation of APP and BACE1 expression, might be a cause or a consequence of AD (Figure 1).

2.2. MicroRNAs in Parkinson's Disease. Parkinson's disease (PD) is associated with progressive neurodegeneration of dopaminergic neurons (DNs) in the substantia nigra and leads to tremor, rigidity, and bradykinesia. Futrhermore, widespread neuronal modifications lead to complex and variable non-motor symptoms. Lewy bodies are a neropathological feature of PD and are cellular inclusions comprising a dense core of filamentous material surrounded by a halo of fibrils, which mainly consists of a-synuclein. Mutations in genes coding for synuclein (SNCA), parkin, pink1, DJ-I, Lrrk2, can explain only a limited number of familial PD cases, while the molecular bases of vast numbers of non familial cases are not yet understood [18]. A recent study shed some light on the role of microRNA in DNs differentiation and raises the question whether microRNA are involved in etiology of PD. Deletion of Dicer impairs the ability of ES cells to differentiate into DNs. Since Dicer deletion was partially rescued by transfection of small RNA derived from embryonic mouse midbrain, it is likely that microRNAs are involved in DNs differentiation and survival [19]. In addition, specific deletion of Dicer in vivo, in mouse midbrain dopaminergic neurons, leads to cell death in the substantia nigra. Behavioural studies of the animals revealed reduced locomotion in an-open-field assay, reminiscent of the phenotype of human patients with PD. MiRNA expression profiles of normal adult midbrain compared with the profiles of midbrain depleted of DNs from PD patients revealed alterations of certain midbrain-enriched miRNA, in PD brain [19]. The role of miR-133b, enriched in midbrain and absent in the brains of PD patients, was further investigated. It was demonstrated that miR-133b constitutes a negative feedback loop with the transcription factor Ptx-3: Ptx-3 transcribes miR-133b which in turn represses Ptx-3 translation. In vitro experiments showed that depletion of miR-133b increases the expression of DN markers and depolarization-induced dopamine release while miR-133b overexpression suppresses the full differentiation of DN neurons and produces a significant decrease in dopamine release [19]. Thus, although miR-133b is involved in differentiation and function of DN neurons, additional microRNAs should be responsible for Dicer deletion phenotype in DN.

Polymorphisms affecting the interactions between microRNAs and their targets are emerging in various studies on neurodegenerative disease. Genetic analyses showed that relevant polymorphic variations in the fibroblast growth factor 20 gene (FGF20) are associated with the risk of developing PD. FGF20 is preferentially expressed in the substantia nigra and promotes survival of dopaminergic neurons. More recently, one SNP (rs 127202208), located within the FGF20 3′UTR, was strongly associated with PD. SNP rs 127202208 lies within a predicted binding site for microRNA 433 which is highly expressed in the brain [20]. Through several functional assays, it was demonstrated that the risk allele rs 127202208 damped a binding site for microRNA 433 and increased translation of FGF20. In cell culture experiments and in PD brains, the increased FGF20 translation was correlated with increased synuclein expression. Synuclein is included into the genes responsible for familial cases of PD and althought the function of this protein is not yet defined, it has been demonstrated that overexpression and point mutations can cause PD.

2.3. MicroRNAs in Polyglutamine Diseases. Polyglutamine (polyQ) disorders constitute a family of dominantly inherited neurodegenerative diseases caused by the expansion of CAG triplet repeats in a specific gene. A common signature is the accumulation of the mutant protein in large intranuclear inclusions. The clinical features include spasticity and cognitive impairments. To date, ten such neurodegenerative disorders known to be caused by expansion of the CAG repeat in the coding region of the respective genes have been identified [21]. These prototypical protein misfolding disorders include Huntington disease (HD), six distinct forms of spinocerebellar ataxia (SCA-1, 2, 3, 6, 7 and 17), dentatorubropallidoluysian atrophy (DRPLA), and spinobulbar muscular atrophy (SBMA).

2.3.1. Huntington Disease. Huntington's disease (HD) is the most common and well-studied polyglutamine neurodegenerative disorder [22]. It is an hereditary autosomal dominant disease characterized by motor, cognitive, and psychiatric symptoms. It affects about 3 in 100 000 individuals. The onset of symptoms typically occurs between the ages 35 and 50 years, though it may appear at any age. The molecular basis of the disease is the expansion of the trinucleotide CAG in the first exon of a gene on chromosome four (4p 16.3). This gene encodes the protein huntingtin (Htt) of 3136 amino acids. The mutation of huntingtin produces an expanded stretch of glutamine (Gln) residues. This CAG/polyGln expansion has 6–39 units in normal individuals and 36 to 180 units in HD patients. Huntingtin appears to be associated to protein trafficking, transcriptional regulation, synaptic signalling, vesicle transport, and apoptosis. HD patients show progressive loss of cortical and striatal neurons associated with choreic movement and dementia. The neuropathological hallmark is the gradual atrophy of the striatum (caudate nucleus and putamen), observed in 95% of the HD brains. Mechanisms of neurodegeneration implicated in HD pathology are excitotoxicity, dopamine toxicity, mithocondrial dysfunction, oxidative stress, apoptosis, and autophagy.

Several observations suggested microRNAs dysregulation in HD. Interaction between wild type Htt and Repressor Element1 Silencing Transcription (REST) factor was described. In pathological conditions Htt mutation inhibits its interaction with REST and provokes REST build-up in the nucleus of HD neurons, decreasing neuronal gene expression. REST is a transcriptional repressor of neural genes, including several microRNAs [23].

Recently, Johnson et al. [24] identified miRNA regulated by REST in neurons, and measured the expression of these miRNAs in the brains of HD mouse models and in postmortem tissue of HD patients. Several changes in microRNAs expression profile were allocated to species-specific differences, and others to the comparative analysis of a specific human cortex, area versus whole mouse cortex. Both in HD mouse model and in human HD cortex, miR-132 was downregulated, and its mRNA target p250 GAP, which modulates dendritic plasticity, was increased. Since REST is highly involved in HD, microRNAs are likely expected to play an important role in the disease pathogenesis. In addition, it was demonstrated that huntingtin protein co-purified with Argonaute proteins, fundamental components of RISC complex. Argonaute proteins have been shown to localize to cytoplasmic foci, named P bodies. Htt, colocalized with Argonaute2 in P bodies, and depletion of Htt showed compromised RNA-mediated gene silencing. Thereafter, in mouse striatal neurons expressing Htt mutation, P bodies formation and translation miRNA-mediated repression were impaired. These data suggest that Htt play a role in miRNA processes [25].

2.3.2. Spinocerebellar Ataxia Type 3. The polyglutamine (polyQ) protein Ataxin-3 is mutated in the human polyglutamine disease spinocerebellar ataxia type 3 (SCA3), resulting in a progressive dysfunction of the cerebellum. SCA3 is typically a late-onset fatal autsosomal dominant neurodegenerative disease that, like all ataxias, is characterized by loss of motor coordination and balance. In SCA-3 Drosophila model, the suppression of miRNA processing by dicer mutation increases ataxin-3 toxicity, inducing a neurodegenerative phenotype. Moreover, depletion of R3D1, a dsRNA-binding protein, that forms a stable complex with Dicer-1, causes accumulation of precursor miRNA, increasing ataxin-3-induced toxicity [26, 27]. In HeLa cell line, dicer reduction by RNAi enhances polyQ protein toxicity only in cells expressing pathogenic Ataxin-3, causing loss of 70% of the cultured cells. These findings suggest a neuroprotective role of miRNAs in these neurodegenerative diseases [27].

2.3.3. Spinocerebellar Ataxia Type 1. Spinocerebellar ataxia type 1 (SCA1) is a dominant inherited disease caused by expanded trinucleotide repeats resulting in an increased

polyglutamine tract in the gene product ataxin-1 (ATXN-1). SCA1 patients loose motor coordination and develop slurred speech, spasticity, and cognitive impairments. A typical feature of SCA1 pathology is the atrophy and loss of Purkinje cells from the cerebellar cortex. Purkinje cells are the major integrative neurons of the cerebellar cortex, projecting their axons onto the deep cerebellar nuclei. A recent study showed that a conditional Purkinje (PK) cell-specific ablation of Dicer leads to PK cell death, cerebellar dysfunction, and ataxia indicating an involvement of miRNAs in cerebellar neurodegeneration [28].

Another line of evidence suggesting a role of miRNAs in SCA1 pathogenesis comes from the observation that miR-19, miR-101, and miR-130 cooperatively regulate ATXN1 levels [29]. When miR-19, miR-101 and miR-130 were inhibited by 2′-O-methyl oligonucleotides, an increase of ATXN1 protein level was observed. Moreover, it was demonstrated that miR-19, miR-101, and miR-130 were expressed in mouse cerebellum and Purkinje cells by Northern blot analysis and in situ hybridization. These miRNAs regulate the cell toxicity of the polyQ-expanded ATXN1, suggesting to investigate miRNAs-mediated regulation in SCA1 neurodegenerative disorder.

2.4. MicroRNAs in Frontotemporal Dementia. Frontotemporal dementia (FTD) is a neurodegenerative disease representing ∼5% of all dementia patients, characterized by the progressive degeneration of the frontal and anterior temporal cortex. Considering the involvement of the frontal lobe, the clinical picture is cognitive and memory impairment, language dysfunction, and/or changes in personality or behavioural disorders. FTD can be divided into two main neuropathological subtypes: frontotemporal lobar degeneration (FTLD) with neuronal and glial tau inclusions (FTLD-tau), and FTLD with neuronal cytoplasmic inclusions (NCIs) that are positive for ubiquitin and TAR DNA-binding protein (TDP-43) (FTLD-U). However, 20%–30% of cases of FTD follow an autosomal dominant pattern of inheritance, and half of them are caused by defects in microtubule-associated protein tau (MAPT), multi-vesicular body protein 2B (CHMP2B), and valosin-containing protein (VCP) [30].

Mutations in the *progranulin* gene (*GRN*), encoding a secreted growth factor, on chromosome 17q21, have recently been identified as a major cause of familial FTLD-U. These cases have a characteristic pattern of neuropathology that is a distinct subtype of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U), with NCIs in layer II of the cortex and lentiform neuronal intranuclear inclusions (NIIs). To date, more than 60 different mutations in *GRN* were mapped, and a recent breakthrough was the identification of a genetic variant (rs5848), located in the 3′UTR of GRN mRNA, in a binding site for miR-659 [32]. This research showed that miR-659 targets GRN suppressing its translation, and demonstrated a decrease of GRN protein levels of ∼30% in FTLD-U rs5848 homozygous TT carriers compared to CC carriers. Consistently, in FTLD-U patients heterozygous for rs5848, an intermediate dosage of GRN protein was determined. miR-659 seems to be a specie-specific human microRNA, which expressed in brain, including frontal and temporal neocortex. In addition "seed" sequence for miR-659 in the GRN 3′UTR is only present in humans, and is not found in other mammals. Although a small number of FTLD-U patients were examined, the enhanced binding of miR-659 to the 3′UTR of the GRN gene is an important risk for TDP-43-positive FTLD-U. Future studies on specific human cortical miRNAs might be relevant to decrypt human neurodegenerative disease.

2.5. MicroRNAs in Prion Disease. Prion diseases or transmissible spongiform encephalopathies (TSEs) are a family of rare progressive neurodegenerative disorders that affect both humans and animals. They are distinguished by long incubation periods, characteristic spongiform changes associated with neuronal loss, and a failure to induce inflammatory response. The causative agent of TSEs is believed to be a prion, a transmissible agent, which is able to induce abnormal folding of normal cellular prion proteins in the brain [33]. According to the protein-only hypothesis, the central event in the pathogenesis of prion diseases is the conversion of a normal cellular protein termed PrP(C) to PrP(Sc), a conformational isoform. Prion diseases impair brain function, causing memory impairment, personality changes, dementia, and movement disorders and the characteristic signs and symptoms of the disease. TSEs begin in adulthood are rapidly progressive and lead to death within a few months to several years. Familial prion diseases of humans include classic Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal insomnia (FI). To explain TSE pathogenesis, it is important to identify disease-associated alterations in gene expression. Recently, by microarrays and RT-PCR, the analysis of miRNA expression was made [31]. Brain miRNAs expression of mice infected with mouse-adapted scrapie showed changes of 15 miRNAs. Among these, only two, miR-338-3p and miR337-3p, were downregulated, whereas the others were up-regulated. Several predictions of the theoretical mRNA targets of changed miRNAs during prion disease were performed, using web-based computational algorithms. From this in silico analysis, genes involved both in transcription, cell cycle, ubiquitin-proteasome pathway, and in normal functioning of synapses, neuronal activity, neurogenesis, and neurites growth were identified. Lastly, only one target, the transcriptional regulator EGR1, was experimentally validated by luciferase assay in vitro. In particular, the authors suggested that the prion disease upregulates miR-191 which represses the EGR1 mRNA translation. The transcriptional regulators EGR1 and CREB1 were already identified as downregulated prion-related genes with a central role in biologically relevant networks in prion infection [34]. As a consequence, miRNAs mediated regulation of these prion-related genes could contribute to neuronal death and neurodegeneration. Finally, the miRNA expression profile was proposed as potential biomarker of prion diseases.

Table 1: MIND: MicroRNAs in neurodegenerative diseases.

miRNA	Neurodegenerative disease	mRNA target	Reference
miR-298; miR328 ↓	Alzheimer's Disease mouse model	BACE1	[15]
miR-107 ↓	Alzheimer's Disease	BACE1	[14]
miR-29a/b-1 ↓	Alzheimer's Disease	BACE1	[11]
miR-133b ↓	Parkinson's disease	Pitx3	[19]
miR-433	Parkinson's disease	FGF20 (SNP rs127202208) ↑	[20]
miR-191 ↑	Prion disease	EGR1	[31]
miR-132 ↓	Huntington disease	P250GAP	[24]
miR-659	Frontotemporal dementia	GRN (SNP s5848) ↓	[32]

3. Concluding Remarks

The studies on miRNA in neurodegenerative diseases (Table 1) are only now coming to light. Until now, both changes of several miRNA expression profiles and polymorphisms affecting the interactions between miRNAs and their targets are emerging in various studies on neurodegenerative disease. It is difficult to determine if the changes in miRNA expression detected in the brains or CSF of patients are primary or secondary events, or both. Nevertheless early or late in the evolution of the disease, they could contribute to the pathogenesis of the observed lesions and neuronal loss. Unique patterns of miRNA expression profile in the CSF of particular neurodegenerative disease could be useful as molecular biomarkers for disease diagnosis and eventually prediction of therapeutic responses. The identification of miRNA causing a specific pathology could open new therapeutic perspectives to block endogenous miRNAs or deliver exogenous miRNAs. Until now either antisense oligonucleotides chemically modified [35] or expressed sequences corresponding to multiple miRNA seed target (miRNA sponge) [36] have been used as microRNA inhibitors. Delivery of these molecules to the CNS, avoiding toxicities, could be the challenge of future research. Furthermore since in several neurodegenerative disorders specific nuclear or cytoplasmic protein accumulation is causative of the neuropathological picture, the identification of microRNAs regulating the translation of these targets could represent the first step aimed to therapeutic applications. The second step might be to evaluate the quantitative effects on the proteome of specific amounts of the "therapeutic" microRNAs.

Acknowledgments

This work was supported by Italian Institute of Technology (IIT), REGIONE LAZIO grant—'Studio delle basi molecolari della neurodegenerazione nella malattia di Alzheimer's—(to C. Cogoni), National Research Council (CNR) grant DG.RSTL.059.012 (to F. Ruberti), and by "Fondazione Alazio Award 2007" (to C. Barbato) (http://www.fondazionealazio.org/). The first and second author contributed equally to the preparation of this manuscript.

References

[1] D. M. Skovronsky, V. M.-Y. Lee, and J. Q. Trojanowski, "Neurodegenerative diseases: new concepts of pathogenesis and their therapeutic implications," *Annual Review of Pathology*, vol. 1, pp. 151–170, 2006.

[2] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[3] K. S. Kosik, "The neuronal microRNA system," *Nature Reviews Neuroscience*, vol. 7, no. 12, pp. 911–920, 2006.

[4] C. Barbato, C. Giorgi, C. Catalanotto, and C. Cogoni, "Thinking about RNA? MicroRNAs in the brain," *Mammalian Genome*, vol. 19, no. 7-8, pp. 541–551, 2008.

[5] M. Selbach, B. Schwanhäusser, N. Thierfelder, Z. Fang, R. Khanin, and N. Rajewsky, "Widespread changes in protein synthesis induced by microRNAs," *Nature*, vol. 455, no. 7209, pp. 58–63, 2008.

[6] D. Baek, J. Villén, C. Shin, F. D. Camargo, S. P. Gygi, and D. P. Bartel, "The impact of microRNAs on protein output," *Nature*, vol. 455, no. 7209, pp. 64–71, 2008.

[7] E. Berezikov, F. Thuemmler, L. W. van Laake, et al., "Diversity of microRNAs in human and chimpanzee brain," *Nature Genetics*, vol. 38, no. 12, pp. 1375–1377, 2006.

[8] C. Haass and D. J. Selkoe, "Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid β-peptide," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 2, pp. 101–112, 2007.

[9] H. Braak and E. Braak, "Neuropathological staging of Alzheimer-related changes," *Acta Neuropathologica*, no. 82, pp. 239–259, 1991.

[10] W. J. Lukiw, "Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus," *NeuroReport*, vol. 18, no. 3, pp. 297–300, 2007.

[11] S. S. Hébert, K. Horré, L. Nicolaï, et al., "Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/β-secretase expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 17, pp. 6415–6420, 2008.

[12] J. P. Cogswell, J. Ward, I. A. Taylor, et al., "Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways," *Journal of Alzheimer's Disease*, vol. 14, no. 1, pp. 27–41, 2008.

[13] P. Formichi, C. Battisti, E. Radi, and A. Federico, "Cerebrospinal fluid tau, Aβ, and phosphorylated tau protein for the diagnosis of Alzheimer's disease," *Journal of Cellular Physiology*, vol. 208, no. 1, pp. 39–46, 2006.

[14] W.-X. Wang, B. W. Rajeev, A. J. Stromberg, et al., "The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of β-site amyloid precursor protein-cleaving enzyme 1," *Journal of Neuroscience*, vol. 28, no. 5, pp. 1213–1223, 2008.

[15] V. Boissonneault, I. Plante, S. Rivest, and P. Provost, "MicroRNA-298 and microRNA-328 regulate expression of mouse β-amyloid precursor protein-converting enzyme 1," *Journal of Biological Chemistry*, vol. 284, no. 4, pp. 1971–1981, 2009.

[16] R. Niwa, F. Zhou, C. Li, and F. J. Slack, "The expression of the Alzheimer's amyloid precursor protein-like gene is regulated by developmental timing microRNAs and their targets in *Caenorhabditis elegans*," *Developmental Biology*, vol. 315, no. 2, pp. 418–425, 2008.

[17] N. Patel, D. Hoang, N. Miller, et al., "MicroRNAs can regulate human APP levels," *Molecular Neurodegeneration*, vol. 3, no. 1, p. 10, 2008.

[18] W. Mandemakers, V. A. Morais, and B. De Strooper, "A cell biological perspective on mitochondrial dysfunction in Parkinson disease and other neurodegenerative diseases," *Journal of Cell Science*, vol. 120, no. 10, pp. 1707–1716, 2007.

[19] J. Kim, K. Inoue, J. Ishii, et al., "A microRNA feedback circuit in midbrain dopamine neurons," *Science*, vol. 317, no. 5842, pp. 1220–1224, 2007.

[20] G. Wang, J. M. van der Walt, G. Mayhew, et al., "Variation in the miRNA-433 binding site of FGF20 confers risk for Parkinson disease by overexpression of α-Synuclein," *American Journal of Human Genetics*, vol. 82, no. 2, pp. 283–289, 2008.

[21] P. Siwach and S. Ganesh, "Tandem repeats in human disorders: mechanisms and evolution," *Frontiers in Bioscience*, vol. 13, no. 12, pp. 4467–4484, 2008.

[22] J. M. Gil and A. C. Rego, "Mechanisms of neurodegeneration in Huntington's disease," *European Journal of Neuroscience*, vol. 27, no. 11, pp. 2803–2820, 2008.

[23] C. Conaco, S. Otto, J.-J. Han, and G. Mandel, "Reciprocal actions of REST and a microRNA promote neuronal identity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 7, pp. 2422–2427, 2006.

[24] R. Johnson, C. Zuccato, N. D. Belyaev, D. J. Guest, E. Cattaneo, and N. J. Buckley, "A microRNA-based gene dysregulation pathway in Huntington's disease," *Neurobiology of Disease*, vol. 29, no. 3, pp. 438–445, 2008.

[25] J. N. Savas, A. Makusky, S. Ottosen, et al., "Huntington's disease protein contributes to RNA-mediated gene silencing through association with Argonaute and P bodies," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 31, pp. 10820–10825, 2008.

[26] F. Jiang, X. Ye, X. Liu, L. Fincher, D. McKearin, and Q. Liu, "Dicer-1 and R3D1-L catalyze microRNA maturation in Drosophila," *Genes and Development*, vol. 19, no. 14, pp. 1674–1679, 2005.

[27] J. Bilen, N. Liu, B. G. Burnett, R. N. Pittman, and N. M. Bonini, "MicroRNA pathways modulate polyglutamine-induced neurodegeneration," *Molecular Cell*, vol. 24, no. 1, pp. 157–163, 2006.

[28] A. Schaefer, D. O'Carroll, C. L. Tan, et al., "Cerebellar neurodegeneration in the absence of microRNAs," *Journal of Experimental Medicine*, vol. 204, no. 7, pp. 1553–1558, 2007.

[29] Y. Lee, R. C. Samaco, J. R. Gatchel, C. Thaller, H. T. Orr, and H. Y. Zoghbi, "miR-19, miR-101 and miR-130 co-regulate ATXN1 levels to potentially modulate SCA1 pathogenesis," *Nature Neuroscience*, vol. 11, no. 10, pp. 1137–1139, 2008.

[30] N. J. Cairns, E. H. Bigio, I. R. A. Mackenzie, et al., "Neuropathologic diagnostic and nosologic criteria for frontotemporal lobar degeneration: consensus of the Consortium for Frontotemporal Lobar Degeneration," *Acta Neuropathologica*, vol. 114, no. 1, pp. 5–22, 2007.

[31] R. Saba, C. D. Goodman, R. L. C. H. Huzarewich, C. Robertson, and S. A. Booth, "A miRNA signature of prion induced neurodegeneration," *PLoS ONE*, vol. 3, no. 11, p. e3652, 2008.

[32] R. Rademakers, J. L. Eriksen, M. Baker, et al., "Common variation in the miR-659 binding-site of GRN is a major risk factor for TDP43-positive frontotemporal dementia," *Human Molecular Genetics*, vol. 17, no. 23, pp. 3631–3642, 2008.

[33] A. Aguzzi, F. Baumann, and J. Bremer, "The prion's elusive reason for being," *Annual Review of Neuroscience*, vol. 31, pp. 439–477, 2008.

[34] G. Sorensen, S. Medina, D. Parchaliuk, C. Phillipson, C. Robertson, and S. A. Booth, "Comprehensive transcriptional profiling of prion infection in mouse models reveals networks of responsive genes," *BMC Genomics*, vol. 9, article 114, 2008.

[35] G. Meister, M. Landthaler, Y. Dorsett, and T. Tuschl, "Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing," *RNA*, vol. 10, no. 3, pp. 544–550, 2004.

[36] M. S. Ebert, J. R. Neilson, and P. A. Sharp, "MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells," *Nature Methods*, vol. 4, no. 9, pp. 721–726, 2007.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 654346, 13 pages
doi:10.1155/2009/654346

Review Article

MicroRNA Implications across Neurodevelopment and Neuropathology

Sabata Martino,[1] Ilaria di Girolamo,[1] Antonio Orlacchio,[2,3] Alessandro Datti,[1] and Aldo Orlacchio[1]

[1] *Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Sezione di Biochimica e Biologia Molecolare, Università di Perugia, Via del Giochetto, 06126 Perugia, Italy*
[2] *Laboratorio di Neurogenetica, CERC-IRCCS Santa Lucia, Via del Fosso di Fiorano 64, 00143 Rome, Italy*
[3] *Dipartimento di Neuroscienze, Università di Roma "Tor Vergata", Viale Oxford 81, 00133 Rome, Italy*

Correspondence should be addressed to Aldo Orlacchio, orly@unipg.it

Received 2 February 2009; Revised 11 May 2009; Accepted 20 July 2009

Recommended by Ravi Sachidanandam

MicroRNAs (miRNAs) have rapidly emerged as biologically important mediators of posttranscriptional and epigenetic regulation in both plants and animals. miRNAs function through a variety of mechanisms including mRNA degradation and translational repression; additionally, miRNAs may guide gene expression by serving as transcription factors. miRNAs are highly expressed in human brain. Tissue and cell type-specific enrichments of certain miRNAs within the nervous system argue for a biological significance during neurodevelopmental stages. On the other hand, a large number of studies have reported links between alterations of miRNA homeostasis and pathologic conditions such as cancer, heart diseases, and neurodegeneration. Thus, profiles of distinct or aberrant miRNA signatures have most recently surged as one of the most fascinating interests in current biology. Here, the most recent insights into the involvement of miRNAs in the biology of the nervous system and the occurrence of neuropathological disorders are reviewed and discussed.

1. Introduction

microRNAs (miRNAs) are small, noncoding oligoribonucleotides of ~21-22 nt which regulate gene expression through the assembly of an RNA-induced silencing complex (RISC). In particular, the downstream effects of miRNAs relate to the fate of target mRNA, which may be subjected to endonucleolytic cleavage, enrolled into a faulty translational process or, as surprisingly shown in most recent studies, translationally enhanced [1–12]. Each of the hundreds of miRNAs present in mammalian genomes can potentially modulate an impressively large number of target genes, thereby depicting a highly versatile network with the capacity to effectively control and modify the biochemical wiring and, in turn, the phenotypic outcome of a cell [1, 8].

It is now well established that miRNAs are involved in disparate physiological functions, such as developmental transitions and neuronal patterning, apoptosis, fat metabolism, and regulation of hematopoietic lineage differentiation. For example, miRNAs are key regulators of the nervous system in the worm and brain morphogenesis in the fish and show distinct expression patterns during mammalian brain development [13].

A clear understanding of the functional impact of miRNAs on brain neurodegeneration is an intriguing, yet rather elusive, matter of study. However, the current literature shows clear evidence that tightly controlled miRNA expression is required for proper neurodevelopment and, conversely, that specific miRNA dysregulation is likely linked to the pathogenesis of neurodisorders.

2. miRNA Biogenesis

Biogenesis and silencing mechanisms of miRNAs were recently revisited by Carthew and Sontheimer, who have highlighted common themes and unique features of both

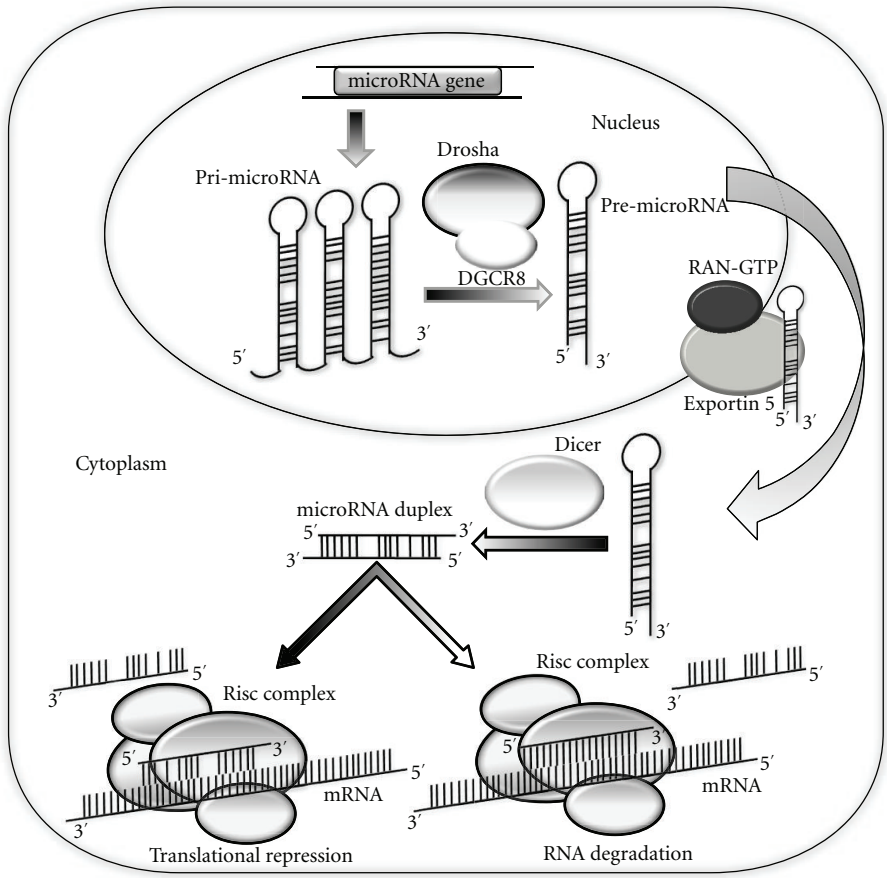


Figure 1: The biogenesis of miRNAs. Shown are the key steps of miRNA biogenesis in mammalian cells (reviewed in [14, 15]).

miRNA- and siRNA-related pathways (see Figure 1 and [14]). In either context, the molecular events that span from miRNA transcription towards RNA degradation are complex and imply an intricate interplay of molecular events to ensure accurate and efficient regulation of gene expression [16].

In mammals, 80% of miRNA genes are located within introns of longer primary transcripts that can be either protein-coding or mRNA-like transcripts; the majority of these are produced by RNA polymerase II [17–20], while a minor group of genes, characterized by Alu sequences, is instead transcribed by Pol III [21]. Thus, Pol II-associated transcription factors may regulate the expression of the majority of miRNA genes in a tissue- and/or cell-specific fashion [22].

While transcription of intergenic miRNA genes implies usage of own promoters, intronic miRNAs are transcribed with their host genes and seem to be cotranscriptionally processed prior to the removal of the host intron [18]. Typically, primary miRNA transcripts or pri-miRNAs are composed of a double-stranded stem of 33 base pairs, a terminal loop, and two flanking, single-stranded segments which are subject to cleavage, in the nucleus, by a protein complex called Microprocessor. This is composed of a nuclear member of the RNA III family (Drosha) associated with a cofactor (DGCR8) for efficient and precise processing of pri-miRNAs into 60–70 nt, hairpin-like precursor miRNAs (pre-miRNAs) [23–27].

Interestingly, several pre-miRNAs, known as mirtrons, originate directly from the splicing of pri-miRNAs and are subsequently processed without a requirement for Microprocessor activity. Evidence suggests that this alternative pathway, although rather uncommon, has emerged throughout metazoans prior to the advent of Drosha [28–30].

Through the exportin-5 pathway, pre-miRNAs are then transferred to the cytoplasm where they are further processed by Dicer, a second RNase III complexed with the human immunodeficiency virus transactivating response RNA-binding protein, TRBP [31, 32]. Dicer binds the 3′ overhang of the dsRNA and then excises the terminal loop to produce a mature, single-stranded miRNA duplex of approximately 22 bp. This duplex is ephemeral, in that it is rapidly unwound as soon as it associates with an Argonaute protein (Ago). Only one strand of the original dsRNA molecules is incorporated into RISC while the ejected strand, unlike during the siRNA unwinding mechanism, is not degraded by the associated Ago [7, 14–32]. Finally, miRNAs trigger gene silencing through partial base-pairing with the 3′-UTRs of protein-coding mRNAs, thereby preventing translation of targeted mRNAs and/or accelerating their degradation [15, 33].

Table 1: miRNAs involved in neurodevelopment.

miRNA	Species	Target	Function	Reference number
miR-iab-4-5p	*Drosophila*	UBX	Regulation of Hox gene involved in the development of halters	[34]
miR10a	Human	HOXA1	Downregulation of HOXA1 geneExpression	[35]
lsy-6	C. elegans	Cog-1	Required to specify ASEL sensory neuron identity	[36, 37]
miR-273	C. elegans	Die-1	Expressed in ASER; suppresses ASEL identity	[36, 38]
miR-196	Rodents	HOXB8	Downregulation of HOXC8, HOXD8 and HOXA7	[39]
miR-124	Rodents	SCP1	Neural induction in the spinal cord of developing embryos	[40]
miR-124	Rodents	SOX9	Regulation of the neurogenesis in the SVZ stem cell niche and neurite outgrowth in neuronal differentiation	[41]
miR-132	Rodents	P250GAP	Regulation of the neuronal morphogenesis and circadian clock	[42, 43]
miR-219	Rodents	SCOP	Regulation of the circadian period length	[36, 43]
miR-133b	Rodents	Pitx3	Regulation of the maturation of midbrain dopaminergic neurons	[44]
miR-134	Rodents	LimK1	Modulation of the size of dendritic spines	[45]

The existence of stringent regulatory mechanisms affecting the biogenesis of miRNAs suggests that this pathway plays a crucial role in the control of gene expression and, further downstream, the definition of biological outcomes. In this regard, several examples of double-negative feedback loops have been described, showing that the expression of miRNA genes can be controlled by their own targets [14].

3. miRNAs Play Major Physiological Roles in Neural Development

In *Drosophila*, multiple miRNAs interact with different 3′ UTR binding sites to play a cooperative role in the post-transcriptional regulation of nerfin-1, a nuclear regulator of axon guidance, within both the developing central nervous system (CNS) and peripheral nervous system. In species of this organism, the high degree of evolutionary conservation of miRNA-binding sites provides evidence that regulation of the onset and extinction dynamics of nerfin-1 expression is common to all members of the *Drosophila* genus [46].

As shown in Table 1, the effects of miRNA-mediated modulation of gene expression during multiple steps of neuronal development, from early neurogenesis to synaptogenesis, have been well documented across the animal kingdom [34–45, 47–61].

Strong evidence for a biological role of miRNAs in neural development emerged from their identification within the HOX gene clusters [49]. When ectopically expressed, these miRNAs induce a homeotic mutant phenotype, as shown in *Drosophila* for miR-iab-4-5p, which reduces endogenous Ubx protein levels, causing halteres to be transformed into wings [34]. In addition, miR-10a targets HOXA1 [35] while miR-196, encoded at three paralogous locations in the A, B, and C mammalian HOX clusters, directs the cleavage of HOXB8 mRNA and, apparently, downregulates HOXC8, HOXD8, and HOXA7 [39].

The capacity of miRNAs to directly control cell fate decisions and, in turn, specify neuron identity was also shown in *Caenorhabditis elegans* [37, 38]. In developing axons, miRNAs may regulate pathfinding, the process by which the circuitry of the nervous system is built. In zebrafish, normal brain morphogenesis is disrupted in the absence of the miRNA-processing enzyme Dicer [50], while the observation that functional RISC complexes can be assembled in rat DRG axons and growth cones is indicative of important roles played by miRNAs in the regulation of axonal mRNA translation [51].

Indeed, the CNS displays a substantial enrichment of miRNA species, of which a considerable number is expressed in a temporally- and/or spatially-controlled fashion, thereby suggesting biological implications for specific developmental stages [52, 53]. Expression profiling revealed that several species of miRNAs, such as miR-9, miR-124, miR-124a, miR-125b, miR-127, miR-128, miR-132, miR-219, and members of the let-7 family, are especially localized in the mouse brain [40–45, 54–61], while the expression of 63 additional miRNAs appears to be widely distributed, although differentially, throughout the CNS [62]. Of these, some are primarily present in the cerebellum (miR-195, miR-497, and miR-30b), others in the medulla oblongata (miR-34a, miR-451, miR-219, miR-338, miR-10a, and miR-10b), while a third group (miR-7, miR-7b miR-218, miR-221, miR-222, miR-26a, miR-128a/b, miR-138, and let-7c) appears to be restricted to the hypothalamus [63–66]. In general, region-specific enrichments reflect expression rates threefold higher compared to average miRNA levels displayed throughout the CNS [67–70].

Conceivably, miRNAs affect patterning mechanisms that specify the fate of neural cells at specific times and within proper locations. For example, an investigation of the expression of 104 miRNAs during murine brain development showed that these were distributed according to specific temporal expression patterns; in particular, the expression of 12 miRNAs was significantly upregulated during embryonic stages while markedly decreased during brain development. The involvement of modulated miRNAs was recapitulated by computational screens aimed at target identification, which revealed that 10 of 12 miRNAs are likely associated with neurogenesis [70].

In some instances, the physiological role of a number of miRNAs in neurodevelopment is well-documented. For example, miR-124 controls neurite outgrowth in differentiating mouse P19 cells [71] and stimulates neuronal differentiation in the developing chick spinal cord by counteracting the antineural activity of one of its targets, namely, the small C-terminal domain phosphatase 1 (SCP1) [40]. Furthermore, functional studies in vivo have recently demonstrated that miR-124 controls adult neurogenesis in the mouse subventricular zone via a time-regulated control of neuroblast generation from transit-amplifying precursors. In particular, neuronal differentiation is promoted through downregulation of the transcription factor Sox9, shown to be one of the targets of miR-124 [41, 72]. A second example relates to miR-132, which oversees dendritic morphogenesis by inhibiting translation of the synaptic protein p250GAP, suggesting a key role of miR132 p250GAP pathway in synapse growth and plasticty [42, 43, 73]. Additionally, miR-133b regulates maturation and function of midbrain dopaminergic neurons through a negative feedback affecting the paired-like homeodomain transcription factor Pitx3 [44], while miR-134 activity leads to dendritic spine development through downregulation of the Lim domain kinase-1 [45].

miRNAs may also play significant roles in apoptosis, which is crucial in neurogenesis and during the subsequent, continued expansion of the brain size following a massive loss of neurons (i.e., 20%–80%) typical of embryonic development [74]. It was proposed that several aspects of neuronal function, for example, the control of plasticity, are directly mediated by miRNAs [75].

Instead, not much evidence has yet become available to define the impact of miRNAs on neural induction, namely, the stage when embryonic cells assume a neuronal identity. However, in light of a specific expression in stem cells, it is possible that miRNAs play a role in self-renewal and differentiation events through the regulation of key genes [76], as suggested by the ability of embryonic stem cell-specific miRNAs to enhance murine stem cell reprogramming [77, 78].

As regards human miRNAs, the miRBase Sequence Database of the Sanger Center (Release 14.0, dated September 2009) contains 706 sequences (http://microrna.sanger.ac.uk/sequences/). It was estimated, based on high-throughput sequencing data, that the number of miRNAs expressed in the human brain may well exceed one-thousand [68]. Interestingly, many miRNAs expressed in the human brain are not conserved beyond primates, suggesting a recent evolutionary origin [68]. Although functions have been assigned to only very few brain-specific miRNAs, increasing evidence suggests key roles in normal development, differentiation events, and homeostasis, as well as in related pathological conditions [11, 13, 33, 36, 46, 47, 52, 100].

4. miRNA and Neurodegenerative Diseases

Neurodegenerative diseases result from dysfunction, progressive deterioration, and extensive loss of neurons in the central and/or peripheral nervous system [10, 100]. In this regard, Alzheimer's disease (AD)[101–104], Parkinson's disease (PD) [105–107], prion diseases [108], amyotrophic lateral sclerosis (ALS) [109, 110], and hereditary spastic paraplegia [111, 112] may have a genetic or sporadic etiology. Instead, Huntington's disease (HD) [113, 114] and metabolic disorders with neurological involvement, such as the GM2-gangliosidoses [115–119], can only be genetically transmitted.

There is now compelling evidence that dysregulation of miRNA networks is implicated in the development and onset of human neurodegenerative diseases (see Table 2 and [12, 120]). This, in turn, may provide the opportunity to elucidate underlying disease mechanisms and open up novel strategies for therapeutic applications.

4.1. Alzheimer's Disease. AD is the most common form of dementia. While several hypotheses have been proposed to explain the disease's etiology, the causes of AD and means of stopping its progression are still elusive matters [101–104, 121–125]. Features of the disease encompass neuronal loss, intraneuronal neurofibrillary tangles (i.e., aggregates of the microtubule-associated protein *tau* following hyperphosphorylation), and extracellular deposits of amyloid plaques (i.e., deposits of Aβ-peptide) [102, 121–125].

Only 10%–15% of AD cases represent an inheritable disease which follows an autosomal dominant Mendelian pattern, while the majority arise sporadically. Apparently, the disease may be caused by a genetic predisposition, as shown by the identification of specific DNA mutations in a large number of families [101, 122–125]. Despite a variable etiology, a common pathogenetic cascade resulting from distinct gene defects and/or unknown environmental factors cannot be ruled out. For example, accumulation of the Aβ peptide, the cause of which is unknown, is consistently observed. In approximately 30% of sporadic AD patient samples, the expression of BACE1 protein, a secretase associated with the formation of Aβ-peptide, is significantly increased [126].

In AD, several miRNAs exhibit abnormal expression levels, suggesting a dysfunctional orchestration of gene expression [79, 80, 127, 128].

Interestingly, Boissonneault et al. have recently found that miR-298 and miR-328 bind to the 3′-UTR of BACE1 mRNA, thereby producing a regulatory effect on enzyme expression in cultured neuronal (N2a) cells. Presence of both miR-298 and miR-328 in the hippocampus of APPSwe/PS1 mice, a well-documented model for AD, and the observation that their levels of expression decrease with aging suggest that altered levels of these miRNAs may deregulate BACE1 and, in turn, lead to increased Aβ formation and disease progression [81].

Moreover, BACE1 can be controlled by the miR-29a/b-1 cluster, consistent with their inverse pattern of expression observed in sporadic AD patients; in addition, a causal correlation was shown in vitro between this cluster and the appearance of the Aβ peptide [12, 79, 80]. miRNAs may also be involved in the neuroinflammatory process associated

Table 2: miRNAs involved in neurological diseases.

microRNA	Neurological disease	Effect	Reference number
miR-29a/b-1	Alzheimer's disease	downregulation	[79]
miR-128a	Alzheimer's disease	upregulation	[80]
miR-298	Alzheimer's disease	downregulation	[81]
miR-328	Alzheimer's disease	downregulation	[81]
miR-146a	Alzheimer's disease	upregulation	[82]
miR-133b	Parkinson's disease	downregulation	[83]
miR-19	Spinocerebellar ataxia type 1	downregulation	[84]
miR-101	Spinocerebellar ataxia type 1	downregulation	[84]
miR-130	Spinocerebellar ataxia type 1	downregulation	[84]
miR-9	Hungtington's disease	downregulation	[85, 86]
miR-1	Tourette syndrome	deregulation	[87]
miR-206	Tourette syndrome	deregulation	[87]
miR-21	Glioblastoma	upregulation	[88]
miR-124	Glioblastoma	downregulation	[89]
miR-137	Glioblastoma	downregulation	[89]
miR-124a	Medulloblastoma	downregulation	[90]
miR-34a	Neuroblastoma	downregulation	[91]
miR-184	Neuroblastoma	downregulation	[92]
miR-15a	Pituitary adenoma	downregulation	[93–95]
miR-16	Pituitary adenoma	downregulation	[93–95]
miR-221	Glioblastoma	upregulation	[88, 96–98]
miR-128	Glioblastoma	upregulation	[88, 96–98]
miR-181a	Glioblastoma	upregulation	[88, 96–98]
miR-181b	Glioblastoma	upregulation	[88, 96–98]
miR-181c	Glioblastoma	downregulation	[88, 96–98]
miR-9	Medulloblastoma	downregulation	[99]
miR-125a	Medulloblastoma	downregulation	[99]

with deposition of the Aβ-peptide. In this regard, the NF-kB-sensitive miRNA-146a, which targets complement factor H, an important repressor of inflammatory responses in the brain, was found to be up-regulated in AD [82].

Finally, a recent work from Carrettiero et al. shows that miR-128a regulates the cochaperone BAG2 and, in turn, a pathway of degradation for microtubule-associated *tau* proteins with a propensity for misfolding. BAG2 would normally direct *tau* toward an ubiquitin-independent pathway and selectively reduce the levels of sarkosyl-insoluble protein [129]. Thus, the observation that miR-128a is upregulated in AD [80] may highlight a molecular mechanism that underlies *tau* inclusions in neurodegeneration. Taken together, these findings suggest a mechanistic involvement of miRNAs in both the amyloid and *tau* hypotheses for AD pathogenesis.

4.2. Parkinson's Disease (PD). PD is the second most common neurodegenerative disorder, characterized by resting tremor, muscular rigidity, bradykinesia, and impaired balance and coordination [105–107, 130–132]. Other symptoms include dysautonomia, dystonic cramps, and dementia. Typical pathological features are loss of dopaminergic neurons in the substantia nigra (SN) and presence of Lewy bodies, which consist of intracellular inclusions affecting surviving neurons in various areas of the brain [130–132]. Several gene loci have been implicated in autosomal, dominant forms of PD. These include PARK1 and PARK4 (due to a mutation or a triplication of the α-synuclein gene [SNCA] on 4q21 and 4p15, resp.), PARK3 on 2p13, PARK5 (due to a mutation in the UCHL1 gene) on 4p14, PARK8 (due to a mutation in the LRRK2 gene) on 12q12, PARK10 on 1p, PARK11 on 2q, and PARK13 (due to a mutation in the HTRA2 gene) on 2p12 [105–107, 133, 134].

The implication of miRNAs in PD is intriguing. In murine models, the competence of embryonic stem cells to differentiate into midbrain dopamine neurons in vitro was shown to be disrupted by Dicer deletion and subsequent suppression of miRNA biogenesis, suggesting a physiological role for miRNAs in cell differentiation and/or survival. These results were confirmed in vivo, using mice conditional for Dicer, which exhibited impaired locomotor activity that recapitulated motility problems observed in PD patients. Through a subtractive approach, performed by comparing miRNA expression profiles in normal human adult *versus* PD patients midbrains, it was shown that miR-133b is specifically missing in PD and that, based on both overexpression and inhibitory tests in vitro, is likely implicated

in the maturation and function of dopaminergic neurons [44, 83]. A markedly reduced expression of miR-133b was found in Aphakia mice [44], a dopaminergic neuron deficiency model, which lack Pitx3 [135], a homeobox transcription factor required for neuron survival and normal motor activity susceptible to polymorphisms associated with sporadic PD [136]. Together, these observations suggest a relationship between miR-133b and Pitx3, which operate through a negative feedback loop, wherein Pitx3 promotes the expression of miR-133b that, in turn, downregulates Pitx3 [83]. While these results point to a functional role of the miR-133b/Pitx3 system in ensuring correct dopaminergic function, miR-133b knock-out mice, which are currently unavailable, would establish the extent of miR-133b impact on PD etiology.

On the other hand, a more recent study showed that deletion of Dicer in dopaminoceptive neurons of the murine striatum led to aberrant anatomical features (smaller brain, reduced neuron size, astrogliosis) and motor impairments (clasping and ataxia) but, surprisingly, not neurodegeneration [137]. As dysfunction, but not necessarily loss, of dopaminoceptive neurons was previously implicated in PD [138], these observations, taken together, suggest that the link between Dicer, miRNAs, and neurodegeneration is restricted to dopaminergic neurons, thereby pointing to distinct functional roles in dopaminoceptive cells.

Finally, Wang et al. found that in PD brains and in vitro cell models disruption of the binding site for miRNA-433 led to increased translation of fibroblast growth factor-20 (FGF20). Notably, an FGF20 polymorphism at 8p21.3–22 was previously identified as a PD risk factor correlated with increased α-synuclein expression, and consequently PD onset [139].

4.3. Spinocerebellar Ataxia. Spinocerebellar ataxia type 1 (SCA1), which is caused by the expansion of a CAG repeat encoding glutamine within the gene ATXN1, is characterized by the death of cerebellar Purkinje cells [140]. In eight-week old mice, depletion of Dicer from murine Purkinje neurons is irrelevant to cell function and survival, whereas 13-week-old animals are affected by a progressive degeneration of Purkinje neurons leading to cell death. Further, these older mice develop a slight tremor and mild ataxia, both of which worsen with advancing age [141].

In 2008, Lee et al. found that miR-19, miR-101, and miR-130 coregulate Ataxin-1 protein levels and that their inhibition enhance the cytotoxic effects of polyglutamine (PolyQ)-expanded Ataxin-1 in human cells. Thus, mutations in the miRNA binding sites or the miRNA genes themselves might be linked to neurodegenerative phenotypes as a result of Ataxin-1 accumulation [84]. Consistent with this possibility are earlier results showing that, in both *Drosophila* and human cells, the elimination of miRNAs via Dicer mutation was followed by enhanced pathogenic polyQ protein toxicity. Altogether, these observations point to a neuroprotective function of miRNAs [142].

4.4. Huntington's Disease (HD). HD is a fatal, hereditary neurodegenerative disorder characterized by involuntary ballistic movements, depression, and dementia [113, 114, 143]. Hallmarks of HD are progressive chorea, rigidity, and frequent accurrence of seizures, emotional problems, loss of cognition, as well as atrophy of the caudate nucleus. The causal factor of HD is a gene mutation consisting of abnormally extended repeats of the CAG sequence within the HTT gene, which translates into a huntingtin protein containing an excessively increased glutamine segment [113, 114, 143].

Disruption of miRNA homeostasis, most likely in connection with an aberrant functionality of the transcriptional repressor REST, was recently shown to play a dynamic role in HD. In fact, levels of several miRNAs with upstream RE1 sites are decreased in HD patient cortices relative to healthy controls. Interestingly, one of these, the bifunctional, brain-enriched miR-9/miR-9* targets two components of the REST complex: miR-9 targets REST and miR-9* targets CoREST [85, 86]. As a consequence of a markedly altered miRNA expression, target mRNAs are subject to dysregulated levels which, in turn, affect the physiological status of forebrain neurons [85, 86].

In 2005, RNA interference was shown to produce therapeutically-relevant effects in HD mouse models [144, 145]. Moreover, McBride et al. reported that RNA interference through miRNA technology, as compared to the shRNA-based approach, is a more appropriate strategy for HD treatment. In particular, shRNAs targeting mutant human HD transgenes were found to cause overt toxicity in the mouse striatum, whereas the same sequences introduced into artificial miRNA expression constructs markedly alleviated the neurotoxic profile without compromising achievement of an efficient silencing effect on the murine HD gene homolog [146].

5. Other Neurological Diseases

5.1. Fragile X Mental Retardation. The Fragile X syndrome is one of the most common forms of inherited, X-linked dominant mental retardation affecting approximately one in every 4000 males and 8000 females [147, 148] with reduced penetrance of 80% and 30%, respectively [148, 149]. The clinical presentations of Fragile X syndrome include mild to severe mental retardation, that is reflected by IQ values ranging between 20 and 70, some abnormal facial features affecting jaw and ears as well as macroorchidism in postpubescent males [149].

The gene responsible for the Fragile X syndrome, FMR1, encodes a protein, FMRP, that interacts with target RNAs [150] and is implicated in mRNA transport and translational control [148]. In particular, FMRP is linked to the miRNA pathway in light of its association with RISC, as shown in *Drosophila* [151, 152], and with Argonaute proteins, Dicer and miRNAs, as shown in mammals [153–158]. Indeed, FMRP can act as a miRNA acceptor for Dicer and facilitate the assembly of miRNAs [154, 159, 160]. Thus, the neurodegenerative outcome caused by mutations in FMR1 may give rise to a host of secondary effects mediated by the action of FMRP on associated RNA targets.

The molecular mechanisms which underlie the pathogenesis of this disorder have yet to be elucidated. However, Xu et al. reported that miR-124a, a nervous-system-specific miRNA, is modulated, at least partially, by the *Drosophila* homolog of mammalian FMRP (dFMR1), which was found to associate with miR-124 in vivo [161]. That FMRP could utilize specific miRNAs to regulate the translation of target mRNAs was also confirmed by a recent *Drosophila* study, in which the bantam miRNA was shown to interact with dFMR1 to regulate the fate of germline stem cells [162].

Further, miR-184 was found to be repressed by MeCP2, a protein that binds to methylated DNA forms and plays an important role in synaptic plasticity [163]. This observation points to a link between miRNA and DNA methylation pathways in the dysregulation of synaptic plasticity, a feature for which there is growing evidence of an important role played by miRNAs [164] and that is observed in the Fragile X syndrome.

5.2. Tourette Syndrome. The paradigm for a disease caused by a specific miRNA is the G to A transition in the 3′ UTR of the myostatin/growth differentiation factor 8 gene in Texel sheep [87]. This mutation creates a target site for miR-1 and miR-206, which are highly expressed in the skeletal muscle. The downstream effect is the translational inhibition of the myostatin gene, which normally limits muscle growth but in the sheep contributes to muscular hypertrophy. Based on this finding, it may be postulated that a search of human SNP databases will reveal mutations that are potentially able to create or destroy putative miRNA target sites and thereby contribute to phenotypic variation.

Conceivably, some of these mutations may affect neuronal miRNAs. One such example is a rare sequence variant of SLIT and Trk-like 1 (SLITRK1), a candidate gene for Tourette syndrome located on chromosome 13q31.1 which is involved in neural development [165]. Two independent instances of the same mutation in the binding site for the miRNA hsa-miR-189 were detected among a population of unrelated individuals with Tourette syndrome, while absent in 3600 control chromosomes. That this mutation may be implicated in Tourette's syndrome is supported by circumstantial evidence showing an overlapping expression pattern of SLITRK1 mRNA and hsa-miR-189 in several brain regions implicated in the disease [165].

5.3. miRNAs and Tumours of the Nervous System. Several studies found that a high proportion of genomic loci containing miRNA genes exhibit DNA copy number alterations in common cancers [166] and miRNA misexpression has also been described in tumours of the nervous system (see Table 2 and [88–92, 96, 167–170]). miRNAs have been shown to act either as tumor suppressors or oncogenes and, depending on the mRNA target, may accelerate the oncogenic process [167]. A suppressor effect was observed in pituitary adenomas, the most common tumors of the central nervous system, in which down-regulation of miR-15a and miR-16 correlates with tumor size [93–95]. Other miRNAs, such as the miR-155 and miR17-92 cluster, have an oncogenic effect [171, 172]. The consequence of an upregulation of miR-21 has been characterised in glioblastoma tumor cells [88], wherein the knockdown of miR-21 led to increased apoptotic cell death, suggesting that this miRNA may act as an antiapoptotic player [88, 96, 173]. In addition, miRNA profiling in glioblastoma cells has shown high levels of miR-221, miR-128, miR-181a, and miR-181b and low levels of miR-181c [88, 97, 98].

miRNA expression analysis may also be used for medulloblastoma prognosis. Down-regulation of miR-9 and miR-125a was observed in aggressive brain malignancy, which results in the activation of medulloblastoma cell growth and arrest of apoptosis by activation of the proproliferative truncated TrkC isoform [99].

Based on these findings, the potential to modulate multiple messages at the same time via miRNA technology would therefore represent an intriguing prospect for cancer treatment.

6. Concluding Remarks and Prospects

Contemporary science has embraced RNA as a central element of cellular biology. In addition to the canonical role as an intermediate carrier of information, this molecule may in fact perform catalytic, structural, and regulatory tasks. Hence, over the last decade, unravelling the unique versatility of RNA has renewed impetus towards the concept of an "RNA world", which refers to a self-sustaining replication system, antecedent to DNA and proteins, that was engaged during a hypothetical stage at the origin of life [174–177]. Along with the most recent, stunning advances in RNA biology on several fronts, the discovery of gene expression regulators has opened up a large window into the RNA world. Three main categories of small RNAs, namely, short-interfering, micro- and piwi-interacting RNAs, have emerged as regulatory players within a structurally and functionally sophisticated, and to some extent overlapping, context [14, 178].

Unlike most of the siRNAs, which silence the same locus from which they derive, the effect of miRNAs is to repress genes unrelated to their own loci. Thus, miRNas are subject to precise sequence requirements for the necessary interaction with heterologous targets. Several approaches exist that can be employed to obtain comprehensive miRNA profiling in cells or tissues [179]; however, the significance of a specific profile may be difficult to interpret, in light of the hundreds of target sequences in the human genome that may be associated with any particular miRNAs. In this regard, computational predictions and simulations have a fundamental impact on experimental miRNA research, considering that the downstream effect of a given miRNA will result from the complex modulation of multiple targets along different pathways prone to cross-talk. Conceivably, experimental and bioinformatic models will continue to evolve to offer large-scale screenings for the identification of the most likely miRNA target(s) under a specific developmental, physiological, environmental, or pathological status.

Currently, functional characterization of specific miRNAs is facilitated by the existence of first-class reagents such as miRNA mimics and inhibitors, available through several specialized vendors. These reagents, appropriately modified to optimize correct strand utilization by RISC (mimics) and ensure tight binding (inhibitors), can be used to either increase or decrease the activity of specific miRNAs. Corresponding applications can be exceptionally informative with respect to studies on gain (loss)-of-function effects, development of high-throughput screens to select species involved in normal and pathological cellular pathways, and the identification of targets.

However, despite the significant progress in miRNA research in the field of neurodevelopment and neurological diseases, it is still elusive as to whether any of the miRNAs implicated in a neuropathological process is directly involved in the etiology or progression of the disorder. Indeed, aberrant expression of a miRNA could simply be circumstantial. This causality issue can be addressed, for example, through an accurate determination of the frequency of specific miRNA mutations, the definition of temporal and spatial miRNA profiles within multiple pathways in vitro, and the development of appropriate in vivo models.

Based on their functional role in fine-tuning metabolic pathways and genetic networks, miRNAs appear to be suitable tools for use in diagnosis, prognosis, and therapy. The problem is to demonstrate which miRNA sequences should be considered drug discovery targets.

Systemic delivery is another hurdle that must be dealt with. Initially, this problem was common to all RNA-based therapeutics, including antisense oligos and siRNAs (reviewed in [180]). However, second-generation antisense technologies have shown that drug delivery issues can be overcome, as shown by systemic drug distribution following subcutaneous administration.

Specific antisense oligos called antagomirs could be used to affect the activity of miRNA. In this regard, treatment of a mouse model of heart disease with an antagomir against miR-21 prevented heart failure [181], and antagomirs to target glioma angiogenesis has recently been proposed [182]. miRNA-based therapeutics have great potential because of their capability to efficiently silence multiple messages concurrently within an entire disease pathway. Instead, conventional therapies directed at single targets require administration of a plurality of drugs giving rise to complex drug interaction and patient compliance issues.

Acknowledgments

The authors thank members of the Neurogenetics Laboratory of the Santa Lucia Foundation (Rome, Italy) for critical reading of the manuscript. This work was supported by the Italian *Ministero della Salute* (Grant no. RF-UMB-2006-339457 to Al.O. and An.O.; Grants nos. EBRI.1O, PS05.11, and PS05.21 to An.O.), the *Consorzio INBB to Al.O.*, and the *Comitato Telethon Fondazione Onlus*, the *Amministrazione Autonoma dei Monopoli di Stato* (*AAMS*), and the *City of Gubbio*, Italy (Grant no. GGP06209 to An. O.).

References

[1] D. P. Bartel, "microRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[2] G. Meister, "miRNAs get an early start on translational silencing," *Cell*, vol. 131, no. 1, pp. 25–28, 2007.

[3] M. Kiriakidou, G. S. Tan, S. Lamprinaki, M. De Planell-Saguer, P. T. Nelson, and Z. Mourelatos, "An mRNA m7G cap binding-like motif within human Ago2 represses translation," *Cell*, vol. 129, no. 6, pp. 1141–1151, 2007.

[4] G. Mathonnet, M. R. Fabian, Y. V. Svitkin, et al., "microRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F," *Science*, vol. 317, no. 5845, pp. 1764–1767, 2007.

[5] R. Thermann and M. W. Hentze, "*Drosophila* miR2 induces pseudo-polysomes and inhibits translation initiation," *Nature*, vol. 447, no. 7146, pp. 875–878, 2007.

[6] M. Wakiyama, K. Takimoto, O. Ohara, and S. Yokoyama, "Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system," *Genes and Development*, vol. 21, no. 15, pp. 1857–1862, 2007.

[7] X. C. Ding, J. Weiler, and H. Grosshans, "Regulating the regulators: mechanisms controlling the maturation of microRNAs," *Trends in Biotechnology*, vol. 27, no. 1, pp. 27–36, 2009.

[8] D. J. Guarnieri and R. J. DiLeone, "microRNAs: a new class of gene regulators," *Annals of Medicine*, vol. 40, no. 3, pp. 197–208, 2008.

[9] A. Köhler and E. Hurt, "Exporting RNA from the nucleus to the cytoplasm," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 10, pp. 761–773, 2007.

[10] A. Orlacchio, G. Bernardi, A. Orlacchio, and S. Martino, "Patented therapeutic RNAi strategies for neurodegenerative diseases of the CNS," *Expert Opinion on Therapeutic Patents*, vol. 18, no. 10, pp. 1161–1174, 2008.

[11] S. S. Hébert and B. De Strooper, "Molecular biology: miRNAs in neurodegeneration," *Science*, vol. 317, no. 5842, pp. 1179–1180, 2007.

[12] S. S. Hébert and B. De Strooper, "Alterations of the microRNA network cause neurodegenerative disease," *Trends in Neurosciences*, vol. 32, no. 4, pp. 199–206, 2009.

[13] S. K. Singh, "miRNAs: from neurogeneration to neurodegeneration," *Pharmacogenomics*, vol. 8, no. 8, pp. 971–978, 2007.

[14] R. W. Carthew and E. J. Sontheimer, "Origins and mechanisms of miRNAs and siRNAs," *Cell*, vol. 136, no. 4, pp. 642–655, 2009.

[15] V. N. Kim, J. Han, and M. C. Siomi, "Biogenesis of small RNAs in animals," *Nature Reviews Molecular Cell Biology*, vol. 10, no. 2, pp. 126–139, 2009.

[16] M. J. Moore and N. J. Proudfoot, "Pre-mRNA processing reaches back to transcription and ahead to translation," *Cell*, vol. 136, no. 4, pp. 688–700, 2009.

[17] A. Rodriguez, S. Griffiths-Jones, J. L. Ashurst, and A. Bradley, "Identification of mammalian microRNA host genes and transcription units," *Genome Research*, vol. 14, no. 10A, pp. 1902–1910, 2004.

[18] Y.-K. Kim and V. N. Kim, "Processing of intronic microRNAs," *EMBO Journal*, vol. 26, no. 3, pp. 775–783, 2007.

[19] X. Cai, C. H. Hagedorn, and B. R. Cullen, "Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs," *RNA*, vol. 10, no. 12, pp. 1957–1966, 2004.

[20] Y. Lee, M. Kim, J. Han, et al., "microRNA genes are transcribed by RNA polymerase II," *EMBO Journal*, vol. 23, no. 20, pp. 4051–4060, 2004.

[21] U. Ohler, S. Yekta, L. P. Lim, D. P. Bartel, and C. B. Burge, "Patterns of flanking sequence conservation and a characteristic upstream motif for microRNA gene identification," *RNA*, vol. 10, no. 9, pp. 1309–1322, 2004.

[22] X. Liu, K. Fortin, and Z. Mourelatos, "microRNAs: biogenesis and molecular functions," *Brain Pathology*, vol. 18, no. 1, pp. 113–121, 2008.

[23] Y. Lee, K. Jeon, J.-T. Lee, S. Kim, and V. N. Kim, "microRNA maturation: stepwise processing and subcellular localization," *EMBO Journal*, vol. 21, no. 17, pp. 4663–4670, 2002.

[24] A. M. Denli, B. B. J. Tops, R. H. A. Plasterk, R. F. Ketting, and G. J. Hannon, "Processing of primary microRNAs by the microprocessor complex," *Nature*, vol. 432, no. 7014, pp. 231–235, 2004.

[25] R. I. Gregory, K.-P. Yan, G. Amuthan, et al., "The microprocessor complex mediates the genesis of microRNAs," *Nature*, vol. 432, no. 7014, pp. 235–240, 2004.

[26] J. Han, Y. Lee, K.-H. Yeom, Y.-K. Kim, H. Jin, and V. N. Kim, "The Drosha-DGCR8 complex in primary microRNA processing," *Genes and Development*, vol. 18, no. 24, pp. 3016–3027, 2004.

[27] M. Landthaler, A. Yalcin, and T. Tuschl, "The human DiGeorge syndrome critical region gene 8 and its D. melanogaster homolog are required for miRNA biogenesis," *Current Biology*, vol. 14, no. 23, pp. 2162–2167, 2004.

[28] E. Berezikov, W.-J. Chung, J. Willis, E. Cuppen, and E. C. Lai, "Mammalian mirtron genes," *Molecular Cell*, vol. 28, no. 2, pp. 328–336, 2007.

[29] K. Okamura, J. W. Hagen, H. Duan, D. M. Tyler, and E. C. Lai, "The mirtron pathway generates microRNA-class regulatory RNAs in *Drosophila*," *Cell*, vol. 130, no. 1, pp. 89–100, 2007.

[30] J. G. Ruby, C. H. Jan, and D. P. Bartel, "Intronic microRNA precursors that bypass Drosha processing," *Nature*, vol. 448, no. 7149, pp. 83–86, 2007.

[31] M. T. Bohnsack, K. Czaplinski, and D. Gorlich, "Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs," *RNA*, vol. 10, no. 2, pp. 185–191, 2004.

[32] E. Lund, S. Guttinger, A. Calado, J. E. Dahlberg, and U. Kutay, "Nuclear export of microRNA precursors," *Science*, vol. 303, no. 5654, pp. 95–98, 2004.

[33] W. Filipowicz, S. N. Bhattacharyya, and N. Sonenberg, "Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?" *Nature Reviews Genetics*, vol. 9, no. 2, pp. 102–114, 2008.

[34] M. Ronshaugen, F. Biemar, J. Piel, M. Levine, and E. C. Lai, "The *Drosophila* microRNA iab-4 causes a dominant homeotic transformation of halteres to wings," *Genes and Development*, vol. 19, no. 24, pp. 2947–2952, 2005.

[35] R. Garzon, F. Pichiorri, T. Palumbo, et al., "microRNA fingerprints during human megakaryocytopoiesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 13, pp. 5078–5083, 2006.

[36] F.-B. Gao, "Posttranscriptional control of neuronal development by microRNA networks," *Trends in Neurosciences*, vol. 31, no. 1, pp. 20–26, 2008.

[37] R. J. Johnston Jr. and O. Hobert, "A microRNA controlling left/right neuronal asymmetry in *Caenorhabditis elegans*," *Nature*, vol. 426, no. 6968, pp. 845–849, 2003.

[38] R. J. Johnston Jr., S. Chang, J. F. Etchberger, C. O. Ortiz, and O. Hobert, "microRNAs acting in a double-negative feedback loop to control a neuronal cell fate decision," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 35, pp. 12449–12454, 2005.

[39] S. Yekta, I.-H. Shih, and D. P. Bartel, "microRNA-directed cleavage of HOXB8 mRNA," *Science*, vol. 304, no. 5670, pp. 594–596, 2004.

[40] J. Visvanathan, S. Lee, B. Lee, J. W. Lee, and S.-K. Lee, "The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development," *Genes and Development*, vol. 21, no. 7, pp. 744–749, 2007.

[41] L.-C. Cheng, E. Pastrana, M. Tavazoie, and F. Doetsch, "MiR-124 regulates adult neurogenesis in the subventricular zone stem cell niche," *Nature Neuroscience*, vol. 12, no. 4, pp. 399–408, 2009.

[42] N. Vo, M. E. Klein, O. Varlamova, et al., "A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 45, pp. 16426–16431, 2005.

[43] H.-Y. M. Cheng, J. W. Papp, O. Varlamova, et al., "microRNA modulation of circadian-clock period and entrainment," *Neuron*, vol. 54, no. 5, pp. 813–829, 2007.

[44] J. Kim, K. Inoue, J. Ishii, et al., "A microRNA feedback circuit in midbrain dopamine neurons," *Science*, vol. 317, no. 5842, pp. 1220–1224, 2007.

[45] G. M. Schratt, F. Tuebing, E. A. Nigh, et al., "A brain-specific microRNA regulates dendritic spine development," *Nature*, vol. 439, no. 7074, pp. 283–289, 2006.

[46] A. Kuzin, M. Kundu, T. Brody, and W. F. Odenwald, "The *Drosophila* nerfin-1 mRNA requires multiple microRNAs to regulate its spatial and temporal translation dynamics in the developing nervous system," *Developmental Biology*, vol. 310, no. 1, pp. 35–43, 2007.

[47] X. Cao, G. Yeo, A. R. Muotri, T. Kuwabara, and F. H. Gage, "Noncoding RNAs in the mammalian central nervous system," *Annual Review of Neuroscience*, vol. 29, pp. 77–103, 2006.

[48] B. L. Davidson and R. L. Boudreau, "RNA interference: a tool for querying nervous system function and an emerging therapy," *Neuron*, vol. 53, no. 6, pp. 781–788, 2007.

[49] J. C. Pearson, D. Lemons, and W. McGinnis, "Modulating Hox gene functions during animal body patterning," *Nature Reviews Genetics*, vol. 6, no. 12, pp. 893–904, 2005.

[50] A. J. Giraldez, R. M. Cinalli, M. E. Glasner, et al., "microRNAs regulate brain morphogenesis in zebrafish," *Science*, vol. 308, no. 5723, pp. 833–838, 2005.

[51] U. Hengst, L. J. Cox, E. Z. Macosko, and S. R. Jaffrey, "Functional and selective RNA interference in developing axons and growth cones," *Journal of Neuroscience*, vol. 26, no. 21, pp. 5727–5732, 2006.

[52] K. S. Kosik, "The neuronal microRNA system," *Nature Reviews Neuroscience*, vol. 7, no. 12, pp. 911–920, 2006.

[53] L. Smirnova, A. Gräfe, A. Seiler, S. Schumacher, R. Nitsch, and F. G. Wulczyn, "Regulation of miRNA expression during neural cell specification," *European Journal of Neuroscience*, vol. 21, no. 6, pp. 1469–1477, 2005.

[54] M. Castoldi, S. Schmidt, V. Benes, et al., "A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA)," *RNA*, vol. 12, no. 5, pp. 913–920, 2006.

[55] S. Griffiths-Jones, "miRBase: the microRNA sequence database," *Methods in Molecular Biology*, vol. 342, pp. 129–138, 2006.

[56] T. Babak, W. Zhang, Q. Morris, B. J. Blencowe, and T. R. Hughes, "Probing microRNAs with microarrays: tissue specificity and functional inference," *RNA*, vol. 10, no. 11, pp. 1813–1819, 2004.

[57] O. Barad, E. Meiri, A. Avniel, et al., "microRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues," *Genome Research*, vol. 14, no. 12, pp. 2486–2494, 2004.

[58] E. A. Miska, E. Alvarez-Saavedra, M. Townsend, et al., "Microarray analysis of microRNA expression in the developing mammalian brain," *Genome Biology*, vol. 5, no. 9, article R68, 2004.

[59] L. F. Sempere, S. Freemantle, I. Pitha-Rowe, E. Moss, E. Dmitrovsky, and V. Ambros, "Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation," *Genome Biology*, vol. 5, no. 3, article R13, 2004.

[60] J. Shingara, K. Keiger, J. Shelton, et al., "An optimized isolation and labeling platform for accurate microRNA expression profiling," *RNA*, vol. 11, no. 9, pp. 1461–1470, 2005.

[61] J. M. Thomson, J. Parker, C. M. Perou, and S. M. Hammond, "A custom microarray platform for analysis of microRNA gene expression," *Nature Methods*, vol. 1, no. 1, pp. 47–53, 2004.

[62] V. G. Tusher, R. Tibshirani, and G. Chu, "Significance analysis of microarrays applied to the ionizing radiation response," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 9, pp. 5116–5121, 2001.

[63] K. K.-H. Farh, A. Grimson, C. Jan, et al., "The widespread impact of mammalian microRNAs on mRNA repression and evolution," *Science*, vol. 310, no. 5755, pp. 1817–1821, 2005.

[64] H. Hohjoh and T. Fukushima, "Expression profile analysis of microRNA (miRNA) in mouse central nervous system using a new miRNA detection system that examines hybridization signals at every step of washing," *Gene*, vol. 391, no. 1-2, pp. 39–44, 2007.

[65] P. Landgraf, M. Rusu, R. Sheridan, et al., "A mammalian microRNA expression atlas based on small RNA library sequencing," *Cell*, vol. 129, no. 7, pp. 1401–1414, 2007.

[66] W. P. Kloosterman and R. H. A. Plasterk, "The diverse functions of microRNAs in animal development and disease," *Developmental Cell*, vol. 11, no. 4, pp. 441–450, 2006.

[67] Y. Zeng and B. R. Cullen, "Recognition and cleavage of primary microRNA transcripts," *Methods in Molecular Biology*, vol. 342, pp. 49–56, 2006.

[68] E. Berezikov, F. Thuemmler, L. W. van Laake, et al., "Diversity of microRNAs in human and chimpanzee brain," *Nature Genetics*, vol. 38, no. 12, pp. 1375–1377, 2006.

[69] S. Griffiths-Jones, "The microRNA registry," *Nucleic Acids Research*, vol. 32, pp. D109–D111, 2004.

[70] D. B. Dogini, P. A. O. Ribeiro, C. Rocha, T. C. Pereira, and I. Lopes-Cendes, "microRNA expression profile in murine central nervous system development," *Journal of Molecular Neuroscience*, vol. 35, no. 3, pp. 331–337, 2008.

[71] J.-Y. Yu, K.-H. Chung, M. Deo, R. C. Thompson, and D. L. Turner, "microRNA miR-124 regulates neurite outgrowth during neuronal differentiation," *Experimental Cell Research*, vol. 314, no. 14, pp. 2618–2633, 2008.

[72] Q. Shen and S. Temple, "Fine control: microRNA regulation of adult neurogenesis," *Nature Neuroscience*, vol. 12, no. 4, pp. 369–370, 2009.

[73] G. A. Wayman, M. Davare, H. Ando, et al., "An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 26, pp. 9093–9098, 2008.

[74] R. R. Buss and R. W. Oppenheim, "Role of programmed cell death in normal neuronal development and function," *Anatomical Science International*, vol. 79, no. 4, pp. 191–197, 2004.

[75] K. C. Martin and K. S. Kosik, "Synaptic tagging—who's it?" *Nature Reviews Neuroscience*, vol. 3, no. 10, pp. 813–820, 2002.

[76] L. Smirnova, A. Gräfe, A. Seiler, S. Schumacher, R. Nitsch, and F. G. Wulczyn, "Regulation of miRNA expression during neural cell specification," *European Journal of Neuroscience*, vol. 21, no. 6, pp. 1469–1477, 2005.

[77] R. L. Judson, J. E. Babiarz, M. Venere, and R. Blelloch, "Embryonic stem cell-specific microRNAs promote induced pluripotency," *Nature Biotechnology*, vol. 27, no. 5, pp. 459–461, 2009.

[78] K. D. Wilson, S. Venkatasubrahmanyam, F. Jia, N. Sun, A. J. Butte, and J. C. Wu, "microRNA profiling of human-induced pluripotent stem cells," *Stem Cells and Development*, vol. 18, no. 5, pp. 749–757, 2009.

[79] S. S. Hébert, K. Horré, L. Nicolaï, et al., "Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/β-secretase expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 17, pp. 6415–6420, 2008.

[80] W. J. Lukiw, "Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus," *NeuroReport*, vol. 18, no. 3, pp. 297–300, 2007.

[81] V. Boissonneault, I. Plante, S. Rivest, and P. Provost, "microRNA-298 and microRNA-328 regulate expression of mouse β-amyloid precursor protein-converting enzyme 1," *Journal of Biological Chemistry*, vol. 284, no. 4, pp. 1971–1981, 2009.

[82] W. J. Lukiw, Y. Zhao, and J. G. Cui, "An NF-κB-sensitive micro RNA-146a-mediated inflammatory circuit in alzheimer disease and in stressed human brain cells," *Journal of Biological Chemistry*, vol. 283, no. 46, pp. 31315–31322, 2008.

[83] S.-L. Ang, "Transcriptional control of midbrain dopaminergic neuron development," *Development*, vol. 133, no. 18, pp. 3499–3506, 2006.

[84] Y. Lee, R. C. Samaco, J. R. Gatchel, C. Thaller, H. T. Orr, and H. Y. Zoghbi, "miR-19, miR-101 and miR-130 co-regulate ATXN1 levels to potentially modulate SCA1 pathogenesis," *Nature Neuroscience*, vol. 11, no. 10, pp. 1137–1139, 2008.

[85] A. N. Packer, Y. Xing, S. Q. Harper, L. Jones, and B. L. Davidson, "The bifunctional microRNA miR-9/miR-9* regulates REST and CoREST and is downregulated in Huntington's disease," *Journal of Neuroscience*, vol. 28, no. 53, pp. 14341–14346, 2008.

[86] R. Johnson, C. Zuccato, N. D. Belyaev, D. J. Guest, E. Cattaneo, and N. J. Buckley, "A microRNA-based gene dysregulation pathway in Huntington's disease," *Neurobiology of Disease*, vol. 29, no. 3, pp. 438–445, 2008.

[87] A. Clop, F. Marcq, H. Takeda, et al., "A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep," *Nature Genetics*, vol. 38, no. 7, pp. 813–818, 2006.

[88] J. A. Chan, A. M. Krichevsky, and K. S. Kosik, "microRNA-21 is an antiapoptotic factor in human glioblastoma cells," *Cancer Research*, vol. 65, no. 14, pp. 6029–6033, 2005.

[89] J. Silber, D. A. Lim, C. Petritsch, et al., "miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells," *BMC Medicine*, vol. 6, article 14, 2008.

[90] J. Pierson, B. Hostager, R. Fan, and R. Vibhakar, "Regulation of cyclin dependent kinase 6 by microRNA 124 in medulloblastoma," *Journal of Neuro-Oncology*, vol. 90, no. 1, pp. 1–7, 2008.

[91] J. S. Wei, Y. K. Song, S. Durinck, et al., "The MYCN oncogene is a direct target of miR-34a," *Oncogene*, vol. 27, no. 39, pp. 5204–5213, 2008.

[92] Y. Chen and R. L. Stallings, "Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis," *Cancer Research*, vol. 67, no. 3, pp. 976–983, 2007.

[93] A. Bottoni, D. Piccin, F. Tagliati, A. Luchin, M. C. Zatelli, and E. C. D. Uberti, "miR-15a and miR-16-1 down-regulation in pituitary adenomas," *Journal of Cellular Physiology*, vol. 204, no. 1, pp. 280–285, 2005.

[94] A. P. Heaney, "Pituitary tumour pathogenesis," *British Medical Bulletin*, vol. 75-76, no. 1, pp. 81–97, 2005.

[95] A. Cimmino, G. A. Calin, M. Fabbri, et al., "miR-15 and miR-16 induce apoptosis by targeting BCL2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 39, pp. 13944–13949, 2005.

[96] S. A. Ciafrè, S. Galardi, A. Mangiola, et al., "Extensive modulation of a set of microRNAs in primary glioblastoma," *Biochemical and Biophysical Research Communications*, vol. 334, no. 4, pp. 1351–1358, 2005.

[97] T. Papagiannakopoulos and K. S. Kosik, "microRNAs: regulators of oncogenesis and stemness," *BMC Medicine*, vol. 6, article 15, 2008.

[98] S. Lawler and E. A. Chiocca, "Emerging functions of microRNAs in glioblastoma," *Journal of Neuro-Oncology*, vol. 92, no. 3, pp. 297–306, 2009.

[99] E. Ferretti, E. De Smaele, A. Po, et al., "microRNA profiling in human medulloblastoma," *International Journal of Cancer*, vol. 124, no. 3, pp. 568–577, 2009.

[100] N. Bushati and S. M. Cohen, "microRNAs in neurodegeneration," *Current Opinion in Neurobiology*, vol. 18, no. 3, pp. 292–296, 2008.

[101] C. Patterson, J. W. Feightner, A. Garcia, G.-Y. R. Hsiung, C. MacKnight, and A. D. Sadovnick, "Diagnosis and treatment of dementia: 1. Risk assessment and primary prevention of Alzheimer disease," *Canadian Medical Association Journal*, vol. 178, no. 5, pp. 548–556, 2008.

[102] A. Orlacchio, G. Bernardi, A. Orlacchio, and S. Martino, "RNA interference as a tool for Alzheimer's disease therapy," *Mini-Reviews in Medicinal Chemistry*, vol. 7, no. 11, pp. 1166–1176, 2007.

[103] E. Costanzi, S. Martino, E. Persichetti, et al., "Effects of vitamin C on fibroblasts from sporadic Alzheimer's disease patients," *Neurochemical Research*, vol. 33, no. 12, pp. 2510–2515, 2008.

[104] E. A. Rogaeva, S. Premkumar, J. Grubber, et al., "An α-2-macroglobulin insertion-deletion polymorphism in Alzheimer disease," *Nature Genetics*, vol. 22, no. 1, pp. 19–22, 1999.

[105] J. W. Freeman, M. Simmons, and E. Matos, "Parkinson disease: an incremental challenge," *South Dakota Medicine*, vol. 60, no. 12, pp. 481–484, 2007.

[106] C. Balducci, L. Pierguidi, E. Persichetti, et al., "Lysosomal hydrolases in cerebrospinal fluid from subjects with Parkinson's disease," *Movement Disorders*, vol. 22, no. 10, pp. 1481–1484, 2007.

[107] W. Stamey and J. Jankovic, "Impulse control disorders and pathological gambling in patients with parkinson disease," *Neurologist*, vol. 14, no. 2, pp. 89–99, 2008.

[108] G. G. Kovacs and H. Budka, "Prion diseases: from protein to cell pathology," *American Journal of Pathology*, vol. 172, no. 3, pp. 555–565, 2008.

[109] V. B. Tripathi and A. Al-Chalabi, "Molecular insights and therapeutic targets in amyotrophic lateral sclerosis," *CNS and Neurological Disorders—Drug Targets*, vol. 7, no. 1, pp. 11–19, 2008.

[110] A. Orlacchio, G. Bernardi, A. Orlacchio, and S. Martino, "Genetics of amyotrophic lateral sclerosis," *Recent Research in Developmental Neuroscience*, pp. 17–54, 2007.

[111] A. Orlacchio, T. Kawarai, A. Totaro, et al., "Hereditary spastic paraplegia: clinical genetic study of 15 families," *Archives of Neurology*, vol. 61, no. 6, pp. 849–855, 2004.

[112] A. Orlacchio, T. Kawarai, F. Gaudiello, P. H. St. George-Hyslop, R. Floris, and G. Bernardi, "New locus for hereditary spastic paraplegia maps to chromosome 1p31.1–1p21.1," *Annals of Neurology*, vol. 58, no. 3, pp. 423–429, 2005.

[113] E. C. Stack and R. J. Ferrante, "Huntington's disease: progress and potential in the field," *Expert Opinion on Investigational Drugs*, vol. 16, no. 12, pp. 1933–1953, 2007.

[114] E. J. Wild and S. J. Tabrizi, "Huntington's disease phenocopy syndromes," *Current Opinion in Neurology*, vol. 20, no. 6, pp. 681–687, 2007.

[115] S. Martino, C. Emiliani, B. Tancini, et al., "Absence of metabolic cross-correction in Tay-Sachs cells: implications for gene therapy," *Journal of Biological Chemistry*, vol. 277, no. 23, pp. 20177–20184, 2002.

[116] S. Martino, C. Cavalieri, C. Emiliani, et al., "Restoration of the GM2 ganglioside metabolism in bone marrow-derived stromal cells from Tay-Sachs disease animal model," *Neurochemical Research*, vol. 27, no. 7-8, pp. 793–800, 2002.

[117] S. Martino, P. Marconi, B. Tancini, et al., "A direct gene transfer strategy via brain internal capsule reverses the biochemical defect in Tay-Sachs disease," *Human Molecular Genetics*, vol. 14, no. 15, pp. 2113–2123, 2005.

[118] S. Martino and A. Orlacchio, "Cellule staminali e GM2 gangliosidosi," *Annali dell'Istituto Superiore di Sanità*, pp. 27–28, 2005.

[119] S. Martino, I. di Girolamo, C. Cavazzin, et al., "Neural precursor cell cultures from GM2 gangliosidosis animal models recapitulate the biochemical and molecular hallmarks of the brain pathology," *Journal of Neurochemistry*, vol. 109, no. 1, pp. 135–147, 2009.

[120] M. S. Weinberg and M. J. A. Wood, "Short non-coding RNA biology and neurodegenerative disorders: novel disease targets and therapeutics," *Human Molecular Genetics*, vol. 18, no. R1, pp. R27–R39, 2009.

[121] M. Goedert and M. G. Spillantini, "A century of Alzheimer's disease," *Science*, vol. 314, no. 5800, pp. 777–781, 2006.

[122] P. T. Lansbury and H. A. Lashuel, "A century-old debate on protein aggregation and neurodegeneration enters the clinic," *Nature*, vol. 443, no. 7113, pp. 774–779, 2006.

[123] R. S. Turner, "Alzheimer's disease," *Seminars in Neurology*, vol. 26, no. 5, pp. 499–506, 2006.

[124] Y. Ohyagi and T. Tabira, "Intracellular amyloid β-protein and its associated molecules in the pathogenesis of Alzheimer's

disease," *Mini-Reviews in Medicinal Chemistry*, vol. 6, no. 10, pp. 1075–1080, 2006.

[125] D. Goldgaber, M. I. Lerman, and O. W. McBride, "Characterization and chromosomal localization of a cDNA encoding-brain amyloid of Alzheimer's disease," *Science*, vol. 235, no. 4791, pp. 877–880, 1987.

[126] C. E. Hunt and A. J. Turner, "Cell biology, regulation and inhibition of β-secretase (BACE-1)," *FEBS Journal*, vol. 276, no. 7, pp. 1845–1859, 2009.

[127] W.-X. Wang, B. W. Rajeev, A. J. Stromberg, et al., "The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of β-site amyloid precursor protein-cleaving enzyme 1," *Journal of Neuroscience*, vol. 28, no. 5, pp. 1213–1223, 2008.

[128] S. Bicker and G. Schratt, "microRNAs: tiny regulators of synapse function in development and disease," *Journal of Cellular and Molecular Medicine*, vol. 12, no. 5A, pp. 1466–1476, 2008.

[129] D. C. Carrettiero, I. Hernandez, P. Neveu, T. Papagiannakopoulos, and K. S. Kosik, "The cochaperone BAG2 sweeps paired helical filament-insoluble tau from the microtubule," *Journal of Neuroscience*, vol. 29, no. 7, pp. 2151–2161, 2009.

[130] M. M. Hoehn and M. D. Yahr, "Parkinsonism: onset, progression, and mortality," *Neurology*, vol. 57, no. 10, pp. S11–S26, 2001.

[131] R. L. Nussbaum and M. H. Polymeropoulos, "Genetics of Parkinson's disease," *Human Molecular Genetics*, vol. 6, no. 10, pp. 1687–1691, 1997.

[132] M. H. Polymeropoulos, C. Lavedan, E. Leroy, et al., "Mutation in the α-synuclein gene identified in families with Parkinson's disease," *Science*, vol. 276, no. 5321, pp. 2045–2047, 1997.

[133] V. M. Miller, H. Xia, G. L. Marrs, et al., "Allele-specific silencing of dominant disease genes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 12, pp. 7195–7200, 2003.

[134] P. Gonzalez-Alegre, V. M. Miller, B. L. Davidson, and H. L. Paulson, "Toward therapy for DYT1 dystonia: allele-specific silencing of mutant Torsin A," *Annals of Neurology*, vol. 53, no. 6, pp. 781–787, 2003.

[135] P. van den Munckhof, K. C. Luk, L. Ste-Marie, et al., "Pitx3 is required for motor activity and for survival of a subset of midbrain dopaminergic neurons," *Development*, vol. 130, no. 11, pp. 2535–2542, 2003.

[136] J. Fuchs, J. C. Mueller, P. Lichtner, et al., "The transcription factor PITX3 is associated with sporadic Parkinson's disease," *Neurobiology of Aging*, vol. 30, no. 5, pp. 731–738, 2009.

[137] T. L. Cuellar, T. H. Davis, P. T. Nelson, et al., "Dicer loss in striatal neurons produces behavioral and neuroanatomical phenotypes in the absence of neurodegeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 14, pp. 5614–5619, 2008.

[138] A. H. Evans and A. J. Lees, "Dopamine dysregulation syndrome in Parkinson's disease," *Current Opinion in Neurology*, vol. 17, no. 4, pp. 393–398, 2004.

[139] G. Wang, J. M. van der Walt, G. Mayhew, et al., "Variation in the miRNA-433 binding site of FGF20 confers Risk for Parkinson disease by overexpression of α-synuclein," *American Journal of Human Genetics*, vol. 82, no. 2, pp. 283–289, 2008.

[140] H. T. Orr and H. Y. Zoghbi, "Trinucleotide repeat disorders," *Annual Review of Neuroscience*, vol. 30, pp. 575–621, 2007.

[141] A. Schaefer, D. O'Carroll, L. T. Chan, et al., "Cerebellar neurodegeneration in the absence of microRNAs," *Journal of Experimental Medicine*, vol. 204, no. 7, pp. 1553–1558, 2007.

[142] J. Bilen, N. Liu, and N. M. Bonini, "A new role for microRNA pathways: modulation of degeneration induced by pathogenic human disease proteins," *Cell Cycle*, vol. 5, no. 24, pp. 2835–2838, 2006.

[143] K. Nakamura and M. J. Aminoff, "Huntington's disease: clinical characteristics, pathogenesis and therapies," *Drugs of Today*, vol. 43, no. 2, pp. 97–116, 2007.

[144] S. Q. Harper, P. D. Staber, X. He, et al., "RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 16, pp. 5820–5825, 2005.

[145] E. Rodriguez-Lebron, E. M. Denovan-Wright, K. Nash, A. S. Lewin, and R. J. Mandel, "Intrastriatal rAAV-mediated delivery of anti-huntingtin shRNAs induces partial reversal of disease progression in R6/1 Huntington's disease transgenic mice," *Molecular Therapy*, vol. 12, no. 4, pp. 618–633, 2005.

[146] J. L. McBride, R. L. Boudreau, S. Q. Harper, et al., "Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 15, pp. 5868–5873, 2008.

[147] Y. Li, L. Lin, and P. Jin, "The microRNA pathway and fragile X mental retardation protein," *Biochimica et Biophysica Acta*, vol. 1779, no. 11, pp. 702–705, 2008.

[148] S. T. Warren and S. L. Sherman, "The fragile X syndrome," in *The Metabolic and Molecular Bases of Inherited Disease*, vol. 1, pp. 1257–1290, McGraw-Hill, New York, NY, USA, 2001.

[149] S. T. Warren and D. L. Nelson, "Advances in molecular analysis of fragile X syndrome," *Journal of the American Medical Association*, vol. 271, no. 7, pp. 536–542, 1994.

[150] G. J. Bassell and S. T. Warren, "Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function," *Neuron*, vol. 60, no. 2, pp. 201–214, 2008.

[151] A. A. Caudy, M. Myers, G. J. Hannon, and S. M. Hammond, "Fragile X-related protein and VIG associate with the RNA interference machinery," *Genes and Development*, vol. 16, no. 19, pp. 2491–2496, 2002.

[152] A. Ishizuka, M. C. Siomi, and H. Siomi, "A *Drosophila fragile* X protein interacts with components of RNAi and ribosomal proteins," *Genes and Development*, vol. 16, no. 19, pp. 2497–2508, 2002.

[153] P. Jin, D. C. Zarnescu, S. Ceman, et al., "Biochemical and genetic interaction between the fragile X mental retardation protein 3nd the microRNA pathway," *Nature Neuroscience*, vol. 7, no. 2, pp. 113–117, 2004.

[154] I. Plante, L. Davidovic, D. L. Ouellet, et al., "Dicer-derived microRNAs are utilized by the fragile X mental retardation protein for assembly on target RNAs," *Journal of Biomedicine and Biotechnology*, vol. 2006, Article ID 64347, 12 pages, 2006.

[155] P. Jin, R. S. Alisch, and S. T. Warren, "RNA and microRNAs in fragile X mental retardation," *Nature Cell Biology*, vol. 6, no. 11, pp. 1048–1053, 2004.

[156] M. P. Perron and P. Provost, "Protein components of the microRNA pathway and human diseases," *Methods in Molecular Biology*, vol. 487, pp. 369–385, 2009.

[157] A. Cheever and S. Ceman, "Translation regulation of mRNAs by the fragile X family of proteins through the microRNA pathway," *RNA Biology*, vol. 6, no. 2, pp. 175–178, 2009.

[158] M. Costa-Mattioli, W. S. Sossin, E. Klann, and N. Sonenberg, "Translational control of long-lasting synaptic plasticity and memory," *Neuron*, vol. 61, no. 1, pp. 10–26, 2009.

[159] R. Duan and P. Jin, "Identification of messenger RNAs and microRNAs associated with fragile X mental retardation protein," *Methods in Molecular Biology*, vol. 342, pp. 267–276, 2006.

[160] A. Cheever and S. Ceman, "Phosphorylation of FMRP inhibits association with Dicer," *RNA*, vol. 15, no. 3, pp. 362–366, 2009.

[161] X.-L. Xu, Y. Li, F. Wang, and F.-B. Gao, "The steady-state level of the nervous-system-specific microRNA-124a is regulated by dFMR1 in *Drosophila*," *Journal of Neuroscience*, vol. 28, no. 46, pp. 11883–11889, 2008.

[162] Y. Yang, S. Xu, L. Xia, et al., "The bantam microRNA is associated with *Drosophila fragile* X mental retardation protein and regulates the fate of germline stem cells," *PLoS Genetics*, vol. 5, no. 4, article e1000444, 2009.

[163] T. Nomura, M. Kimura, T. Horii, et al., "MeCP2-dependent repression of an imprinted miR-184 released by depolarization," *Human Molecular Genetics*, vol. 17, no. 8, pp. 1192–1199, 2008.

[164] N. R. Smalheiser and G. Lugli, "microRNA Regulation of Synaptic Plasticity," *NeuroMolecular Medicine*, vol. 11, no. 3, pp. 133–140, 2009.

[165] J. F. Abelson, K. Y. Kwan, B. J. O'Roak, et al., "Sequence variants in SLITRK1 are associated with Tourette's syndrome," *Science*, vol. 310, no. 5746, pp. 317–320, 2005.

[166] L. Zhang, J. Huang, N. Yang, et al., "microRNAs exhibit high frequency genomic alterations in human cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 24, pp. 9136–9141, 2006.

[167] A. Esquela-Kerscher and F. J. Slack, "Oncomirs—microRNAs with a role in cancer," *Nature Reviews Cancer*, vol. 6, no. 4, pp. 259–269, 2006.

[168] T. Papagiannakopoulos, A. Shapiro, and K. S. Kosik, "microRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells," *Cancer Research*, vol. 68, no. 19, pp. 8164–8172, 2008.

[169] M. S. Nicoloso and G. A. Calin, "microRNA involvement in brain tumors: from bench to bedside," *Brain Pathology*, vol. 18, no. 1, pp. 122–129, 2008.

[170] G. A. Calin, C. Sevignani, C. D. Dumitru, et al., "Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 9, pp. 2999–3004, 2004.

[171] S. Volinia, G. A. Calin, C.-G. Liu, et al., "A microRNA expression signature of human solid tumors defines cancer gene targets," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 7, pp. 2257–2261, 2006.

[172] L. He, J. M. Thomson, M. T. Hemann, et al., "A microRNA polycistron as a potential human oncogene," *Nature*, vol. 435, no. 7043, pp. 828–833, 2005.

[173] G. A. Calin and C. M. Croce, "microRNA signatures in human cancers," *Nature Reviews Cancer*, vol. 6, no. 11, pp. 857–866, 2006.

[174] C. R. Woese, *The Genetic Code*, Harper and Row, New York, NY, USA, 1968.

[175] T. A. Lincoln and G. F. Joyce, "Self-sustained replication of an RNA enzyme," *Science*, vol. 323, no. 5918, pp. 1229–1232, 2009.

[176] P. A. Sharp, "The Centrality of RNA," *Cell*, vol. 136, no. 4, pp. 577–580, 2009.

[177] T. R. Cech, "Crawling out of the RNA world," *Cell*, vol. 136, no. 4, pp. 599–602, 2009.

[178] C. D. Malone and G. J. Hannon, "Small RNAs as guardians of the genome," *Cell*, vol. 136, no. 4, pp. 656–668, 2009.

[179] W. Kong, J.-J. Zhao, L. He, and J. Q. Cheng, "Strategies for profiling microRNA expression," *Journal of Cellular Physiology*, vol. 218, no. 1, pp. 22–25, 2009.

[180] L. Bonetta, "RNA-based therapeutics: ready for delivery?" *Cell*, vol. 136, no. 4, pp. 581–584, 2009.

[181] T. Thum, C. Gross, J. Fiedler, et al., "microRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts," *Nature*, vol. 456, no. 7224, pp. 980–984, 2008.

[182] T. Würdinger and B. A. Tannous, "Glioma angiogenesis: towards novel RNA therapeutics," *Cell Adhesion and Migration*, vol. 3, no. 2, pp. 230–235, 2009.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 659028, 9 pages
doi:10.1155/2009/659028

Methodology Report

Quantification of Small Non-Coding RNAs Allows an Accurate Comparison of miRNA Expression Profiles

Andrea Masotti,[1] Viviana Caputo,[2,3] Letizia Da Sacco,[1] Antonio Pizzuti,[2,3] Bruno Dallapiccola,[2,3] and Gian Franco Bottazzo[4]

[1] *Gene Expression and Microarrays Laboratory, Bambino Gesú Children's Hospital, P. za S. Onofrio 4, 00165 Rome, Italy*
[2] *IRCCS-CSS, San Giovanni Rotondo and CSS-Mendel Institute, Viale Regina Margherita 261, 00198 Rome, Italy*
[3] *Department of Experimental Medicine and Pathology, University La Sapienza, Viale Regina Elena 324, 00161 Rome, Italy*
[4] *Scientific Directorate, Bambino Gesú Children's Hospital, P. za S. Onofrio 4, 00165 Rome, Italy*

Correspondence should be addressed to Andrea Masotti, andrea.masotti@opbg.net

Received 1 January 2009; Revised 17 April 2009; Accepted 4 June 2009

Recommended by Daniel Gautheret

MicroRNAs (miRNAs) are highly conserved ~22-mer RNA molecules, encoded by plants and animals that regulate the expression of genes binding to the 3′-UTR of specific target mRNAs. The amount of miRNAs in a total RNA sample depends on the recovery efficiency that may be significantly affected by the different purification methods employed. Traditional approaches may be inefficient at recovering small RNAs, and common spectrophotometric determination is not adequate to quantify selectively these low molecular weight (LMW) species from total RNA samples. Here, we describe the use of qualitative and quantitative *lab-on-a-chip* tools for the analysis of these LMW RNA species. Our data emphasize the close correlation of LMW RNAs with the expression levels of some miRNAs. We therefore applied our result to the comparison of some miRNA expression profiles in different tissues. Finally, the methods we used in this paper allowed us to analyze the efficiency of extraction protocols, to study the small (but significant) differences among various preparations and to allow a proper comparison of some miRNA expression profiles in various tissues.

1. Introduction

In the last few years, a new class of highly conserved ~21-mer non-coding RNAs, microRNAs (miRNAs), has emerged as an important player in post-transcriptional gene expression control in different physiological and pathological conditions: metabolism, proliferation, cell death, differentiation and development, viral infection, and cancer [1–3]. They specifically bind the 3′-untranslated regions (3′-UTR) of target mRNAs, promoting either mRNA degradation or translation arrest [4–6]. Each miRNA may control the activity of many genes, and almost 30% of the genome could be regulated in such a way, which renders these small molecules as important as the transcription factors [7, 8].

Owing to their extreme importance as regulators, the isolation and the precise quantification of these tiny molecules are therefore fundamental. As purity and integrity are essential requirements not only for total RNA but also for these small species, RNA extraction protocols must also account for the maximizing of their recovery. Sometimes, the handling and recovery of small RNAs are not straightforward. Nowadays, it is well established that the traditional glass-fiber total RNA extraction protocol may be inefficient at recovering small RNAs. In addition, the common spectrophotometric determination of total RNA is not adequate to quantify low molecular weight (LMW) species selectively.

We and other authors found previously that the recovery of LMW RNA species is significantly affected by the specific purification process [3, 9]. Our results showed that different extraction strategies lead to significantly different recovery of LMW species including miRNAs. We also demonstrated that using the same amount of total RNA (from different tissues), different amounts of miRNAs may be obtained. Moreover, the amount of LMW RNA species does not perfectly parallel that of miRNAs: even with the same extraction protocol,

the concentration of miRNAs may differ significantly among various tissues. Nevertheless, this may also be because of a different global expression of miRNA by different tissues. All of these considerations are extremely important when one compares the relative expression of many miRNAs in different tissues. Commonly, some small non-coding RNAs (i.e., U6, snoZ30, and others) are used as normalization controls to compare miRNAs expression in different tissues. The choice of a proper control should be conditioned to its real "endogenous behavior", that is, it should have a constant and equal expression in all tissues. If the amount of that control small non-coding RNA is different, a relative quantification is not feasible. In this latter case, only the comparison between various miRNAs and the control gene in the same specimen (tissue) is methodologically acceptable. Therefore, a method allowing a reliable estimation of the goodness of an endogenous control is highly desirable.

In order to address all of these issues we used a *lab-on-a-chip* technology to characterize the LMW RNA fractions obtained with different RNA extraction protocols and evaluated miRNAs recovery with specific quantitative real-time PCR (qRT-PCR).

In this paper we report and discuss the methods that allowed us to analyze the efficiency of extraction protocols, to study the small (but significant) differences between various preparations and to obtain a correct comparison of some miRNA expression profiles in various tissues.

2. Materials and Methods

2.1. Total RNA Extraction and Small RNAs Enrichment Protocols. Total RNA was extracted using three different methods: an acid phenol/guanidine isothiocyanate solution (TRIzol Reagent, Invitrogen), a glass-fiber filtration protocol (MirVana miRNA Isolation Kit, Ambion) that provides also a procedure to isolate and enrich low molecular weight (LMW) RNAs from higher molecular weight (HMW) RNAs, and another common glass-fiber purification protocol (RNEasy Mini Kit, Qiagen). All extractions were performed according to manufacturer's instructions.

2.2. Cell Lines. Three different cell lines (HeLa, COS-1 and a lymphoblastoid cell line (LCL) obtained following an already reported method [10]) were cultured using standard procedures, trypsinized (if necessary), and pelleted by centrifugation. Approximately 10^7 cells for each extraction were resuspended in the appropriate lysis solution contained in the RNA extraction kit and treated according to manufacturer's instructions.

2.3. Electrophoresis and RNA Elution from Gel. The integrity of RNA samples was checked by gel electrophoresis (agarose 1%) stained with ethidium bromide. Gel images were acquired and analyzed with the Quantity One (software Ver.2.0, Biorad). Two micrograms of each RNA samples were resolved into a 15% polyacrylamide TBE-Urea gel and the image acquired. A second polyacrylamide gel, performed with the same experimental conditions, was used for RNA bands extraction. The visible bands were separately excised using a modified "*crush-and-soak*" method [11]. Briefly, each band was gently disrupted with a pin and incubated in a soaking solution (0.5 M ammonium acetate, 2 mM EDTA, 0.1% SDS) for 3 hours at 37°C. The mixture was centrifuged at 14000 g for 5 minutes, and the supernatant was collected in a new tube. The solution was centrifuged at 12000 g for 10 minutes through a YM-50 spin column, washed with water ($3 \times 300\ \mu l$), and centrifuged at 12000 g for 5 minutes. Purified fractions were collected in new tubes by inverting the columns and spinning at 1000 g for 3 minutes. These RNA samples were then separately run on the Agilent Bioanalyzer.

2.4. Agilent 2100 Bioanalyzer Chip Preparation. Total RNA samples were analyzed with the Total RNA 6000 Nano Kit (Vers. II), specifically optimized for total RNA analysis with the Agilent 2100 Bioanalyzer. RNA samples obtained after extraction from polyacrylamide gel were run with the same kit. For miRNA quantification we used the dedicated Small RNA kit. The instrument uses fluorescence detection, monitoring the emission between 670 nm and 700 nm. The run was performed according to manufacturer's instructions. Electropherograms were analyzed using the Agilent 2100 Expert B.02.06 software that includes data collection, presentation, and interpretation functions.

2.5. Real-Time PCR. For the small RNAs recovery efficiency evaluation of different extraction procedures, hsa-miR-21 and small nucleolar Z30 (snoZ30) were assayed by Real-Time PCR (TaqMan, Applied Biosystems), according to manufacturer's instructions [12]. For the analysis of miRNA expression in different tissues, three miRNAs (hsa-miR-26a, hsa-miR-26b, and hsa-miR-134) and two controls (U6 and snoZ30) were arbitrarily chosen as model miRNAs. A panel of five tissues (brain, skeletal muscle, heart, liver, and uterus) was chosen for evaluation, and the corresponding total RNA (Clontech, BD Biosciences) was analyzed with Agilent 2100 Bioanalyzer. Briefly, from five to ten nanograms of each RNA samples were retrotranscribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) with specific primers. The principle of TaqMan MicroRNA Assays is a specific stem-loop reverse transcription (RT) primer. The short length of mature miRNAs (~22 nt) prohibits conventional design of a random-primed RT step followed by a specific real-time assay. In the former case, the resulting RT amplicon is a suitable template for standard real-time PCR with TaqMan assays. Reactions were performed incubating samples for 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and finally cooled on ice. PCR products were assayed with specific probes using the TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) following the manufacturer's protocol. PCR reactions were performed incubating samples for 10 minutes at 95°C, then for 15 seconds at 95°C and 60 seconds at 60°C for 45 cycles by means of ABI PRISM 7900HT Sequence Detection System. Data were analyzed using the SDS software (Ver. 2.1).

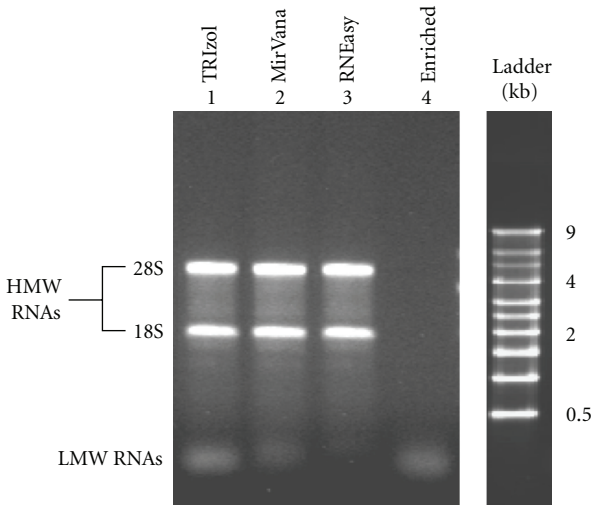


Figure 1: Gel electrophoresis (agarose 1% stained with ethidium bromide) of RNA samples (from COS-1) extracted with TRIzol reagent (lane 1), MirVana kit (lane 2), RNEasy kit (lane 3), and LMW RNA fraction enriched with MirVana kit (lane 4).

2.6. Deconvolution with PeakFit Software. The early electropherogram region (from 23 to 29 seconds), obtained from LCL RNA sample extracted with TRIzol reagent, was exported in ASCII format with the Agilent 2100 Expert B.02.06 and imported in PeakFit V.4.12. The curve was automatically fitted using the least-squares method. Data were further adjusted using the Savitzky-Golay smoothing algorithm. The overall model was fitted with chromatographic Gaussian curves of variable amplitudes. The fitting procedure was iterated until a constant r^2 value (r^2 = 0.998693) was obtained.

3. Results

3.1. The Recovery of Low Molecular Weight (LMW) RNAs Is Affected by Different Extraction Protocols. RNA samples extracted from HeLa, COS-1, and LCL were run on agarose gel to visualize the differences between various extraction methods. COS-1 RNA samples, extracted with TRIzol reagent and MirVana kit, clearly showed the High Molecular Weight (HMW) 28S and 18S rRNA bands, while LMW RNAs are visualized as faint, smeary bands (Figure 1, lanes 1 and 2, resp.). COS-1 extracted with RNeasy kit displayed only the HMW RNA bands (28S and 18S) (Figure 1, lane 3), while the enriched LMW fraction obtained with MirVana kit is clearly displayed in the lowest part of the gel (Figure 1, lane 4). Similar results (not shown) were also obtained with the other cell lines.

The same samples were also checked with Agilent 2100 Bioanalyzer which is one of the most versatile microfluidics-based platforms for the analysis of DNA, RNA, proteins and cells. In all electropherograms the 28S and 18S RNAs are represented on the right side (Figure 2), and the smaller species (LMW RNAs) are present at a very low concentration

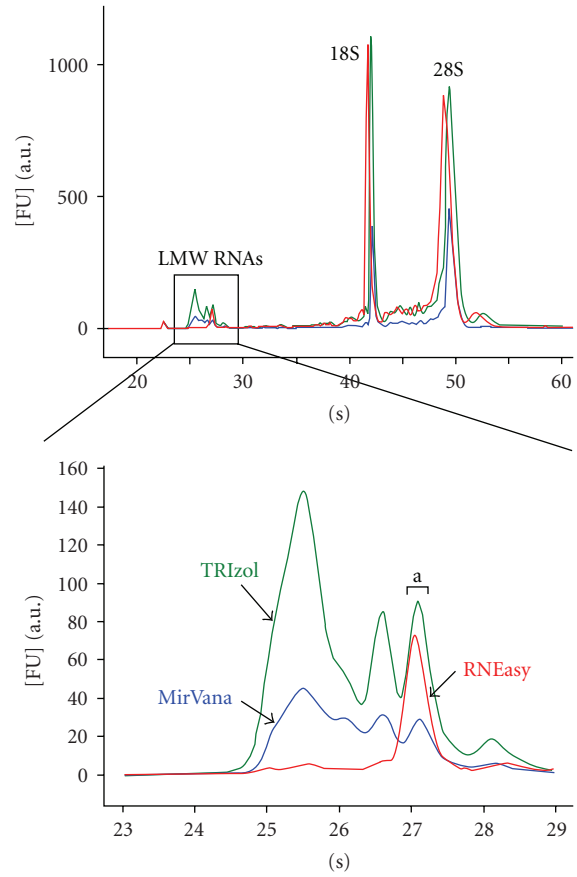


Figure 2: Agilent 2100 Bioanalyzer electropherogram profiles of total RNA samples (HeLa cells) extracted with TRIzol reagent (green), MirVana kit (blue), and RNEasy kit (red). Inbox: magnification of small RNA profiles for the three samples (between 23 and 29 seconds).

and distinguishable on the left side of the profile (see the magnification of the LMW RNAs region in Figure 2). In the electropherogram, all samples present a similar HMW profile region, but the major differences are recognizable in the LMW region. While total RNA recovery is quite good, and similar for the three protocols (> 1.2 μg/μl), the LMW fractions are substantially different (Table 1). In particular, TRIzol reagent allowed the highest LMW RNA recovery (22%–34% of total RNA), while RNEasy Mini Kit the lowest (2.5%–3%). MirVana miRNA Isolation Kit gives good yields for LMW RNA species (16%–19%) even before the enrichment step. While LMW RNA species extracted with TRIzol and MirVana have comparable profiles (Figure 2); RNEasy kit retains only one RNA peak in comparable concentrations to the others (peak *a* in the magnification of Figure 2). Therefore, the *lab-on-a-chip* analysis is a useful tool to quantify precisely the amount of LMW RNAs of samples extracted with different protocols.

3.2. Low Molecular Weight (LMW) RNAs Characterization. Total RNAs from LCL extracted with TRIzol reagent, and RNEasy kit and enriched with MirVana kit were loaded on

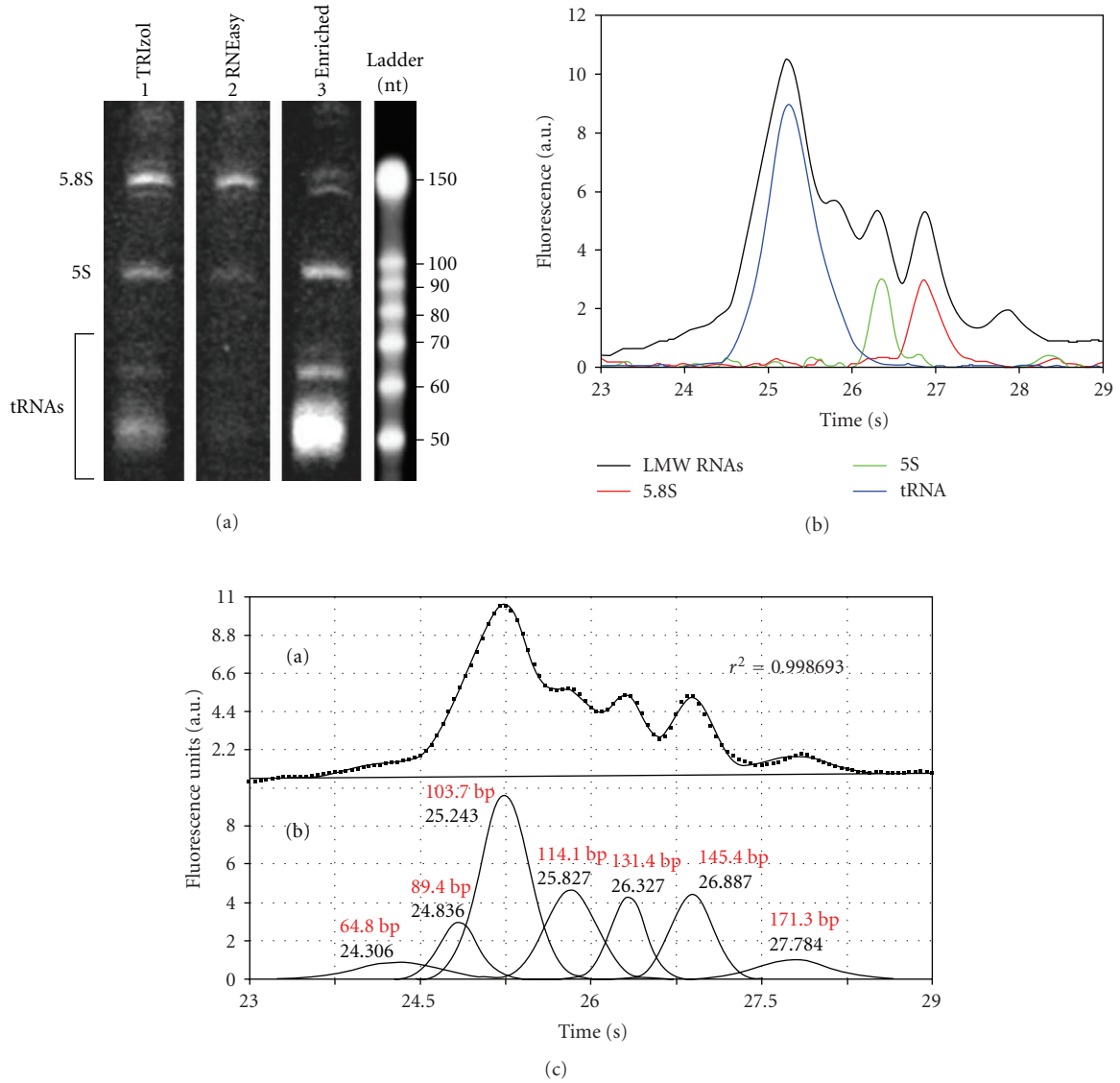


Figure 3: (a) Gel electrophoresis (polyacrylamide 15% stained with ethidium bromide) of LMW RNA samples (LCL) extracted with TRIzol reagent (lane 1), RNEasy kit (lane 2), and small RNA fraction enriched with MirVana kit (lane 3). LMW profile obtained with MirVana kit extraction is similar to that obtained with TRIzol reagent which is not shown for clarity. (b) Agilent 2100 Bioanalyzer electropherogram profile of LMW RNAs (LCL) extracted with TRIzol (black) superimposed on 5.8S (red), 5S (green), and tRNA (blue) bands eluted from polyacrylamide gel. (c) Lymphoblastoid (LCL) LMW RNA profile obtained after plotting the exported raw data from Agilent electropherogram (■) together with the PeakFit fitted curve (solid line) and the component peak functions. Seven peaks below the LMW RNA profile were fitted by the software ($r^2 = 0.998693$).

a polyacrylamide gel (Figure 3(a)) to analyze the differences between various extraction protocols. All the RNA bands were cut out from the gel, and the RNA was extracted as described. Each species coming from excised bands was run on Bioanalyzer, and the corresponding electropherograms (Figure 3(b)) were superimposed on the large and unresolved LMW RNA profile (black line). This allowed us to localize unambiguously and identify precisely each RNA species previously identified on polyacrylamide gel.

The electropherogram profile in the LMW RNAs region is made of several peaks (Figure 3(b)), the greatest part belonging to the 5.8S rRNA (red line), the 5S rRNA (green line), and the tRNAs fractions (blue line). In order to obtain the principal number of peaks under the curve, PeakFit software was used for the deconvolution of the LMW RNA region. Seven peaks underlying the LMW RNA profile were obtained with a good fit ($r^2 = 0.998693$). The upper part of Figure 3(c) reports raw data (■) together with the fitted curve (solid line) and the calculated baseline, while the lower part of Figure 3(c) shows the component peak functions. Calculated peak-peaking (expressed in bp) is also reported (Figure 3(c)). Three of the calculated peaks centered at 145 bp (26.89 seconds), 131 bp (26.33 seconds), and 104 bp (25.24 seconds) are located in the correspondence

Table 1: Low molecular weight (LMW) RNA mean concentration (% with respect to total RNA) for HeLa, COS-1 and LCL extracted with different RNA extraction protocols evaluated with Agilent 2100 Bioanalyzer. Standard deviations of at least three independent extractions are reported in parentheses.

	LMW RNA Mean concentration (%)		
Extraction method (total RNA)	HeLa	COS-1	LCL
TRIzol reagent (acid phenol/guanidine isothiocyanate)	24 (±3)	34 (±2)	22 (±3)
MirVana kit	16 (±1.5)	19 (±1)	19 (±1)
RNEasy kit	2.5 (±0.5)	3 (±0.5)	3 (±0.5)

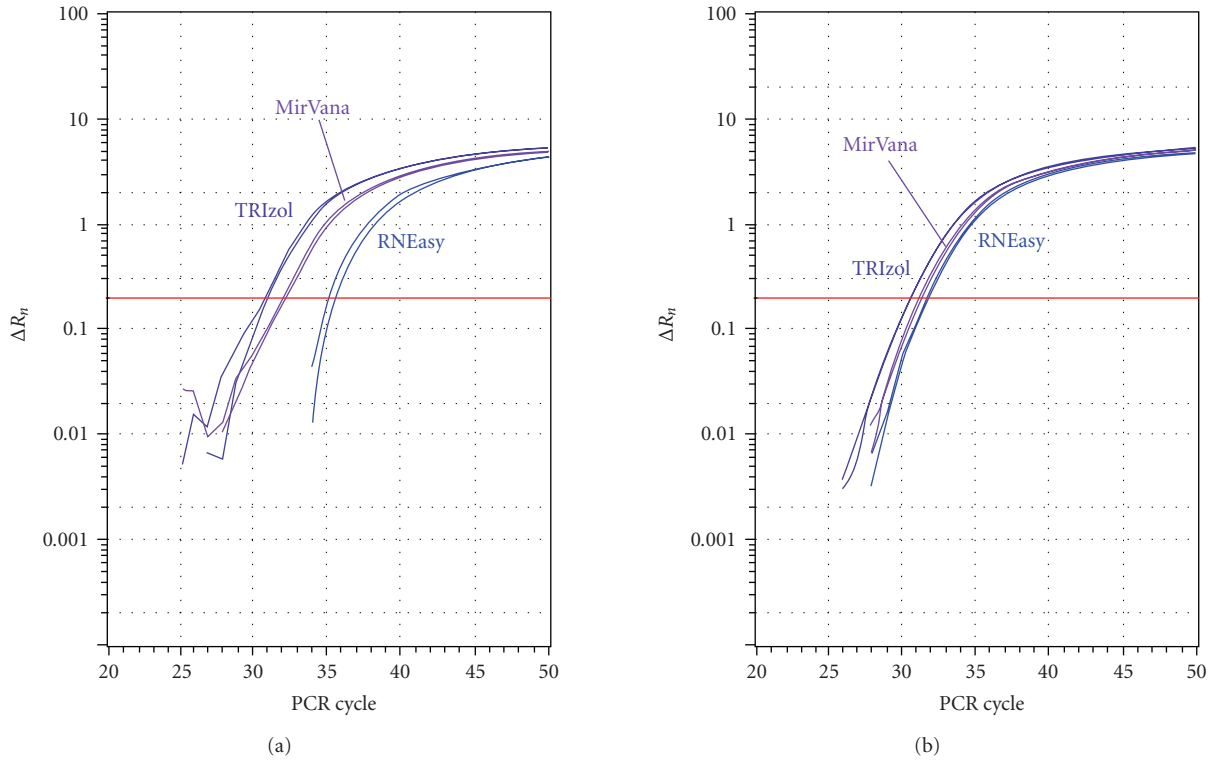


Figure 4: Small nucleolar Z30 real-time PCR assay (two replicas) of RNA samples (LCL) obtained starting from 10 ng of total RNA samples (a) and from 10 ng of LMW RNA (b) extracted with different protocols. In the latter case Ct values of samples from different extractions are more reproducible, indicating a strict correlation between the amount of miRNAs and the LMW RNA fraction.

of 5.8S, 5S, and tRNA peaks, respectively. Another less intense peak is located at 114 bp (25.83 seconds) and may be described as a further tRNA peak. Other peaks found at 171 bp (27.78 seconds), 89 bp (24.84 seconds) and 65 bp (24.31 seconds) might belong to other LMW RNA species, such as smaller tRNAs, pre-miRNAs, and small nuclear or nucleolar RNAs. These small peaks are difficult to extract from polyacrylamide gel owing to their low abundance, and therefore it was not possible to run them on the chip.

Therefore, determination of small RNA species by Bioanalyzer (Table 1) and by polyacrylamide gel electrophoresis (Figure 3(a)) showed that the LMW RNA samples significantly differ from each other. This could reasonably pertain also to the miRNAs fraction.

3.3. MicroRNAs Amount Correlates with the LMW RNA Fraction. In order to quantify miRNAs extracted with different protocols and to study the correlation between LMW RNAs and miRNAs, we carried out a TaqMan quantitative assay for two representative small RNAs: a control non-coding RNA (snoZ30) and a miRNA (hsa-miR-21). A target-specific stem-loop adapter technology was employed to obtain the corresponding cDNA [12].

We started the RT reaction with 10 ng of total RNA from each sample and quantified the absolute expression level of each miRNAs through the analysis of cycle threshold (Ct) values. Ct is the PCR cycle at which the sample reaches the level of detection above the background. LCL RNA samples extracted with different protocols showed different Ct values for snoZ30 (Figure 4(a)) and for miR-21. A similar behavior was also obtained by using HeLa and COS-1 cell lines with both probes (data not shown).

Then, we repeated the RT reaction using 10 ng of LMW RNA calculated from the Bioanalyzer electropherogram. As

Table 2: Real-time PCR data obtained for HeLa and LCL were compared calculating the ΔCt differences between Ct (10 ng of total RNA) and Ct (10 ng of LMW RNA) values for miR-21 and snoZ30. Standard deviations of at least three independent assays are reported in parentheses. LMW RNA concentrations (%) calculated using the formula $2^{-\Delta Ct} \cdot 100$ are also reported where ΔCt are referred to snoZ30 values. Standard deviations of at least three independent assays are reported in parentheses.

		ΔC_t (total RNA-LMW RNA)		Calculated LMW RNA (%) ($2^{-\Delta Ct(snoZ30)} \cdot 100$)
Cell Type	Extraction Method	miR-21	snoZ30	
HeLa	TRIzol reagent	1.8 (2)	2.1 (1)	23 (±2)
	MirVana kit	2.5 (1)	2.9 (1)	13 (±1)
	RNEasy kit	3.5 (4)	5.1 (3)	3 (±0.5)
LCL	TRIzol reagent	2.3 (1)	2.4 (1)	19 (±1)
	MirVana kit	2.2 (1)	2.1 (4)	23 (±5)
	RNEasy kit	4.2 (2)	4.6 (4)	4 (±1)

expected, using the same LMW RNA amount similar Ct values both for miR-21 and snoZ30 between the various samples were obtained (Figure 4(b)). This demonstrates that the amount of miR-21 and snoZ30 correlates only to the LMW RNA fraction and not to total RNA amount. In fact, Figure 4(a) clearly shows that using the same amount of total RNA different Ct values may be obtained.

Ct differences (ΔC_t) between total RNA and LMW RNA Ct values for each extraction protocol and for each cell line are very similar both for hsa-miR-21 and snoZ30 (Table 2). This demonstrates that ΔC_t differences parallel the amount of input RNA that is ultimately linked to the amount extracted by various protocols. Table 2 reports also the calculated LMW RNA concentration (expressed in %) calculated from ΔC_t values applying the formula $2^{-\Delta Ct}$. MiRNA concentrations calculated from real-time assays are, as expected, in perfect agreement with LMW RNAs concentrations evaluated with the *lab-on-a-chip* technology.

3.4. Different Tissues Express Different Amounts of Small- and MicroRNAs. Here, we showed that LMW RNAs and miRNAs amount are closely correlated. Then, we asked if different tissues might also express different amounts of LMW and miRNAs and if it might be possible to quantify them individually with *lab-on-a-chip* technology. For this reason we selected five commercial RNA samples from different tissues extracted with the same protocol (according to manufacturer information). Total RNAs from brain, skeletal muscle, heart, liver, and uterus were run on RNA 6000 Nano kit to quantify the total and the LMW RNA fractions. Total RNA concentrations were quite homogeneous and resulted: brain (1159 ng/μL) > heart (1106 ng/μL) > uterus (1080 ng/μL) > liver (963 ng/μL) > skeletal muscle (890 ng/μL). These values are in good agreement with the nominal manufacturer's concentration of 1000 ng/μL. For all samples the concentration of LMW RNA fraction was quite homogeneous and varied in the order: liver (43 ng/μL) > brain (29 ng/μL) > heart (26 ng/μL) > uterus (25 ng/μL) > skeletal muscle (23 ng/μL). Electropherograms of LMW RNAs of different tissues are displayed in Figure 5(a). Calculating the percentage of LMW RNAs with respect to the whole total RNA amount we found the following: liver (4.4%) > skeletal muscle (2.6%) > brain (2.5%) > heart (2.3%) > uterus (2.3%). Only some minor differences may be observed among various tissues. Given that the extraction protocol employed by the manufacturer was the same for all samples, we could hypothesize that the only observed difference for liver might depend on the different global expression of small RNAs in this tissue.

To evaluate if the difference in LMW RNA amount may also pertain to the miRNA fraction, we ran the total RNA samples on Agilent Small RNA kit, specifically designed for the evaluation of miRNAs. Figure 5(b) shows a magnification of the electropherogram profile obtained by running total RNA samples from different tissues. The displayed region (from 35 to 45 seconds) is specific to the miRNA region (as indicated by the manufacturer). MiRNA concentrations were liver (700 pg/μL) > brain (510 pg/μL) > uterus (404 pg/μL) > heart (226 pg/μL) > skeletal muscle (71 pg/μL). The miRNA concentration, expressed in percentage, respect to LMW RNAs resulted brain (1.7%) > liver (1.6%) = uterus (1.6%) > heart (0.9%) > skeletal muscle (0.3%). From this *lab-on-a-chip* quantification it was possible to conclude that the miRNAs amount does not parallel that of LMW RNAs in the same tissue. Moreover, the variation is greater than that displayed by LMW RNAs among different tissues. Again, assuming that the extraction efficiency is the same for all samples, the observed differences are only due to the different global expression of miRNAs in these tissues.

3.5. Evaluation of Endogenous Controls' Reliability and Their Use for Expression Profile Comparison. The Small RNA kit, specifically designed for the identification and quantification of miRNA species, allowed us to know the exact amount of miRNAs present in the samples used for retrotranscription (RT) reactions. This information is useful, since the Ct values obtained from real-time assays are directly proportional to the amount of miRNAs. In fact, the Ct differences observed for the same miRNA in different tissues may be due essentially (1) to a real difference between different samples and/or (2) to a different sample dilution that generates a shift in Ct absolute values. To eliminate the dilution problem, that occurs when absolute quantification is performed, a relative quantification with respect to an endogenous control is commonly followed. In fact, the endogenous control must

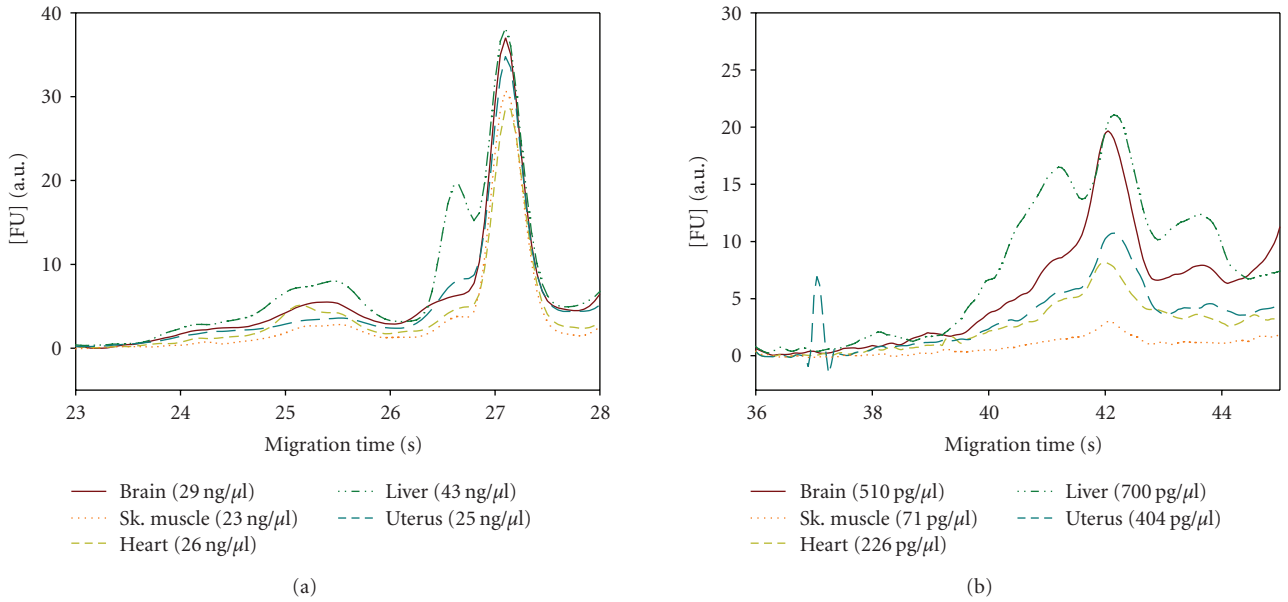


Figure 5: (a) Electropherogram profile of LMW RNA species of five different tissues (brain, skeletal muscle, heart, liver, and uterus). (b) Electropherogram profile of miRNA species of the same tissues.

have a constant expression in all samples, and hence it may be used to normalize the expression of the other miRNAs. For a methodologically correct comparison, however, one must be confident that the control (i.e., nuclear and/or nucleolar small RNAs) really does have a constant expression in considered samples. The *lab-on-a-chip* technology that we used is able to give an estimate of this variation and let the researcher choose the right endogenous control (the one that does not significantly vary) from an adequate selection. Therefore, this validation ensures that the miRNA expression comparison among considered samples is methodologically correct.

The expression of three miRNAs (hsa-miR-26a, hsa-miR-26b, and hsa-miR-134) and two small RNAs (U6 and snoZ30) from the same five tissues analyzed before, was assayed with real-time PCR. According to the manufacturer's suggestion, we started the RT reactions with 5 ng of total RNA. Cycle threshold values for all tissues are reported in Table 3. As expected, different Ct values for endogenous and other miRNAs were obtained. These values reflect the absolute concentrations of these miRNAs in various samples. Since most studies aim to discover differences in expression levels of miRNAs and not absolute levels of expression, the use of an endogenous control is needed. In order to assess if the differences in Ct values of the controls we used (U6 and snoZ30) are because of a different starting concentration or a real differential expression, we corrected the obtained values by taking into account the concentration of miRNAs previously obtained with the *lab-on-a-chip* technology. Hence, we considered that for double the concentration a correction of one Ct value should be applied. This preliminary correction eliminated the intrinsic variability owing to different sample concentrations and allowed us to estimate the reliability of the selected endogenous controls. We observed slight variation for Ct values of U6 (Average Ct = 33.5 ± 0.8) while snoZ30 displayed a higher variability (Average Ct = 34.1 ± 1.1) even after the applied correction. This means that U6 is constitutively expressed, at least in these tissues. Certainly, the lower the Ct difference is, the more reliable are the results. To facilitate this, more *lab-on-a-chip* quantifications and real time assays are needed.

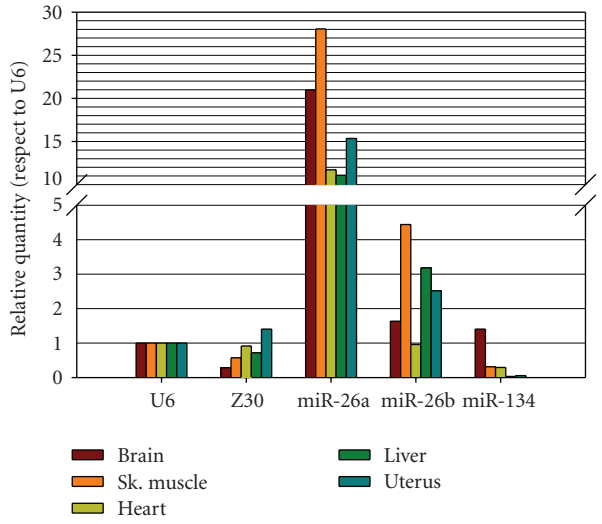


Figure 6: Corrected comparison of the relative expression of a miRNA selection in five different tissues (brain, skeletal muscle, heart, liver, and uterus) with respect to the U6 endogenous control.

Therefore, we concluded that U6 is a more reliable endogenous control than snoZ30 for miRNA expression profile comparison in the analyzed tissues. The miRNA expression profile comparison is reported in Figure 6.

Table 3: List of miRNAs expressed in different tissues with their average Ct values. Corrected Ct values represent the correction made after the precise quantification of miRNA species with the *lab-on-a-chip*technology.

MicroRNA	Average Ct	Corrected Ct
	Brain	
U6	34.16	33.70
snoZ30	35.98	35.52
miR-26a	29.77	29.31
miR-26b	33.45	32.99
miR-134	33.67	33.21
	Liver	
U6	32.37	32.37
snoZ30	32.85	32.85
miR-26a	28.90	28.90
miR-26b	30.70	30.70
miR-134	37.30	37.30
	Muscle	
U6	36.55	33.24
snoZ30	37.35	34.04
miR-26a	31.74	28.43
miR-26b	34.40	31.09
miR-134	38.23	34.92
	Uterus	
U6	34.61	33.81
snoZ30	34.12	33.32
miR-26a	30.67	29.87
miR-26b	33.28	32.48
miR-134	38.77	37.97
	Heart	
U6	36.15	34.52
snoZ30	36.28	34.65
miR-26a	32.60	30.97
miR-26b	36.21	34.58
miR-134	37.93	36.30

We then compared our data with those reported in literature [13]. Figure 7 shows the expression values of hsa-miR-26a, hsa-miR-26b and hsa-miR-134 compared with the expression values of some tissue-specific miRNAs (miR-1 for heart and muscle, miR-122a for liver, miR-124a for brain). Expression values of miRNAs are expressed as copies per ng of RNA.

4. Discussion

One of the most difficult problems with miRNAs experimental studies concerns the efficiency of their quantitative and qualitative recovery after total RNA extraction from cells or tissues. In some cases, traditional total RNA extraction protocols are not efficient methods for extracting both high molecular weight (HMW) and low molecular weight (LMW)

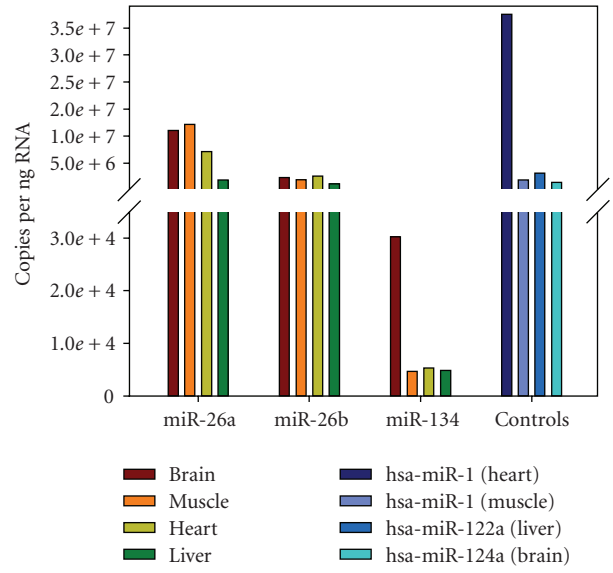


Figure 7: Expression values of hsa-miR-26a, hsa-miR-26b, and hsa-miR-134 reported in literature [13] compared with the expression values of tissue-specific miRNAs.

RNAs. Moreover, conventional quantification methods (i.e., spectrophotometric measurements) giving an overall quantification of total RNA concentration are therefore inadequate for these small RNA species. Northern blotting is the only current viable technique to visualize and evaluate small RNA molecules, particularly miRNAs. However, their low abundance sometimes prevents the use of such techniques. On the other hand, the *lab-on-a-chip* technology we employed is an alternative and valuable tool for the precise quantification of small RNAs and miRNAs present in total RNA samples.

In this work, to characterize in detail all the LMW RNA species visible in the Bioanalyzer electropherogram (5.8S, 5S, tRNAs, etc.), we ran RNA samples on polyacrylamide gels to clearly separate each of the predominant species present in the small RNA region (Figure 3(a)). We also optimized an elution protocol from excised polyacrylamide bands after gel electrophoresis. Then, we monitored the eluted fractions by means of a *lab-on-a-chip* technology that is, at present, the only technique that allows for the comparison of small RNA profiles and the exact quantification of these species (Figure 3(b)). Several other species were present under the peak of LMW RNAs, even if it was not possible to distinguish each of them. For these reasons, we applied a mathematical algorithm (the Fourier transform) for the deconvolution of the profile coming from the Bioanalyzer into different Gaussian peaks. After the fitting procedure, we obtained a mathematical model comprising seven peaks that define the overall profile (Figure 3(c)) . These peaks range from a minimum of 65 bp to a maximum of 171 bp, which is well in agreement with the results obtained with Agilent automatic peak-peaking that relies on the comparison of RNA species with an RNA ladder included in the running buffer. Interestingly, after this procedure was performed

some other non-assigned bands remained undetermined. These species may belong to smaller RNA species like miRNAs. To clarify this, the Small RNA kit, specifically designed for the analysis of miRNA species, was used to study these unidentified species in more detail.

We initially compared the three commonest RNA extraction protocols, the classic acid phenol/guanidine isothiocyanate solution, and two glass fiber filtration protocols to examine and quantify the recovery of HMW and LMW RNA species. The acid phenol/guanidine isothiocyanate solution (Table 1) maximized not only the recovery of HMW RNA fractions but also the LMW RNAs (from 22% to 36%), as previously observed [3]. Then, we assessed by real-time PCR how significantly affected the recovery of miRNA species was as a function of the total RNA extraction protocol used. The expression levels of a miRNA (hsa-miR-21) and a small nucleolar RNA (snoZ30) were evaluated by real-time PCR specific assays [12]. This technique represents an effective alternative to Northern blotting for miRNA detection and quantification. Real-time quantification results (Table 2) demonstrated that the amount of miRNAs correlates better with the amount of LMW RNAs than with total RNA.

Another aspect that we critically analyzed concerned the correlation between LMW RNA species and miRNAs. The presence of LMW RNAs is not always directly correlated with that of miRNAs. In fact, we proved that even with the same extraction protocol the concentration of miRNAs may differ considerably between various preparations (i.e., extraction from different tissues).

As a potential application of our results, we applied our findings to the comparison of some miRNA expression in different tissues. We assessed the expression of hsa-miR-26a, hsa-miR-26b, and has-miR-134 as model miRNAs and two small RNAs (U6 and snoZ30) as controls. The expression profile comparison is correct only if we assume that the endogenous control (U6 or others) has a constant and equal expression in all the considered tissues. The *lab-on-a-chip* technology we used allowed precise quantification of input miRNAs, ultimately leading to a fine correction of real-time PCR Ct data for eventual variations. Therefore, we obtained a reliable and correct estimate of the relative quantities of miRNAs present in various tissues. Surprisingly, the expression of miR-26a was the highest of all the miRNAs in all the tissues considered (fold change > 10 with respect to U6). Although our data are in good agreement with those reported in literature [13] (Figure 7), the high expression values found for miR-26a will deserve future investigations. However, the expression values of miR-26a reported in literature [13] are also very high (and higher than those of miR-26b) and above the values of those tissue-specific miRNAs considered as highly expressed (i.e., miR-1 for muscle, miR-124a for brain, and miR-122a for liver) (see Figure 7).

In conclusion, all the methods we used in this paper allowed us to study accurately the efficiency of extraction protocols, analyze the small (but significant) differences between various preparations, and suggest a methodologically correct method for the comparison of miRNA expression profiles in various tissues.

Acknowledgments

The authors thank editors and reviewers for their useful suggestions and comments to improve the manuscript. This work was supported by Grants of the Italian Ministry of Health. The first and the second authors equally contributed to the work.

References

[1] J. S. Mattick and I. V. Makunin, "Non-coding RNA," *Human Molecular Genetics*, vol. 15, no. 1, pp. R17–R29, 2006.

[2] J. S. Mattick and I. V. Makunin, "Small regulatory RNAs in mammals," *Human Molecular Genetics*, vol. 14, no. 1, pp. R121–R132, 2005.

[3] A. Masotti and T. Preckel, "Analysis of small RNAs with the Agilent 2100 bioanalyzer," *Nature Methods*, vol. 3, no. 8, pp. 3–4, 2006.

[4] T. W. Nilsen, "Mechanisms of microRNA-mediated gene regulation in animal cells," *Trends in Genetics*, vol. 23, no. 5, pp. 243–249, 2007.

[5] R. S. Pillai, S. N. Bhattacharyya, and W. Filipowicz, "Repression of protein synthesis by miRNAs: how many mechanisms?" *Trends in Cell Biology*, vol. 17, no. 3, pp. 118–126, 2007.

[6] A. Eulalio, E. Huntzinger, and E. Izaurralde, "Getting to the root of miRNA-mediated gene silencing," *Cell*, vol. 132, no. 1, pp. 9–14, 2008.

[7] B. P. Lewis, I.-H. Shih, M. W. Jones-Rhoades, D. P. Bartel, and C. B. Burge, "Prediction of mammalian microRNA targets," *Cell*, vol. 115, no. 7, pp. 787–798, 2003.

[8] B. John, A. J. Enright, A. Aravin, T. Tuschl, C. Sander, and D. S. Marks, "Human microRNA targets," *PLoS Biology*, vol. 2, no. 11, article e363, 2004.

[9] R. A. Ach, H. Wang, and B. Curry, "Measuring microRNAs: comparisons of microarray and quantitative PCR measurements, and of different total RNA prep methods," *BMC Biotechnology*, vol. 8, article 69, 2008.

[10] P. D. Ling and H. M. Huls, "Isolation and immortalization of lymphocytes," in *Current Protocols in Molecular Biology*, chapter 28, Unit 28.2, 2005.

[11] Z. Chen and D. E. Ruffner, "Modified crush-and-soak method for recovering oligodeoxynucleotides from polyacrylamide gel," *BioTechniques*, vol. 21, no. 5, pp. 820–822, 1996.

[12] C. Chen, D. A. Ridzon, A. J. Broomer, et al., "Real-time quantification of microRNAs by stem-loop RT-PCR," *Nucleic Acids Research*, vol. 33, no. 20, article e179, 2005.

[13] P. W. Hsu, H. D. Huang, S. D. Hsu, et al., "miRNAMap: genomic maps of microRNA genes and their target genes in mammalian genomes," *Nucleic Acids Research*, vol. 34, pp. D135–D139, 2006.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 419539, 18 pages
doi:10.1155/2009/419539

Review Article

Silencing Viral MicroRNA as a Novel Antiviral Therapy?

Ugo Moens

Department of Microbiology and Virology, University of Tromsø, N-9037 Tromsø, Norway

Correspondence should be addressed to Ugo Moens, ugom@fagmed.uit.no

Received 28 December 2008; Accepted 20 March 2009

Recommended by Bibekanand Mallick

Viruses are intracellular parasites that ensure their existence by converting host cells into viral particle producing entities or into hiding places rendering the virus invisible to the host immune system. Some viruses may also survive by transforming the infected cell into an immortal tumour cell. MicroRNAs are small non-coding transcripts that function as posttranscriptional regulators of gene expression. Viruses encode miRNAs that regulate expression of both cellular and viral genes, and contribute to the pathogenic properties of viruses. Hence, neutralizing the action of viral miRNAs expression by complementary single-stranded oligonucleotides or so-called anti-miRNAs may represent a strategy to combat viral infections and viral-induced pathogenesis. This review describes the miRNAs encoded by human viruses, and discusses the possible therapeutic applications of anti-miRNAs against viral diseases.

1. Introduction

Viruses are common habitants of the human population, where they establish different forms of infection, including an acute, a chronic, or a persistent infection with production of low levels of virions. Some viruses can exist in a true latent state in which infectious particles are only produced upon reactivation stimuli. Viruses that reside harmlessly in their host can under certain conditions or in immunocompromised persons be responsible for malignant and non-malignant diseases, which may even lead to the death of the host. A causal role for human polyomaviruses (HPyV), papillomaviruses (HPV), herpesviruses (HHV), hepatitis B virus (HBV), hepatitis C virus (HCV), and human T-cell lymphotropic virus type-I (HTLV-I) and cancer is accepted (for recent reviews see [1–7]). It is estimated that oncoviruses are associated with 15% of the human cancers [8], while non-malignant infections from human immunodeficiency virus (HIV), HBV and HCV alone cause more than 3 million deaths annually worldwide [9]. Other viral infections (HIV no included) were responsible for the death of more than 6000 patients in Japan in 2006, ∼7000 individuals in the USA in 2005, and 555 people in United Kingdom in 2006 according the statistics of the World Health Organization [10]. Thus the pathogenic properties of viruses necessitate the development of efficient antiviral therapies.

Viruses attempt to create a favorable cellular environment allowing viral replication or survival by establishing a lifelong latent infection through evading the immune system of their hosts. Viruses can hide within a cell by restricting their activity to a minimum so as not to conceal their presence to the immune system and at the same time they will also try to avoid apoptosis. For these purposes, viruses have developed different strategies, one of which includes the posttranscriptional regulation of both cellular and viral gene expressions through modulating the host's RNA-interference (RNAi) machinery. Viruses can suppress the RNAi pathway by viral microRNA (vmiRNA) targeting cellular or viral transcripts, or by viral proteins (e.g., human immunodeficiency virus Tat protein, influenza virus NS1/NS2 protein, Ebola VP35 protein, and vaccinia virus E3L protein) or viral RNA (Adenovirus VA transcripts) that counteract the host's RNAi machinery (for recent reviews see [11–17]). This review summarizes the recent findings on virus-encoded miRNAs and their described functions and briefly discusses the potential of antiviral miRNA as a novel therapeutic strategy in combating virus infections.

2. MicroRNA (miRNA)

MiRNAs are noncoding small RNA molecules that act as posttranscriptional regulators. They seem to be an inherent part of the genomes of most living organisms as they have been described in plants, unicellular and lower invertebrates, all vertebrates, and in viruses. Their exact functions start to emerge and include control of cellular processes such as differentiation, morphogenesis, organogenesis, and metabolism [18–22]. MiRNAs are typically generated by RNA polymerase II. The primary transcript (pri-miRNA) is processed by the RNase III enzyme Drosha, in concert with double-stranded (ds) RNA-binding protein DGCR8 into a ~60 pre-miRNA hairpin. This nuclear pre-miRNA is then transported into the cytoplasma by exportin 5/Ran-GTP and cleaved by the cytoplasmic RNase III Dicer to generate an imperfect ds RNA of 21–25 nucleotides. One of the strands, the mature miRNA strand or guide strand, is loaded in the RNA induced silencing complex (RISC), and directs RISC to the target mRNA, where the complex hybridizes to (partially) complementary sequences resulting in cleavage or translational inhibition of the target mRNA. The unincorporated strand, called the passenger strand, is degraded. The seed region, which encompasses nucleotides 2 to 8 of the 5' ends of miRNA, plays a pivotal role in target selection by RISC-bound miRNA (for recent reviews see [23–25]). In animals, mature miRNAs do not require complete complementarity to their target mRNAs, enabling them to bind to and prevent translation of several mRNAs. Experimental evidence suggests that a single miRNA can potentially target as many as 200 different mRNAs [26–28]. As such, miRNAs have merged as pivotal posttranscriptional regulators of gene expression in multicellular eukaryotes and aberrant expression can contribute to diseases ([28] and references therein).

3. Silencing of miRNA by Anti-miRNA Oligonucleotides

Anti-miRNA oligonucleotides (AMOs) are chemically modified synthetic oligonucleotides that are complementary to their target sequence and this will silence the action of the target. AMOs are modified with the dual purpose to stabilize them and to improve their affinity for their targets. One modification is the 2' sugar modification which implies a chemical modification of the 2'-O of the ribose residue (Figure 1(a)). The 2'-O -methyl AMOs have a methyl group linked to the 2'-O of the ribose residue, while the 2'-O - methoxyethyl AMOs contain a methoxygroup. This modification provides improved RNase resistance and binding affinity to RNA compared to unmodified antioligonucleotides. However, 2'-O -methoxyethyl AMOs possess a higher affinity and specificity to RNA than their 2'-O -methyl AMOs. Other 2' sugar modifications that have been used include 2'-fluor and locked nucleic acid (LNA). In LNA-modified oligonucleotides, the 2'-O -oxygen is bridged to the 4'-position via a methylene linker to form a rigid bicycle, locked into a C3'-end (RNA) sugar conformation (Figure 1(a)). LNAs give very strong duplex formation with their target sequences and they display excellent mismatch discrimination, hence avoiding off-target effects (for recent reviews see [28] and [29]). LNA injections against miR-122, a cellular miRNA involved in lipid metabolism, resulted in efficient and long-lasting decrease in plasma cholesterol in African green monkeys without any evidence for toxicities [30]. A second type of modification is the phosphorothioate backbone which reduces the affinity to the target somewhat, but it confers significant stability to nuclease degradation (Figure 1(b)). A third generation of antisense oligonucleotides are phosphodiamidate morpholino oligomers (PMO) in which the ribose ring is replaced with a morpholine ring. Adding an arginine-rich peptide $(RXR)_4$ further increased the stability and tissue retention of the PMO (reviewed in [31]). An additional modification can be made to improve the cellular uptake of the AMO. Krützfeldt et al. linked a cholesterol moiety to their AMOs and referred to these anti-miRNAs as antagomirs. Antagomirs should be >19 nucleotides in length to provide highest efficiency in silencing target miRNA [32–34]. The putative therapeutic potentials of antagomirs were recently demonstrated in treatment of lipid metabolic disease in animals [35]. An alternative class of AMOs is peptide nucleic acids (PNA), which are synthetic oligonucleotides with N-(2-aminoethyl)-glycine replacing the deoxyribose or ribose backbone [36]. A study published in 2008 reported that PNA can efficiently block the action of cellular miRNAs [37]. Finally, another approach in silencing miRNA is the use of so-called microRNA sponge, a synthetic mRNA that contains multiple binding sites for a particular miRNA and that is transcribed from a plasmid containing a strong promoter (reviewed in [28]). In conclusion, different classes of AMO have been shown to be efficient in silencing miRNA and may be useful therapeutic tools (reviewed in [29, 32–34, 38]).

4. Viral miRNAs Encoded by Human Viruses

4.1. Human polyomaviruses. (HPyV) are nonenveloped viruses with a circular ds DNA genome of approximately 5000 base-pairs. The members BK virus and JC virus were first isolated in 1971 from the urine of a renal transplant patient with the initials B.K., and from the brain of a Hodgkin's lymphoma patient with initials J.C. who suffered from progressive multifocal leukoencephalopathy (PML), respectively. Human infections with the rhesus macaque (*Macaca mulatta*) Simian vacuolating virus 40 (SV40) were considered as accidental transmission of the virus from monkeys to people living in close contact with these animals or through vaccination with the contaminated poliovirus vaccines. However, recent observations support the possibility that SV40 can spread in humans by horizontal infection and even vertical transmission, suggesting that man may be a natural host for this virus. The human polyomaviruses seem to be implicated in tumours of the brain, bone, colon, mesothelium, pancreas, stomach, urogenital tract, and lymphomas and leukaemias (reviewed in [39, 40]). In 2007, two independent research groups reported the isolation of two new human polyomaviruses from nasopharyngeal

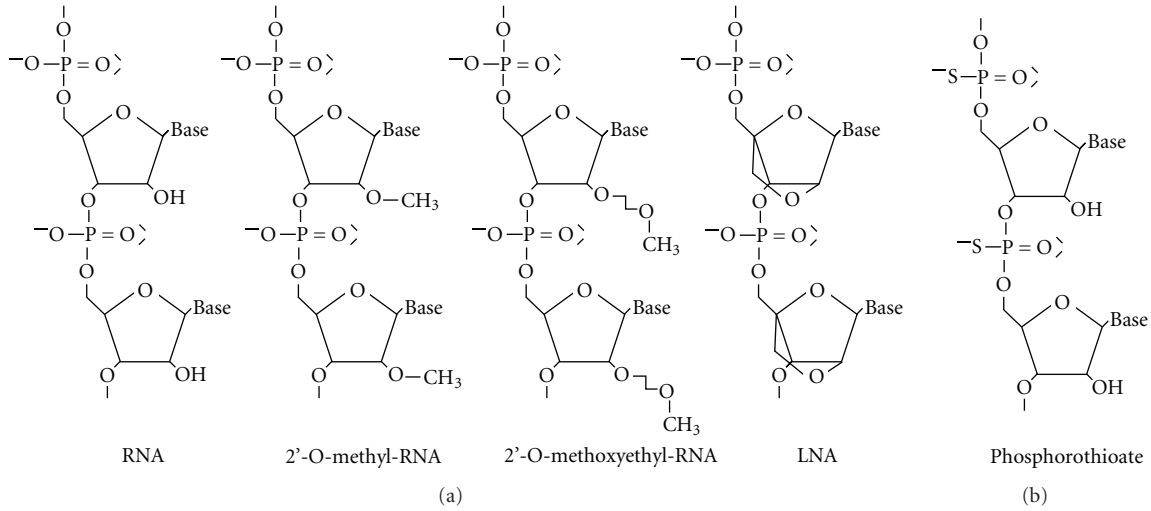


Figure 1: Common chemical modifications used for anti-miRNA oligonucleotides (AMO). (A) Modifications of the 2'-O residue in ribose. (B) Modification of the phosphate-ribose backbone. LNA = locked nucleic acid. See text for details.

samples and they are referred to as KIPyV WUPyV [41, 42]. This year a novel human polyomavirus, Merkel cell polyomavirus (MCPyV), was identified that is associated with Merkel cell carcinoma [43].

The HPyV genome can be divided into three functional regions. The early region encodes the early proteins large T-antigen (LT-ag) and small t-antigen (st-ag), while the late region encodes the capsid proteins VP1-VP3 and the regulatory protein agnoprotein. Both regions are separated by the noncoding control region that encompasses the origin of replication and the promoter/enhancer sequences for the early and late genes (reviewed in [44]). The SV40 genome encodes a viral miRNA (vmiRNA) of which both arms are complementary to the early viral mRNAs and reduces expression of the early proteins (Figure 2(a); Table 1). Cells infected with mutant SV40 lacking this miRNA or with wild-type SV40 yielded comparable levels of infectious viruses, but the latter were less sensitive to lysis by cytotoxic T cells and produced less interferon-γ. Thus SV40-encoded miRNA allows the virus to evade the immune system [45]. The SV40 miRNA is conserved in BKV and JCV and both miRNAs generated from the precursor hairpin bind to the same target, that is, the early transcripts. The BKV and JCV miRNAs serve the same role as SV40 miRNA, that is, downregulation of early expression. JCV miRNA, miR-J1, was readily detected in brain samples of PML patients, suggesting a biological role of this miRNA [46]. The group of Sullivan has also identified a MCPyV-encoded miRNA, miR-M1, which does not share sequence identity with the known miRNAs of the other polyomaviruses. MCPyV miR-M1 is located in the early region (Figure 2(a)) and can downregulate early gene expression. In accordance with SV40, this may allow the virus to evade the immune system. However, MCPyV is associated with Merkel cell carcinoma and the viral genome is integrated in these tumours [43]. Blocking miR-M1 by, for example, antagomirs will increase the expression of the viral oncoprotein LT-antigen and as such have little beneficial therapeutic effect [47]. Expression of a corresponding or other viral-encoded miRNA for the other HPyV WU and KI is lacking so far.

4.2. Human Papillomaviruses. Human papillomaviruses (HPV) are nonenveloped viruses with a circular dsDNA genome of approximately 8000 base-pairs. These viruses are associated with benign and malignant lesions of the skin and the genital tract. More than 100 different HPV genotypes have been identified and based on their association with benign warts or cancer, they are classified as low-risk and high-risk variants, respectively [1].

One study with HPV type 31 failed to clone vmiRNA from virus-infected cells [58]. However, this does not exclude that other strains may encode vmiRNA. Moreover, the expression of vmiRNA may be regulated in a temporal and spatial manner, so that the experimental conditions for capturing vmiRNA may be tricky. In addition, the high mutation rate of HPV genome sequences may impede the prediction of the presence of putative vmiRNA.

4.3. Human Adenovirus. Adenoviruses are naked dsDNA viruses that can cause mild respiratory, gastrointestinal, urogenital, and ocular disease. More than 50 serotypes have been described in human. Although there is no proof for a causative role in malignancies, adenoviruses can induce cancer in animal models and have been extensively studied to scrutinize viral mechanisms for cellular transformation [59].

Adenovirus encodes small noncoding RNAs, known as virus-associated RNA or VAI and VAII RNA, which are generated by RNA polymerase III. This noncoding RNA plays an important role for viral replication and neutralizes the antiviral action of interferon by blocking the dsRNA-induced protein kinase (PKR), which phosphorylates and thereby inactivates the eukaryotic translational initiation

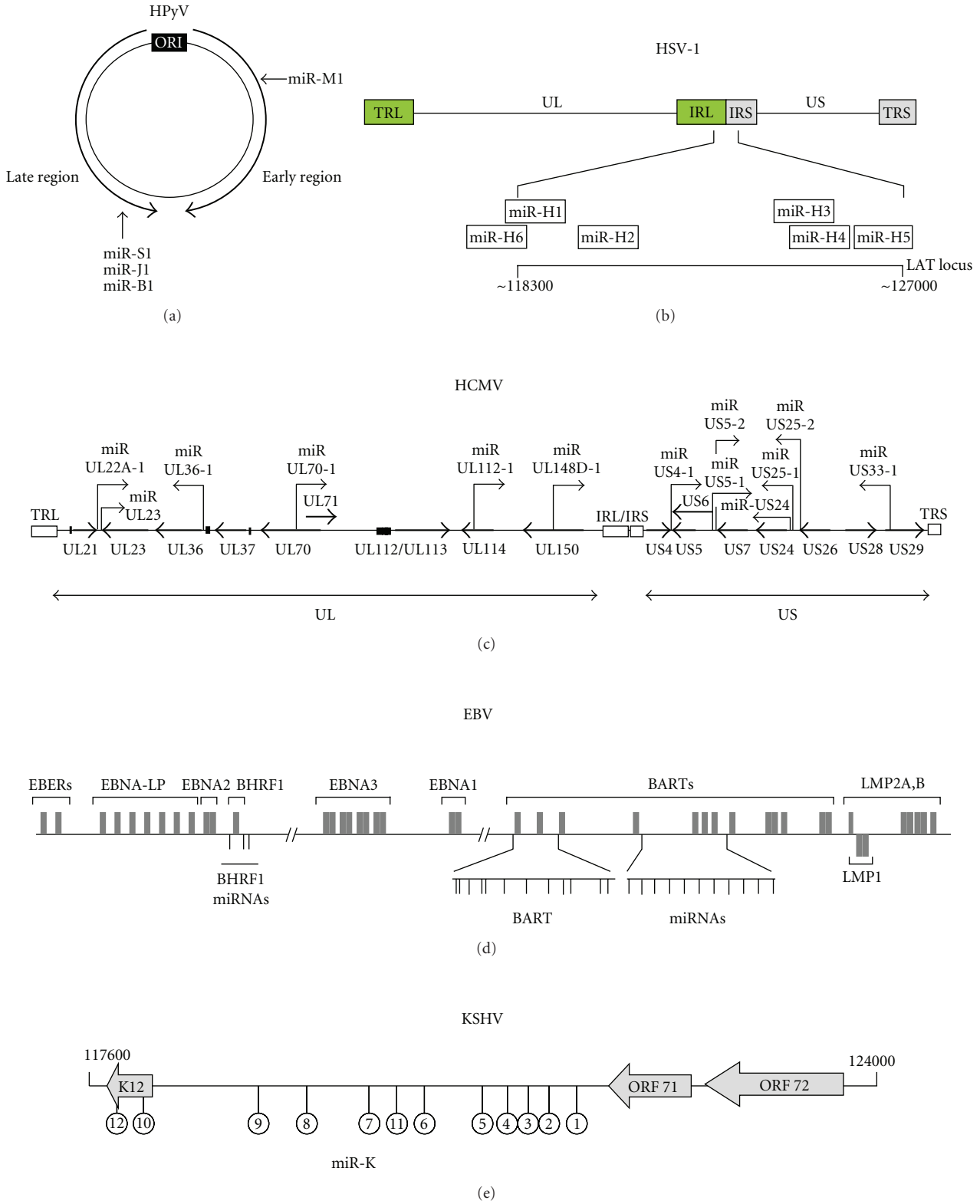


Figure 2: Continued.

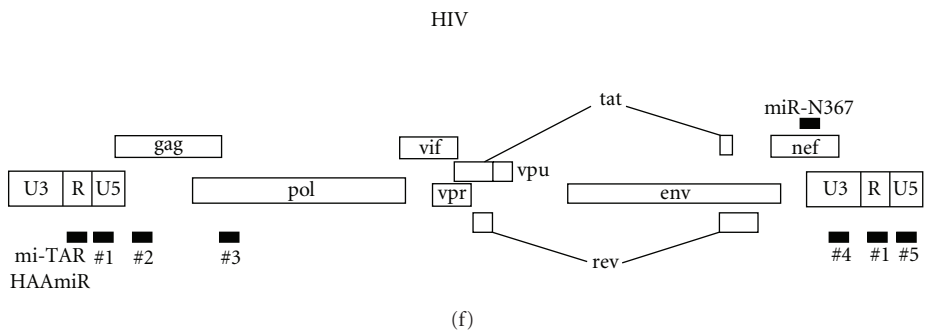


Figure 2: Schematic representation of the genomes of different human viruses and location of viral-encoded miRNAs. (A) The human polyomavirus (HPyV) BK virus, JC virus, SV40, and Merkel Cell polyomavirus encode the viral miRNAs miR-B1, miR-J1, miR-S1, and miR-M1, respectively [45–47]. (B) Herpes simplex virus-1 genome with detail of the *LAT*locus. The numbers refer to the approximate sequence coordinates of the LAT locus. L= long, S= short, U= unique, TR= terminal repeat, and IR= internal repeat. The figure is modified after [48]. (C) Genomic map of HCMV with relative position of some of the genes. The open reading frames are depicted by thick arrows, while the position of the viral miRNAs are indicated by narrow arrows. L= long, S= short, U= unique, TR= terminal repeat, and IR= internal repeat. Modified after [49] and [50]. (D) Location of the miRNAs in the EBV genome. The latency genes are shown as grey boxes, while the miRNAs are indicated by vertical lines. (E) Kaposi's sarcoma-associated herpes virus (KSHV or HHV-8). Most KSHV miRNAs are clustered between the *kaposin*(K12) gene and the v-*FLIP*(ORF71) gene, while two are located within the K12 open reading frame (ORF). Adapted from [51]. (F) The HIV-1 genome and location of the viral-encoded miRNAs. The figure is based on a compilation of the studies by [52–57].

factor 2 [60]. A recent study showed that a minor fraction of VAI RNA is processed by Dicer into functional RISC-associated ssRNA which can act as miRNA (Table 1). Blocking of VAI-derived small RNA by 2'-O -methyl AMO complementary to VAI decreased virion production [61–63]. In an attempt to identify potential targets for VAI-derived miRNA, computational analysis was used. Putative targets include genes encoding apoptosis-related protein NAPOR and PKR-activating protein PACT. Therefore VAI RNA-derived miRNA may help adenovirus to escape the actions of the host defense mechanisms [64].

4.4. Human Herpesviruses. Herpesviruses are enveloped dsDNA viruses with a genome size ranging between ~130 to ~250 kilobase-pairs. They are divided into three subfamilies denoted α, β, and γ. Approximately 130 different herpesviruses have been identified to date, including the human α-herpesviruses herpes simplex virus 1 (HSV-1 or HHV-1), herpes simplex virus 2 (HSV-2 or HHV-2), Varicella-zoster virus (VZV or HHV-3), the β-herpesviruses cytomegalovirus (HCMV or HHV-5), HHV-6A, HHV-6B, and HHV-7, the γ-herpesviruses Epstein-Barr virus (EBV or HHV-4), and Kaposi's sarcoma-associated virus (KSHV or HHV-8). All human herpesviruses are able to establish latent infections with only a small subset of viral genes expressed (reviewed in [64]). As will be discussed in the next section, among the viral transcripts that can be detected in latently infected cells are viral-encoded miRNAs, which seem to be required to maintain a latent state of infecton, but may also contribute to the pathogenic properties of the virus.

4.4.1. HSV-1. Herpes simplex virus-1 (HSV-1 or human herpes virus 1; HHV1) infects the majority of the human population, but remains a latent cohabitant in most people. Reactivation of the viruses usually results in cold sores, but it can also cause a spectrum of diseases from sight-threatening ocular infections in immunocompetent adults to more severe infections in newborns and immunosuppressed patients (reviewed in [89]).

An important gene in HSV latency is the LAT gene. This gene encodes the latency-associated transcript (LAT), which does not code for a protein. LAT seems to promote cell survival of the infected cells [90]. Gupta and coworkers proposed that the antiapoptotic activity of LAT was achieved by an miRNA-entrapped in the LAT (miR-LAT), which downregulates the expression of transforming growth factor-β (TGF-β) and SMAD3. The latter is a mediator of the signalling pathway induced by TGF-β, while TGF-β can prevent cell proliferation and induce cell death [67]. However, a later study revealed that the described miR-LAT was not viral encoded, but in fact a cellular miRNA expressed in SH-SY-5Y cells [67] and the report describing miR-LAT was retracted [91]. Work by Umbach et al. could also not confirm the existence of this miRNA in HSV-1 infected SH-SY-5Y cells [48]. Later, it was shown that the HSV-1 LAT exon encodes HSV-1 miR-LAT-ICP34.5, which can be detected in HSV-1 infected cells [89]. Circa 120 base-pairs upstream in this region, a sequence with 77% homology to the HSV-2 miRNA miR-I (see further) is present, but no mature miRNA was detected in HSV-1 infected cells. However, the existence of this miRNA during HSV-1 latency in vivo remains to be confirmed [68].

Computational analysis predicted 24-miRNA candidates in the HSV-1 genome, 8 of which were conserved in HSV-2, suggesting they may be functional miRNAs [69]. The authors confirmed the expression of one mature miRNA, designated miR-H1, in HSV-1 infected Vero cells, where it was expressed late in productive replication. This miRNA is encoded approximately 450 bp upstream of the transcription start site of the LAT transcript, but a corresponding sequence is not conserved in the HSV-2. The function of miR-H1

Table 1: MicroRNAs encoded by human viruses and their targets. See text for details.

Virus	miRNA	Target/function	Reference
Human polyomaviruses			
SV40	miR-S1	Downregulation early expression; Immunomodulating	[45]
BKV	miR-B1	Downregulation early expression	[46]
JCV	miR-J1	Downregulation early expression	[46]
MCPyV	miR-M1	Downregulation early expression	[47]
Human papillomavirus	not predicted		[65]
	not detected		[66]
Human adenovirus	unnamed		[61–63]
Human parvovirus	not predicted		[65]
HSV-1 (HHV1)	miR-LAT (within exon 1)	Prevents apoptosis by targeting translation of the genes encoding TGF-β1 and SMAD3	[67](retracted),[48, 68, 69],[70]
	miR-LAT-ICP34.5		
	miR-H1	Repression of the expression of the viral protein ICP0, which promotes viral replication	
	miR-H2	Downregulation of ICP34.5, a key viral neurovirulence factor	
	miR-H3	Downregulation of ICP34.5, a key viral neurovirulence factor	
	miR-H4		
	miR-H5	Downregulation of ICP4, a viral transcriptional activator	
	miR-H6	Reduced expression of ICP34.5, a key viral neurovirulence factor	
HSV-2 (HHV2)	miR-I		
	miR-II		
	miR-III		
VZV (HHV3)	not predicted		[65, 71]
HCMV (HHV5)	miR-UL23	Immunomodulating	[12, 49, 65, 72–74]
	miR-UL36-1		
	miR-UL54-1		
	miR-UL70-1		
	miR-UL22A-1		
	miR-UL112-1	Downregulates the expression of CMV genes involved in its own replication process, for example, transactivators IE72 and IE86; UL120/121; UL114MHC class I-related chain B (MICB), a cellular ligand for the activating receptor NKG2D; downregulation of IE-1	
	miR-UL148D-1		
	miR-US4-1		
	miR-US5-1		
	miR-US5-2		
	miR-US24		

Table 1: Continued.

Virus	miRNA	Target/function	Reference
	miR-US25-1		
	miR-US25-2		
	miR-US33-1		
	RNAβ2.7	Bnds components of the mitochondrial respiratory chain complex I and thus preventing apoptosis	
EBV (HHV4)	miR-BART1-1 to -3	Inhibition LMP1 expression	[24], [51, 75–79]
		Inhibit EBV DNA polymerase BALF5	
	miR-BART2	Inhibition LMP1 expression	
	miR-BART3		
	miR-BART4	Antiapoptotic by downregulation of PUMA	
	miR-BART5		
	miR-BART6	Inhibition LMP1 expression	
	miR-BART7		
	miR-BART8		
	miR-BART9		
	miR-BART10		
	miR-BART11		
	miR-BART12		
	miR-BART13		
	miR-BART14	Inhibition LMP1 expression	
	miR-BART15	Inhibition LMP1 expression	
	miR-BART16		
	miR-BART17		
	miR-BART18		
	miR-BART19		
	miR-BART20	Downregulation chemokine CXCL-11	
	miR-BHFR-1		
	miR-BHFR-2		
	miR-BHFR-3		
KSHV (HHV-8)	miR-K12-1	Downregulation of thrombospondin 1 and BACH	[51, 66, 75, 80–82]
	miR-K12-2	Downregulation of thrombospondin 1	
	miR-K12-3		
	miR-K12-4		
	miR-K12-5	Downregulation of thrombospondin 1 and BACH	
	miR-K12-6		
	miR-K12-7		
	miR-K12-8		
	miR-K12-9		
	miR-K12-10a		
	miR-K12-10b	Downregulation of thrombospondin 1 and BACH-1;	
	miR-K12-11	Identical to miR-155	

Table 1: Continued.

Virus	miRNA	Target/function	Reference
	miR-K12-12		
Poxvirus vaccinia virus	3 predicted		[65]
Poxvirus variola virus	1 predicted		[65]
Hepatitis B virus			[54]
Hepatitis C virus			
Human immunodeficiency virus	miR-H1		
Type 1 (HIV-1)	miR-TAR	HDAC-1-mediated repression of viral gene expression	[83]
	miR-TAR-5p		[84]
	miR-TAR-p		
	HAAmiRNA	Downregulation IL-15, IL-2 receptor γ chain, IRAK1, and FMRP	[56]
	VmiRNA#1-5	Proteins involved in, for example, signal transduction, protein synthesis, and degradation, DNA methylation	[85]
	miR-N367	HIV promoter interference	[86]
HIV-2	miR-TAR2-5p		[87]
	miR-TAR2-3p		
HTLV-I	not detected		[88]
Paramyxoviridae (measles virus)	1 predicted		[65]

remains to be established and no cellular target mRNAs were identified [69]. Umbach and coworkers detected in addition to miR-H1 five novel viral miRNAs in trigeminal ganglia of mice latently infected by HSV-1, as well as in HSV-1-infected Vero cells. These miRNAs, that is, miR-H1 to miR-H6, are encompassed in the LAT locus (Figure 2(b)). By quantitative RT-PCR, the authors were able to roughly estimate the number of copies of each miRNA during productive infection of Vero cells. MiR-H1 and miR-H6 were expressed at ~1200 and 300 copies, respectively, while the other miR-Hs were present at less than 40 copies per infected cell. In latently infected trigeminal ganglia, much higher levels were monitored with 63 000 copies of miR-H2, 8000 copies of miR-H3, 800 000 copies of miR-H4, 80 000 copies of miR-H5, and 40 000 copies of miR-H6. The large difference in numbers of miRNA transcripts in latently infected cells compared to cells with productive HSV-1 infection indicates that miR-Hs play an important role in establishing latent HSV-1 infection. Indeed, miR-H2 expression diminished the protein levels of ICP0, a viral transcriptional activator that promotes viral replication, while miR-H6 inhibits expression of ICP4, which is required for expression of most HSV-1 genes during reproductive infection [48].

4.4.2. HSV-2. HSV-2 typically infects the genital region and establishes a lifelong latent infection. The prevalence of latent HSV-2 infection varies between 10–60%. Reactivation can cause oro-facial and genital herpes, but HSV-2 infection can also cause encephalitis and neonatal herpes, and forms a risk factor for HIV acquisition [89]. The only detectable viral transcript during HSV-2 latency is the LAT, but the molecular function of this transcript remains largely unknown [92]. HSV-2 LAT exon encodes an miRNA, referred to as miR-I, which is expressed during latent, as well as during acute infection. Remarkably, several promoters regulate miR-I expression in different stages of the viral life cycle. This HSV-2 miRNA efficiently diminishes the expression of the viral neurovirulence factor ICP34.5, a multifunctional protein required for viral replication in neuronal cells in vivo, and with intrinsic neurovirulent properties [93]. Thus, miR-I may affect the outcome of infection (latent versus productive) by modulating the protein levels of ICP34.5. Whether miR-I has other targets remains to be investigated, but an miR-I analogue is also expressed by HSV-1, indicating the importance of this miRNA for these viruses [68]. Tang and colleagues identified two new HSV-2 miRNAs, miR-II, which includes miR-II-5p and miR-II-3p, and miR-III, both encoded by exon 2 of LAT. The expression of miR-I, -II, and –III increased during infection of cells, but miR-III displayed slower kinetics than the two other miRNAs. Similar to miR-I, miR-II silences the expression of ICP34.5,while

miR-III functionally resembles HSV-I miR-H2 in that it can downregulate the expression of ICP0 [70].

4.4.3. Varicella-Zoster Virus (VZV). VZV is a common virus that causes chickenpox or varicella during primary infection. The virus establishes a latent infection, but reactivation leads to herpes zoster, commonly referred to as shingles. Acute VZV reactivation may lead to post-herpetic neuralgia [94]. No putative miRNAs could be predicted in the VZV genome [65, 71], but experimental studies to unambiguously proof the existence of VZV miRNA are lacking.

4.4.4. Human Cytomegalovirus (HCMV). HCMV causes mild or subclinical diseases in immunocompetent adults, but can lead to life-threatening complications in immunocompromised patients such as organ transplant recipients and AIDS patients. In addition, HCMV is one of the leading viral causes of birth defects and has been implicated in the acceleration of long-term vascular diseases such as atherosclerosis. Depending on the tissue type and the host's immune state, HCMV can establish acute, persistent, or latent infections characterized by different viral gene expression [95, 96]. Because other herpesviruses encode miRNAs, it was assumed that HCMV may encode miRNAs which could be involved in determining the type of infection. To date, 15 HCMV vmiRNAs, scattered throughout the viral genome, have been identified (see Table 1 and Figure 2(c)). Three viral miRNAs, designated as UL23-5p, miR-UL23-3p, and miR-US24, were identified that are expressed during productive HCMV infection of permissive cells (human foreskin fibroblasts, astrocytoma U373MG cells, retinal pigment epithelial cells, and human microvascular endothelial cells). Their putative cellular target genes include genes encoding transcription factors (e.g., HNF3 and TGIF2), receptors (e.g., IL-18 receptor 1 precursor; CD206), proteins implicated in T-cell activation (AHNAK1), in signal transduction (e.g., RAB2L), and in biosynthesis of leukotrienes that sustain inflammatory reactions (coactosin-like protein). Whether these genes represent *bona fide* targets as well as the biological relevance of HCMV miRNA-mediated silencing of these genes remains elusive [49].

HCMV usually establishes a lifelong persistent or latent state in healthy individuals by ensuring that infected cells avoid immune recognition. The HCMV-encoded miRNA miR-UL112-1 seems to play a central role in helping the virus to hide from the host's immune system. This viral miRNA targets mRNA for MHC class I-related chain B (MICB), and to a lesser extend MICA. These proteins are cellular ligands for the activating receptor NKG2D, which is expressed on some natural killer (NK) cells, γ/δ T cells, and CD8+ T cells. During cellular stress (such as viral infection) MICB is induced, thus activating NK-cells and T cells that can lead to the killing of infected cells. Cells infected with mutant virus lacking this miRNA were more susceptible to being killed in an NKG2D-dependent manner by NK cells [72].

The miR-UL112-1 also represses the expression of HCMV genes involved in its own replication process, in part by targeting mRNA encoding immediate early proteins. One of them is the viral transactivator protein called immediate early 72 (IE72) that regulates the transcription of viral genes required for acute replication [50]. IE72 plays a pivotal role in controlling latency and reactivation. miR-UL-112-1 can thus restrict reactivation of the virus through negative regulation of IE72 expression [50, 95]. Two separate studies showed that miR-UL112-1 also inhibited expression of the immediate early protein IE1. Murphy et al. detected increased IE1 levels in cells infected with either a virus lacking miR-UL112-1 or with mutations in the seed sequence of the *ie1* gene compared to cells infected with wild-type HCMV [73]. Grey and coworkers demonstrated that addition of miR-UL112-1 RNA prior to infection reduced IE1 protein levels and blocked viral replication [74]. IE1 is a crucial protein to ascertain lytic replication of HCMV, thus downregulation of IE1 may help the virus to establish latency. This strategy may be a common feature for herpesviruses because immediate-early genes may be putative targets for HSV-1 miR-LAT, EBV miR-BART15 and miR-BHRF1-3, and KSHV miR-K12-6-3p [73]. Yet another target for miR-UL112-1 is the viral protein UL114, a homologue of the mammalian DNA repair enzyme uracyl-DNA glycosylase. UL114 is required for efficient viral DNA replication. Hence, miR-UL112-1-mediated downregulation of UL114 may prevent viral DNA replication and favor a latent infection state [65]. Taken together, the actions of miR-UL112-1 seem to be associated with latent viral infection, a state which allows the virus to hide from the immune system. Ablating expression of this viral-encoded miRNAs by AMOs may therefore force the virus into a lytic cycle and provide the immune system the opportunity to get ride of the viral infection.

4.4.5. EBV. Epstein-Barr virus (EBV) or human herpsesvirus-4 (HHV-4) is a γ-herpesvirus that establishes a lifelong latent infection in B-lymphocytes in more than 90% of the human population. EBV is associated with infectious mononucleosis and has been implicated in the pathogenesis of several malignancies including Burkitt's and Hodgkin's lymphomas, posttransplant and T-cell lymphomas, X-linked lymphoproliferative syndrome, nasopharyngeal, and gastric carcinomas [97]. Latently EBV-infected cells are classified in stage I, II, or III, each of them characterized by distinct EBV gene expression [98]. Among the latency stage-specific EBV transcripts are miRNAs. More than 20 different EBV miRNAs have been identified that are transcribed during latent infection (Table 1; [25, 51, 75–79]). The EBV miRNAs are organized in two major clusters within the EBV genome (Figure 2(d)). One cluster resides in the BART (abbreviation for BamHI-A rightward transcripts) region. The BART region gives rise to multispliced transcripts and is highly expressed in EBV-positive cancers and in epithelial tissues, while there is low BART expression in B lymphocytes. The exact function of BART mRNAs remains obscure [76]. The intronic region of BART also encodes the miRNAs miR-BART1 to miR-BART20. The second region that encodes multiple miRNAs is the untranslated region of the gene encoding BHRF1 (BamHI fragment H rightward open reading frame 1), a viral Bcl-2 homologue that prevents

apoptosis. BHRF1 encompasses the miRNAs miR-BHRF-1-1 to miR-BHRF-1-3 [51, 75].

The expressions of EBV-encoded miRNAs in clinical samples and computational analysis to predict putative targets were applied to unravel the biological functions of EBV miRNAs. These approaches showed that the miR-BARTs are abundantly expressed in latently infected epithelial cells, nasopharyngeal carcinomas, EBV-associated gastric carcinoma cell lines and tissues, Burkitt's lymphomas latency type I, EBV positive primary effusion lymphomas, and diffuse large B-cell lymphomas, but at a significantly lower level in B cells. This corresponds well with the expression pattern of BART multispliced transcripts (see above). Higher levels of BHRF1-3 were measured in latency type III Burkitt's lymphomas and in diffuse large B-cell lymphomas [66, 79, 98–100]. Another study demonstrated that induction of EBV replication in latency I-infected cells was associated with increased expression of miR-BHRF1-1, -2, and -3, but expression levels of miR-BART-1 and -2 did not change. On the other hand, induction of EBV replication in latency III-infected cells did hardly change the expression levels of BHRF1-1, -2, and -3 [98]. These observations suggest that EBV miRNAs may be implicated in the oncogenic properties of the virus, but also in regulating its replication. Moreover, a precise knowledge of the latency state of EBV and the expression pattern of viral miRNAs may improve the successful treatment of EBV infection with AMOs.

The function of most EBV vmiRNA remains poorly understood, but some targets of EBV miRNAs have been recently identified. Several mi-BARTs prevent expression of viral latent infection membrane protein 1 (LMP1) protein (see Table 1). LMP1 functions as a constitutively active tumour necrosis receptor [101], and can activate several signalling pathways including NFκB, AP1, JAK/STAT, MEK/ERK, and PKC. LMP1 can also interact with p53 and affects cyclins, cyclin-dependent protein kinases (CDK), and the CDK inhibitors p16 and p27 (reviewed in [102]). Furthermore, LMP1 is expressed in all the EBV related malignancies and promotes cellular transformation. Its oncogenic property makes LMP1 an attractive target for EBV therapy. Interestingly, overexpression of LMP1 results in growth-inhibitory and sensitization to apoptosis induced by stress or chemotherapeutic agents ([76] and references therein). Thus AMO-mediated neutralization of mi-BARTs may lead to elevated LMP1 protein levels and render EBV-positive tumour cells more susceptible to chemotherapy. The viral miRNA miR-BART2 can inhibit expression of viral DNA polymerase BALF5 and may thus interfere with viral replication and prevent lytic infection [51, 77]. Silencing miR-BART2 could thus allow the virus the complete its life cycle and produce new infectious virus particles, which then could offer the immune system the opportunity to detect and eliminate EBV. Using computational prediction programs such as miRanda and RNAhybrid (reviewed in [103]) allowed Choy and coworkers to envisage the cellular protein p53 up-regulator of apoptosis (PUMA) as a target for miR-BART5 [78]. The authors demonstrated that PUMA levels were decreased in cells expressing miR-BART5 compared to cells lacking miR-BART5. In accordance, when miR-BART5 was specifically inhibited with an anti-miR-BART5 oligonucleotide, PUMA protein levels decreased and apoptosis was triggered. Thus, EBV may promote survival of infected epithelial cells by modulating the expression of an apoptotic protein through an miRNA-mediated mechanism. This finding may have important implications in the development of anti-EBV agents such as AMOs directed against miR-BART5.

Fewer studies have been directed to determine the targets of miR-BHRF1s. miR-BHRF1-2 is involved in the cleavage of BHRF1 RNA in the cytoplasm, but the biological relevance remains to be determined [98]. In another study, Xia et al. observed that high levels of miR-BHRF1-3 were correlated with low levels CXCL-11, a potent interferon-inducible T-cell attracting chemokine. MiRNA-mediated suppression of CXCL-11 may serve as an immunomodulating mechanism allowing the virus to survive in the host [79]. On the other hand, enhancing CXCL-11 expression in EBV-positive tumours by AMOs against miR-BHRF1-3 may increase susceptibility of the tumour cells to the immune system. In agreement with this, two recent studies reported antitumour activity for CXCL-11 in animal models [104, 105].

4.4.6. Kaposi's Sarcoma Virus (KSHV or Human Herpes Virus Type 8; HHV-8). Kaposi's sarcoma-associated virus, so named because it was detected in Kaposi's sarcoma, belongs to the γ-herpesviruses and is also known as human herpesvirus-8 or HHV-8. HHV-8 is associated with Kaposi's sarcoma, as well as with two rare forms of B-cell malignancy: primary effusion lymphoma (PEL) and the plasma cell variant of multicentric Castleman's disease. Like other herpesviruses, KSHV can establish a lifelong latent infection characterized by a limited viral gene expression [106].

A total of 17 KSHV miRNAs encoded by 12 distinct miRNA genes have been reported and their sequences are highly conserved between different KSHV genomes in PEL cell lines and in clinical samples. However, some polymorphism was observed, particularly in miR-K12-5 and miR-K12-9 [80, 107, 108]. The entire KSHV miRNA cluster resides within an approximately 4 kilobase-pairs region between open reading frames ORF K12 (kaposin) and ORF 71 (Figure 2(e)).

To elucidate the functions of the KSHV miRNAs, transcriptome analysis was performed from cells stably expressing the miR-K12 cluster. Among the differentially expressed genes were genes encoding proteins implicated in proliferation, immune modulation, angiogenesis, and apoptosis. The gene encoding thrombospondin-1 was targeted by all ten KSHV miRNAs, but especially by miR-K12-1, miR-K12-3-3p, miR-K12-6-3p, and miR-K12-11. Thrombospondin-1 possesses antiproliferative and anti-angiogenic properties. Other transcripts that were reduced corresponded to the genes for osteopontin, S100 calcium binding protein, plasticity related gene 1 product, and integral membrane protein 2A [80, 81]. The mRNA for the Bcl-2 interacting protein BCLAF1 was identified as a target for miR-K12-5. Additional inhibition of BCLAF1 expression was obtained in the presence of miR-K9, -10a, and -10b. The

exact biological relevance is not yet understood, but siRNA-mediated depletion of BCLAF1 enhanced the frequency of spontaneous lytic reactivation of KSHV. MiRNA-mediated reduction of BCLAF1 expression would prevent permanent latency of the virus, a type of infection that represents a dead-end pathway of viral spreading [108, 109].

A KSHV miRNA that has gained special interest is miR-K12-11 because its seed sequence, known to be critically important for mRNA target recognition is 100% conserved with the cellular miR-155, suggesting that these miRNAs may regulate common targets. The exact role of miR-155 remains unclear, but a number of B-cell lymphomas and solid organ tumors overexpress miR-155, while miR-155 transgenic mice develop B lymphomas [110, 111]. Work by the groups of Skalsky and Cullen confirmed that miR-K12-11 indeed is an orthologue of miR-155, and that they target common transcripts [81, 82]. Comparing the gene expression profiles in cells stably expressing either miR-155 or miR-K12-11 revealed that they regulate an analogous set of mRNAs. The products of these transcripts include proteins involved in B-cell function (e.g., Src-like adaptor or SLA), innate immunity (e.g., IκB kinase and phosphoinositide-3-kinase), apoptosis (XIAP associated factor-1; LDOC1), cell cycle regulation (e.g., FOS), and gene expression (e.g., FOS and BACH1). BACH-1 (Btb and CNC homolog 1) is a bZip protein that can repress transcription through heterodimerization with the small Maf proteins [112], while c-Fos can heterodimerize with the JUN proteins to form the AP-1 complex. AP-1 is a multifunctional protein involved in cellular proliferation, transformation, and apoptosis [113]. For a complete list of miR-155/miR-K12-11 regulated genes, the reader is referred to the work of Skalsky et al., and of Gottwein et al. [81, 82]. Treatment of latently-infected KSHV with an antagomir against miR-K12-11 enhanced Fos protein levels about 2.5-fold compared to untreated cells [81]. Computational analyses further revealed seed sequence homology between the viral miRNAs KSHV miR-K12-6-5p, EBV-BART5, and HCMV UL70-5p with human miRNAs miR-15a plus 16, miR-18a/b, and miR-340, respectively [82]. Both miR-15a and miR-16 are believed to possess tumour suppressor activity and to induce apoptosis by silencing Bcl2 expression, while miR-18 was demonstrated to be oncogenic [52, 114]. KSHV-encoded miRNAs seem to be crucial both in survival of the virus in its host, but also to play a causal role in viral-associated pathologies. AMO-mediated silencing of KSHV-encoded miRNAs may thus be a strategy to counteract viral infection, but may also undesirably target cellular miRNAs with identical seed sequences as the viral miRNAs.

4.5. Poxvirus. Poxviruses are dsDNA viruses that replicate in the cytoplasm and have as such no access to the nuclear proteins involved in the biogenesis of miRNA. Nevertheless, miRNA precursor sequences have been predicted in the genomes of the human poxviruses vaccinia virus and variola virus, but their existence has not been validated [25, 65]. Whether the other human poxvirus, molluscum contagiosum virus, encodes miRNA remains to be established.

4.6. Hepatitis B Virus. Hepatitis B Virus (HBV), an enveloped virus with a circular partial dsDNA genome, persistently infects more than 300 million people worldwide. HBV can cause a spectrum of liver diseases ranging from mild liver dysfunctions to chronic hepatitis, cirrhosis, and hepatocellular carcinoma [53]. This makes efficient anti-HBV therapy highly vital. AMO-based vmiRNA silencing is probably no option since no miRNAs could be detected by computational analysis [65], and expression of HBV miRNAs has not been reported so far. One study identified a putative HBV-encoded miRNA, but in vivo expression of this HBV miRNA was not tested. Computational screening for complementary sequences in the 3' untranslated regions of cellular mRNA to this HBV miRNA did not reveal putative target transcripts [54]. It therefore seems unlike that this is a *bona fide* viral miRNA.

4.7. Human Immunodeficiency Virus (HIV). HIV is the causative agent of acquired immune deficiency syndrome (AIDS), and it is estimated that >30 million people worldwide are infected with this virus. Two species, HIV-1 and HIV-2, infect humans (for a recent review see [55]).

HIV utilizes reverse transcriptase to convert its ssRNA genome into a dsDNA provirus. During this process, the 5' and 3' ends of the viral RNA genome are converted into long terminal repeats (LTRs). The LTRs play a pivotal regulatory role in establishing, maintaining, and overriding the latent state of the virus [115]. The central domain of the LTR is referred to the R region, which encompasses the (transactivation-response region) TAR. TAR binds the viral protein Tat, a transactivator that plays an important role in the transcriptional activation of the provirus genome (reviewed in [116]). The TAR encodes proven and putative miRNAs (Figure 2(f)). Klase and coworkers described an miRNA encoded by the HIV-1 TAR element. This miRNA causes HDAC-1 to associate with the viral LTR, resulting in diminished viral gene expression. This suggests a role for HIV-1 miRNA in maintaining viral latency [83]. In another report, Ouellet and colleagues demonstrated the expression of two TAR element-derived miRNAs by Northern blotting, primer extension, and RNase protection assay. The miRNA derived from the left arm of the TAR stem has been named miR-TAR-5p, while the miRNA originating from the right arm was designated miR-TAR-3p. The latter appears to preferentially accumulate in HIV-positive cells [84]. The biological role of these miRNAs remains to be elucidated, but they may contribute to modulating viral and/or cellular gene expression, with a potential impact on viral replication and/or host antiviral defense efficiency. The miR-TAR-5p described by Ouellet overlaps with the vmiRNA no. 1, while miR-TAR-3p partially overlaps with vmiRNA no. 5 described by Bennasser and coworkers [85]. They predicted by computer-directed analyses 5 pre-miRNAs in the HIV-1 genome, which in principle could yield 10 mature miRNAs. Their expression has not been validated, nor has their biological role been addressed, but deduction of potential target transcripts resulted in the indentification of cellular genes encoding protein kinases, ion channels,

proteins involved in protein synthesis and degradation, growth factors, and DNA methylation [85]. TAR DNA of the long terminal repeat of HIV-1 encompasses an antisense RNA (HIVaINR), which encodes HIV proteins, but that can also form a duplex with the 5' end of all sense HIV mRNA, enabling the virus to control the expression of its gene [117]. HIVaINR can potentially code for several miRNAs, referred to as HAAmiRNAs. Putative targets for these miRNAs are the mRNAs for interleukin (IL)-15, IL-2 receptor γ chain, human fragile X mental retardation protein (FMRP), and IL-1 receptor-associated kinase 1 (IRAK1). IL-15 is important in the regulation of T-cell maturation, development and survival of natural killer cells, and survival of long-lived memory T cells, while the IL-2 receptor γ chain is a common component of the receptors for IL-2, -4, -7, -9, -15, and -21. Aberant expression of this receptor leads to severe T-cell and NK-cell deficiencies. IRAK1 is a critical signalling mediator of innate immunity. Downregulation the expression of IL-15, IL-2 receptor γ chain, and IRAK1 by HIV miRNA would impair the immune system and favor survival of the virus in the host. FMRP is an RNA-binding protein that is implicated in protein synthesis and miRNA processing. Thus HIV could use HAAmiRNA to deregulate the host miRNA mechanism to dispose the virus by depleting FMRP ([56] and references therein.) Although the existence of these HAAmiRNAs has not been proven, it is tempting to speculate that, in accordance with other viruses, HIV encodes miRNAs allowing HIV to survive in the host. A recent study examined the possibility of HIV-2 TAR to encode miRNAs. Two putative miRNAs, miR-TAR2-5p and miR-TAR-3p were identified, but their expression awaits validation [87].

Besides TAR, other regions of the HIV genome have been shown to contain miRNA sequences. The *nef* gene of HIV-1 is located at the 3' end of the viral genome and is highly expressed during the early stages of virus replication. Nef is a multifunctional accessory protein that is important for viral replication, but that also plays a key role in pathogenesis as Nef can downmodulate CD4, CD28, and the class I major histocompatibility complex [86]. HIV-1 encodes a *nef*-derived miRNA referred to as miR-N367 (Figure 2(f)). Unlike classical miRNA, this miRNA does not affect gene expression at the post-transcription level, but rather at the transcription level by promoter interference. MiR-N367 suppresses HIV-1 promoter activity via a negative responsive element in the 5'-long terminal region and via Nef sequences in the 3' untranslated region [57, 118]. Future studies are required to elucidate the precise mechanism by which miR-N3667 represses HIV-1 promoter activity. Downregulation of Nef expression may suppress HIV-1 replication and allow persistently low pathogenic or latent viral infection [57]. As the *nef* gene is conserved in HIV-2, HIV-2 may also apply a similar mechanism to maintain a low profile in the host.

The identity and action of HIV miRNAs remains to be scrutinized before AMOs-based therapy can be considered as anti-HIV drugs. However, computational alignment of the potential HIV-1 miRNAs with specific human T-cell mRNAs identified potential cellular targets including genes encoding CD4, CD28 and interleukin-2, IL-3, and IL-12 [119]. Viral miRNA-caused inhibition of the expression of these proteins seems advantageous for the virus, and therefore counteracting vmiRNA by AMO may help the host to clear HIV infection.

4.8. Human T-cell leukaemia virus type 1 (HTLV-I). HTLV-I persistently infects 10–20 million humans worldwide and is the etiological agent for adult T-cell leukaemia [5]. One study reported that T cells persistently infected with HTLV-1 did not express viral microRNAs [88], but meticulous studies should be performed to rule out the existence of HTLV-I miRNA.

4.9. RNA Viruses. Pfeffer et al. tried to identify miRNA in the genomes of human RNA viruses like Marburg and Ebola virus (*Filoviridae*), measles virus (*Paramyxoviridae*), poliovirus (*Picornaviridae*), yellow fever virus and hepatitis C virus (HCV; *Flaviviridae*), and Sindbis virus (*Togaviridae*). None of these viral genomes seem to contain putative miRNA sequences, except for measles virus and yellow fever virus, which each possesses a single putative miRNA. However, the miRNA in yellow fever virus could not be validated, while the existence of miRNA in measles virus was not tested [25, 65]. Intriguingly, the liver-specific miR-122 facilitates the replication of the oncovirus HCV, but the mechanism for this function of miR-122 in HCV replication is still unknown [120, 121].

5. Conclusions

Numerous human diseases are caused by viral infections, but the intimate relation with the host makes the development of antiviral drugs difficult. Vaccination has been proven to be very succesful to combat some viral infections, but mutations and diversity of virus strains has hampered the development of efficient vaccines against other viruses. New antiviral treatments are based on drugs that inhibit specific viral activities such as viral proteases or polymerases (for recent reviews see [122, 123]). Viral-encoded miRNA that may be implicated in the viral life cycle and the pathogenic properties of the virus offers a novel attractive target for antiviral therapy. Silencing the action of viral miRNAs may enable the host cell or the immune system to gain control over the virus and even to eliminate the virus. The idea of targeting viral transcripts is not new, and RNA interference has been demonstrated to efficiently mediate inhibition of replication of human pathogenic viruses such as HIV-1, HCV, dengue virus, severe acute respiratory syndrome (SARS) coronavirus, poliovirus, human rhinovirus, influenza A virus, hepatitis D virus, HBV, HSV-1, HPV, JCV, EBV, and CMV in cell culture (reviewed in [12]). Besides recent studies have proven the potential of this RNA interference as antiviral therapy in animal models [124, 125], and even in clinical trials such as Alnylam against respiratory syncytial virus and NUC B1000 against HBV (reviewed in [126, 127]). However, anti-HIV RNA interference studies revealed that escape virus variants could appear which could evade the inhibitory action of siRNA [128, 129].

The use of AMO to neutralize viral miRNA adds a new twist to RNA interference. AMOs are easy to produce and relatively cheap, and easy to administer locally (but not systemically). Moreover, they possess low toxicity and are highly specific. Most viral miRNAs identified so far have little homology to each other and to known host cell miRNAs (reviewed in [130]). This reduces the risk of off-target effects of anti-miRNA oligonucleotides and increases the therapeutic potentials of miRNA silencing. The miRNA silencing action of both LNA and antagomirs is sensitive to single nucleotide exchanges. For antagomirs, it was shown that this effect depends on the position of the mismatch. Nucleotide substitutions at the very 5' end or in the centre did not prevent downregulation of miR-122 [33]. These data indicate that changes in the 3' end of the antagomir may abrogate its ability to destroy target miRNA and should be taken into account when designing and testing antagomirs. Another advantage of AMOs is that they probably can be used against all serotypes of a specific virus. Although not meticulously investigated, miRNA sequences between different viral strains seem to be conserved because of their importance for the viral life-cycle (see e.g., KSHV-encoded miRNAs; [80, 107, 108]). However, polymorphism in viral miRNAs has also been observed (see next paragraph).

Although AMOs may provide an attractive novel antiviral therapy, practical problems and other pitfalls may hamper the use of them. For example, antagomirs directed against miR-UL-112-1 could drive the virus towards acute replication and disrupted the inhibition of MCIB expression, resulting in possible clearance of the virus by the immune system. However, there is a potential risk of severe pathological effects caused by acutely replicating HCMV, especially in immunocompromised patients [50]. Another disadvantage of AMOs may be off-target effects. As miRNA do not require full complimentarity to bind their target sequence, it can be imagined that an AMO not only binds to its predicted miRNA but also to other miRNAs and even mRNAs. In addition, ssRNA oligonucleotides may interact with Toll-like receptors 7 and 8 thereby stimulating the immune system. Similar side effects have been reported for siRNA (reviewed in [126]). Polymorphism in viral-encoded miRNAs has been described in viral-infected cell lines and in clinical samples. For instance, miR-K12-5 of different KSHV isolates contains mutations, which can affect maturation and biological activity of this miRNA [25, 100, 107]. Thus the miRNA may not be expressed, in which case the AMO will have no effect or the AMO may not bind because of the mutations in its target miRNA. Another problem facing the use of AMOs in antiviral therapy is that the expression of a specific gene may be regulated by several viral miRNAs. For example, translation of the transcript of the*BACH*gene is prevented by three KSHV viral miRNAS: miR-K12-11, miRNA-K12-1, and miR-K12-6. Thus the effectiveness of an AMO against, for example, miR-12-11 can be compromised. Indeed, treatment of latently infected KSHV virus with a specific antagomir against miR-K12-12 alone only modestly increased the amount of BACH protein [81]. A cocktail of different AMOs directed against distinct viral miRNA may help to overcome this problem. So far, such studies are lacking, but a recent study successfully applied sponge miRNA to silence HBV transcripts. An expression vector encoding multiple miRNAs targeting HBV HBsAg mRNA strongly reduced the expression of this protein [131]. Another challenge is to improve in vivo delivery of the AMOs to viral infected cells and obtain long-lasting action of the antagomirs. Aerosol delivery devices similar to the ones used for delivery of asthma therapeutics could be used for respiratory viruses [15]. Other delivery strategies include intravenal or systemic injection, viral vectors, and lipid- and polymer-based vehicles [131–134]. Recently, sustained inhibition of HCV replication in cell-culture was obtained when cell-degradable multilayered polyelectrolyte film (MPF) coated with siRNA was delivered to infected cells. By this approach, a single regime of MPF-mediated siRNA treatment was sufficient to inhibit HCV replication for 12 days. Moreover, MPF-mediated delivery of siRNA also protected uninfected cells from HCV infection. Another advantage is the very low toxicity of MPF [135]. These promising observations in cell culture put MPF-based delivery of AMOs forward as an efficient antiviral tool. Another limitation of anti-miRNAs is the site of application. Studies with antagomirs against miR-16 in mice revealed that when injected into tail veins, antagomirs were incapable of silencing miR-16, whereas local injection into the mouse cortex efficiently induced degradation of the target miRNA [33]. Another drawback of the use of AMOs is that the chemical modification can exert antiproliferative or other off-target effects such as been demonstrated for the phosphorothioate backbone, which can associate with cellular proteins [29].

Antisense oligonucleotides such as LNA and PMO have proven to efficiently inhibit RNA and DNA virus replication in cell culture and animal models, without toxicity for the cell or animal. However, these PMO were directed against viral protein-encoding mRNA, and studies of PMO-mediated silencing of viral miRNA have not been reported so far (see e.g., [31, 136–138]). Future viral miRNA research is faced with important challenges before AMOs may enter the clinic. Our comprehension on the functions of viral miRNA and the interplay between viral infection and cellular miRNA expression is just beginning to emerge. Studies aimed at the identification of viral miRNA and elucidation of their functions should be pursued. Difficulties facing computational-based prediction are false positives, but also the shortcoming to detect genuine miRNAs. Moreover confirmation of expression of miRNA by, for example, Northern blot may fail to monitor miRNA. Expression levels of vmiRNA may be cell-specific, for example, EBV miR-BHFR1-2 had considerably lower expression levels in Jijoye cells than in B95-8 cells [7]. Dose- and time-dependent studies are required to determine the optimal therapeutic regime. Such pharmacokinetics and pharmacodynamic studies are largely lacking [133], but recent in studies in mice revealed that a single cerebral or tail-vein injection of 240 mg/kg body mass anti-miR122 had a silencing effect for at least 8 days and as long as 23 days in the tissues examined [32, 33]. In another study, a five-week regime of two intraperitonal or subcutaneous injection a week with different concentrations of anti-miR-122 clearly silenced miR-122 and no untoward

effects were observed [35]. Despite the obstacles facing antiviral miRNA silencing as viral therapy, the coming years will certainly see the daylight of intensified research and even the initiation of clinical trials.

References

[1] H. zur Hausen, "Papillomaviruses and cancer: from basic studies to clinical application," *Nature Reviews Cancer*, vol. 2, no. 5, pp. 342–350, 2002.

[2] L. Coscoy, "Immune evasion by Kaposi's sarcoma-associated herpesvirus," *Nature Reviews Immunology*, vol. 7, no. 5, pp. 391–401, 2007.

[3] B. Damania, "DNA tumor viruses and human cancer," *Trends in Microbiology*, vol. 15, no. 1, pp. 38–44, 2007.

[4] E. Klein, L. L. Kis, and G. Klein, "Epstein-Barr virus infection in humans: from harmless to life endangering virus-lymphocyte interactions," *Oncogene*, vol. 26, no. 9, pp. 1297–1305, 2007.

[5] M. Matsuoka and K.-T. Jeang, "Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation," *Nature Reviews Cancer*, vol. 7, no. 4, pp. 270–280, 2007.

[6] V. E. Gurtsevitch, "Human oncogenic viruses: hepatitis B and hepatitis C viruses and their role in hepatocarcinogenesis," *Biochemistry*, vol. 73, no. 5, pp. 504–513, 2008.

[7] U. Moens and M. Johannessen, "Human polyomaviruses and cancer: expanding repertoire," *Journal of the German Society of Dermatology*, vol. 6, no. 9, pp. 704–708, 2008.

[8] V. Brower, "Connecting viruses to cancer: how research moves from association to causation," *Journal of the National Cancer Institute*, vol. 96, no. 4, pp. 256–257, 2004.

[9] D. M. Morens, G. K. Folkers, and A. S. Fauci, "The challenge of emerging and re-emerging infectious diseases," *Nature*, vol. 430, no. 6996, pp. 242–249, 2004.

[10] World Health Organization, 2008, http://www.who.int/healthinfo/morttables/en/index.html.

[11] B. R. Cullen, "Viruses and microRNAs," *Nature Genetics*, vol. 38, supplement 6, pp. S25–S30, 2006.

[12] B. Berkhout and J. Haasnoot, "The interplay between virus infection and the cellular RNA interference machinery," *FEBS Letters*, vol. 580, no. 12, pp. 2896–2902, 2006.

[13] B. Berkhout and K.-T. Jeang, "RISCy business: microRNAs, pathogenesis, and viruses," *Journal of Biological Chemistry*, vol. 282, no. 37, pp. 26641–26645, 2007.

[14] J. Haasnoot, E. M. Westerhout, and B. Berkhout, "RNA interference against viruses: strike and counterstrike," *Nature Biotechnology*, vol. 25, no. 12, pp. 1435–1443, 2007.

[15] W. de Vries and B. Berkhout, "RNAi suppressors encoded by pathogenic human viruses," *International Journal of Biochemistry & Cell Biology*, vol. 40, no. 10, pp. 2007–2012, 2008.

[16] B. R. Cullen, "Viral and cellular messenger RNA targets of viral microRNAs," *Nature*, vol. 457, no. 7228, pp. 421–425, 2009.

[17] Z. Ghosh, B. Mallick, and J. Chakrabarti, "Cellular versus viral microRNAs in host-virus interaction," *Nucleic Acid Research*, vol. 37, no. 4, pp. 1035–1048, 2009.

[18] M. N. Poy, M. Spranger, and M. Stoffel, "MicroRNAs and the regulation of glucose and lipid metabolism," *Diabetes, Obesity and Metabolism*, vol. 9, supplement 2, pp. 67–73, 2007.

[19] Y. Zhao and D. Srivastava, "A developmental view of microRNA function," *Trends in Biochemical Sciences*, vol. 32, no. 4, pp. 189–197, 2007.

[20] D. Baltimore, M. P. Boldin, R. M. O'Connell, D. S. Rao, and K. D. Taganov, "MicroRNAs: new regulators of immune cell development and function," *Nature Immunology*, vol. 9, no. 8, pp. 839–845, 2008.

[21] F.-B. Gao, "Posttranscriptional control of neuronal development by microRNA networks," *Trends in Neurosciences*, vol. 31, no. 1, pp. 20–26, 2008.

[22] S. Griffiths-Jones, H. K. Saini, S. van Dongen, and A. J. Enright, "miRBase: tools for microRNA genomics," *Nucleic Acids Research*, vol. 36, database issue, pp. D154–D158, 2008.

[23] V. Ambros, "The functions of animal microRNAs," *Nature*, vol. 431, no. 7006, pp. 350–355, 2004.

[24] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[25] E. Gottwein and B. R. Cullen, "Viral and cellular microRNAs as determinants of viral pathogenesis and immunity," *Cell Host & Microbe*, vol. 3, no. 6, pp. 375–387, 2008.

[26] J. Brennecke, A. Stark, R. B. Russell, and S. M. Cohen, "Principles of microRNA-target recognition," *PLoS Biology*, vol. 3, no. 3, article e85, pp. 1–15, 2005.

[27] A. Krek, D. Grün, M. N. Poy, et al., "Combinatorial microRNA target predictions," *Nature Genetics*, vol. 37, no. 5, pp. 495–500, 2005.

[28] J. Mattes, A. Collison, and P. S. Foster, "Emerging role of microRNAs in disease pathogenesis and strategies for therapeutic modulation," *Current Opinion in Molecular Therapeutics*, vol. 10, no. 2, pp. 150–157, 2008.

[29] C. C. Esau, "Inhibition of microRNA with antisense oligonucleotides," *Methods*, vol. 44, no. 1, pp. 55–60, 2008.

[30] J. Elmén, M. Lindow, S. Schütz, et al., "LNA-mediated microRNA silencing in non-human primates," *Nature*, vol. 452, no. 7189, pp. 896–899, 2008.

[31] K. B. Spurgers, C. M. Sharkey, K. L. Warfield, and S. Bavari, "Oligonucleotide antiviral therapeutics: antisense and RNA interference for highly pathogenic RNA viruses," *Antiviral Research*, vol. 78, no. 1, pp. 26–36, 2008.

[32] J. Krützfeldt, N. Rajewsky, R. Braich, et al., "Silencing of microRNAs in vivo with 'antagomirs'," *Nature*, vol. 438, no. 7068, pp. 685–689, 2005.

[33] J. Krützfeldt, S. Kuwajima, R. Braich, et al., "Specificity, duplex degradation and subcellular localization of antagomirs," *Nucleic Acids Research*, vol. 35, no. 9, pp. 2885–2892, 2007.

[34] J. Mattes, M. Yang, and P. S. Foster, "Regulation of microRNA by antagomirs: a new class of pharmacological antagonists for the specific regulation of gene function?" *American Journal of Respiratory Cell and Molecular Biology*, vol. 36, no. 1, pp. 8–12, 2007.

[35] C. Esau, S. Davis, S. F. Murray, et al., "miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting," *Cell Metabolism*, vol. 3, no. 2, pp. 87–98, 2006.

[36] M. Egholm, O. Buchardt, L. Christensen, et al., "PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules," *Nature*, vol. 365, no. 6446, pp. 566–568, 1993.

[37] M. M. Fabani and M. J. Gait, "miR-122 targeting with LNA/2′-O-methyl oligonucleotide mixmers, peptide nucleic acids (PNA), and PNA-peptide conjugates," *RNA*, vol. 14, no. 2, pp. 336–346, 2008.

[38] J. Weiler, J. Hunziker, and J. Hall, "Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease?" *Gene Therapy*, vol. 13, no. 6, pp. 496–502, 2006.

[39] U. Moens, M. Van Ghelue, and M. Johannessen, "Human polyomaviruses: molecular mechanisms for transformation and their association with cancers," in *New Research on Oncogenic Viruses*, E. I. Tuneley, Ed., pp. 1–63, Nova Science, New York, NY, USA, 2008.

[40] M. Jiang, J. R. Abend, S. F. Johnson, and M. J. Imperiale, "The role of polyomaviruses in human disease," *Virology*, vol. 384, no. 2, pp. 266–273, 2009.

[41] T. Allander, K. Andreasson, S. Gupta, et al., "Identification of a third human polyomavirus," *Journal of Virology*, vol. 81, no. 8, pp. 4130–4136, 2007.

[42] A. M. Gaynor, M. D. Nissen, D. M. Whiley, et al., "Identification of a novel polyomavirus from patients with acute respiratory tract infections," *PLoS Pathogens*, vol. 3, no. 5, article e64, pp. 1–10, 2007.

[43] H. Feng, M. Shuda, Y. Chang, and P. S. Moore, "Clonal integration of a polyomavirus in human Merkel cell carcinoma," *Science*, vol. 319, no. 5866, pp. 1096–1100, 2008.

[44] U. Moens and O. P. Rekvig, "Molecular biology of BK virus and clinical aspects of BK virus renal infection," in *Human Polyomaviruses: Molecular and Clinical Perspectives*, K. Khalili and G. L. Stoner, Eds., pp. 359–408, Wiley Liss, New York, NY, USA, 2001.

[45] C. S. Sullivan, A. T. Grundhoff, S. Tevethia, J. M. Pipas, and D. Ganem, "SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells," *Nature*, vol. 435, no. 7042, pp. 682–686, 2005.

[46] G. J. Seo, L. H. L. Fink, B. O'Hara, W. J. Atwood, and C. S. Sullivan, "Evolutionarily conserved function of a viral microRNA," *Journal of Virology*, vol. 82, no. 20, pp. 9823–9828, 2008.

[47] G. J. Seo, C. J. Chen, and C. S. Sullivan, "Merkel cell polyomavirus encodes a microRNA with the ability to autoregulate viral gene expression," *Virology*, vol. 383, no. 2, pp. 183–187, 2009.

[48] J. L. Umbach, M. F. Kramer, I. Jurak, H. W. Karnowski, D. M. Coen, and B. R. Cullen, "MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs," *Nature*, vol. 454, no. 7205, pp. 780–783, 2008.

[49] W. Dunn, P. Trang, Q. Zhong, E. Yang, C. van Belle, and F. Liu, "Human cytomegalovirus expresses novel microRNAs during productive viral infection," *Cellular Microbiology*, vol. 7, no. 11, pp. 1684–1695, 2005.

[50] F. Grey and J. Nelson, "Identification and function of human cytomegalovirus microRNAs," *Journal of Clinical Virology*, vol. 41, no. 3, pp. 186–191, 2008.

[51] S. Pfeffer, M. Zavolan, F. A. Grässer, et al., "Identification of virus-encoded microRNAs," *Science*, vol. 304, no. 5671, pp. 734–736, 2004.

[52] L. He, J. M. Thomson, M. T. Hemann, et al., "A microRNA polycistron as a potential human oncogene," *Nature*, vol. 435, no. 7043, pp. 828–833, 2005.

[53] C. Seeger, F. Zoulin, and W. S. Mason, "Hepadnaviruses," in *Fields' Virology*, D. M. Knipe and P. M. Howeley, Eds., pp. 2847–2888, Lippincott Williams and Wilkins, Philadelphia, Pa, USA, 2007.

[54] W.-B. Jin, F.-L. Wu, D. Kong, and A.-G. Guo, "HBV-encoded microRNA candidate and its target," *Computational Biology and Chemistry*, vol. 31, no. 2, pp. 124–126, 2007.

[55] M. A. Wainberg and K.-T. Jeang, "25 years of HIV-1 research—progress and perspectives," *BMC Medicine*, vol. 6, article 31, pp. 1–7, 2008.

[56] L. B. Ludwig, "RNA silencing and HIV: a hypothesis for the etiology of the severe combined immunodeficiency induced by the virus," *Retrovirology*, vol. 5, article 79, pp. 1–13, 2008.

[57] S. Omoto, M. Ito, Y. Tsutsumi, et al., "HIV-1 nef suppression by virally encoded microRNA," *Retrovirology*, vol. 1, p. 44, 2004.

[58] X. Cai, G. Li, L. A. Laimins, and B. R. Cullen, "Human papillomavirus genotype 31 does not express detectable microRNA levels during latent or productive virus replication," *Journal of Virology*, vol. 80, no. 21, pp. 10890–10893, 2006.

[59] W. S. M. Wold and M. S. Horowitz, "Adenoviruses," in *Fields' Virology*, D. M. Knipe and P. M. Howeley, Eds., pp. 2395–2436, Lippincott Williams and Wilkins, Philadelphia, Pa, USA, 2007.

[60] M. B. Mathews, "Structure, function and evolution of adenovirus virus-associated RNAs," *Current Topics in Microbiology and Immunology*, vol. 199, part 2, pp. 173–187, 1995.

[61] M. G. Andersson, P. C. J. Haasnoot, N. Xu, S. Berenjian, B. Berkhout, and G. Akusjärvi, "Suppression of RNA interference by adenovirus virus-associated RNA," *Journal of Virology*, vol. 79, no. 15, pp. 9556–9565, 2005.

[62] O. Aparicio, N. Razquin, M. Zaratiegui, I. Narvaiza, and P. Fortes, "Adenovirus virus-associated RNA is processed to functional interfering RNAs involved in virus production," *Journal of Virology*, vol. 80, no. 3, pp. 1376–1384, 2006.

[63] M. Sano, Y. Kato, and K. Taira, "Sequence-specific interference by small RNAs derived from adenovirus VAI RNA," *FEBS Letters*, vol. 580, no. 6, pp. 1553–1564, 2006.

[64] P. E. Pellet and B. Roizman, "The family Herpesviridae: a brief introduction," in *Fields' Virology*, D. M. Knipe and P. M. Howeley, Eds., pp. 2479–2500, Lippincott Williams and Wilkins, Philadelphia, Pa, USA, 2007.

[65] S. Pfeffer, A. Sewer, M. Lagos-Quintana, et al., "Identification of microRNAs of the herpesvirus family," *Nature Methods*, vol. 2, no. 4, pp. 269–276, 2005.

[66] X. Cai, A. Schäfer, S. Lu, et al., "Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed," *PLoS Pathogens*, vol. 2, no. 3, article e23, pp. 1–12, 2006.

[67] A. Gupta, J. J. Gartner, P. Sethupathy, A. G. Hatzigeorgiou, and N. W. Fraser, "Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript," *Nature*, vol. 442, no. 7098, pp. 82–85, 2006.

[68] S. Tang, A. S. Bertke, A. Patel, K. Wang, J. I. Cohen, and P. R. Krause, "An acutely and latently expressed herpes simplex virus 2 viral microRNA inhibits expression of ICP34.5, a viral neurovirulence factor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 31, pp. 10931–10936, 2008.

[69] C. Cui, A. Griffiths, G. Li, et al., "Prediction and identification of herpes simplex virus 1-encoded microRNAs," *Journal of Virology*, vol. 80, no. 11, pp. 5499–5508, 2006.

[70] S. Tang, A. Patel, and P. R. Krause, "Novel less-abundant viral miRNAs encoded by herpes simplex virus 2 latency-associated transcript and their roles in regulating ICP34.5 and ICP0 mRNAs," *Journal of Virology*, vol. 83, no. 3, pp. 1433–1442, 2009.

[71] R. Grassmann and K.-T. Jeang, "The roles of microRNAs in mammalian virus infection," *Biochimica et Biophysica Acta*, vol. 1779, no. 11, pp. 706–711, 2008.

[72] N. Stern-Ginossar, N. Elefant, A. Zimmermann, et al., "Host immune system gene targeting by a viral miRNA," *Science*, vol. 317, no. 5836, pp. 376–381, 2007.

[73] E. Murphy, J. Vaníček, H. Robins, T. Shenk, and A. J. Levine, "Suppression of immediate-early viral gene expression by herpesvirus-coded microRNAs: implications for latency," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 14, pp. 5453–5458, 2008.

[74] F. Grey, H. Meyers, E. A. White, D. H. Spector, and J. Nelson, "A human cytomegalovirus-encoded microRNA regulates expression of multiple viral genes involved in replication," *PloS Pathogens*, vol. 3, article e163, no. 11, pp. 1–10, 2007.

[75] A. Grundhoff, C. S. Sullivan, and D. Ganem, "A combined computational and microarray-based approach identifies novel microRNAs encoded by human gamma-herpesviruses," *RNA*, vol. 12, no. 5, pp. 733–750, 2006.

[76] A. K. F. Lo, K. F. To, K. W. Lo, et al., "Modulation of LMP1 protein expression by EBV-encoded microRNAs," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 41, pp. 16164–16169, 2007.

[77] S. Barth, T. Pfuhl, A. Mamiani, et al., "Epstein-Barr virus-encoded microRNA miR-BART2 down-regulates the viral DNA polymerase BALF5," *Nucleic Acids Research*, vol. 36, no. 2, pp. 666–675, 2008.

[78] E. Y. Choy, K. L. Siu, K. H. Kok, et al., "An Epstein-Barr virus-encoded microRNA targets PUMA to promote host cell survival," *The Journal of Experimental Medicine*, vol. 205, no. 11, pp. 2551–2560, 2008.

[79] T. Xia, A. O'Hara, I. Araujo, et al., "EBV microRNAs in primary lymphomas and targeting of CXCL-11 by ebv-mir-BHRF1-3," *Cancer Research*, vol. 68, no. 5, pp. 1436–1442, 2008.

[80] M. A. Samols, R. L. Skalsky, A. M. Maldonado, et al., "Identification of cellular genes targeted by KSHV-encoded microRNA," *PLoS Pathogens*, vol. 3, no. 5, article e65, pp. 1–8, 2007.

[81] E. Gottwein, N. Mukherjee, C. Sachse, et al., "A viral microRNA functions as an orthologue of cellular miR-155," *Nature*, vol. 450, no. 7172, pp. 1096–1099, 2007.

[82] R. L. Skalsky, M. A. Samols, K. B. Plaisance, et al., "Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155," *Journal of Virology*, vol. 81, no. 23, pp. 12836–12845, 2007.

[83] Z. Klase, P. Kale, R. Winograd, et al., "HIV-1 TAR element is processed by Dicer to yield a viral micro-RNA involved in chromatin remodeling of the viral LTR," *BMC Molecular Biology*, vol. 8, article 63, pp. 1–19, 2007.

[84] D. L. Ouellet, I. Plante, P. Landry, et al., "Identification of functional microRNAs released through asymmetrical processing of HIV-1 TAR element," *Nucleic Acids Research*, vol. 36, no. 7, pp. 2353–2365, 2008.

[85] Y. Bennasser, S.-Y. Le, M. L. Yeung, and K.-T. Jeang, "HIV-1 encoded candidate micro-RNAs and their cellular targets," *Retrovirology*, vol. 1, article 43, pp. 1–5, 2004.

[86] J. L. Foster and J. V. Garcia, "HIV-1 Nef: at the crossroads," *Retrovirology*, vol. 5, article 84, pp. 1–13, 2008.

[87] K. J. Purzycka and R. W. Adamiak, "The HIV-2 TAR RNA domain as a potential source of viral-encoded miRNA. A reconnaissance study," *Nucleic Acids Symposium Series*, vol. 52, no. 1, pp. 511–512, 2008.

[88] J. Lin and B. R. Cullen, "Analysis of the interaction of primate retroviruses with the human RNA interference machinery," *Journal of Virology*, vol. 81, no. 22, pp. 12218–12226, 2007.

[89] B. Roizman, D. M. Knipe, and R. J. Whitley, "Herpes simplex viruses," in *Fields' Virology*, D. M. Knipe and P. M. Howeley, Eds., pp. 2501–2602, Lippincott Williams and Wilkins, Philadelphia, Pa, USA, 2007.

[90] B. Sugden, "Virology: micro mystery solution," *Nature*, vol. 442, no. 7098, pp. 33–34, 2006.

[91] A. Gupta, J. J. Gartner, P. Sethupathy, A. G. Hatzigeorgiou, and N. W. Fraser, "Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript," *Nature*, vol. 451, no. 7178, p. 600, 2008.

[92] R. Gupta, T. Warren, and A. Wald, "Genital herpes," *The Lancet*, vol. 370, no. 9605, pp. 2127–2137, 2007.

[93] A. Orvedahl, D. Alexander, Z. Tallóczy, et al., "HSV-1 ICP34.5 confers neurovirulence by targeting the beclin 1 autophagy protein," *Cell Host & Microbe*, vol. 1, no. 1, pp. 23–35, 2007.

[94] J. I. Cohen, S. E. Straus, and A. M. Arvin, "Varicella-zoster virus," in *Fields' Virology*, D. M. Knipe and P. M. Howeley, Eds., pp. 2733–2818, Lippincott Williams and Wilkins, Philadelphia, Pa, USA, 2007.

[95] F. Grey, L. Hook, and J. Nelson, "The functions of herpesvirus-encoded microRNAs," *Medical Microbiology and Immunology*, vol. 197, no. 2, pp. 261–267, 2008.

[96] J. A. Nelson, "Small RNAs and large DNA viruses," *The New England Journal of Medicine*, vol. 357, no. 25, pp. 2630–2632, 2007.

[97] L. S. Young and A. B. Rickinson, "Epstein-Barr virus: 40 years on," *Nature Reviews Cancer*, vol. 4, no. 10, pp. 757–768, 2004.

[98] L. Xing and E. Kieff, "Epstein-Barr virus BHRF1 micro- and stable RNAs during latency III and after induction of replication," *Journal of Virology*, vol. 81, no. 18, pp. 9967–9975, 2007.

[99] D. N. Kim, H.-S. Chae, S. T. Oh, et al., "Expression of viral microRNAs in Epstein-Barr virus-associated gastric carcinoma," *Journal of Virology*, vol. 81, no. 2, pp. 1033–1036, 2007.

[100] R. H. Edwards, A. R. Marquitz, and N. Raab-Traub, "Epstein-Barr virus BART microRNAs are produced from a large intron prior to splicing," *Journal of Virology*, vol. 82, no. 18, pp. 9094–9106, 2008.

[101] M. M. Brinkmann and T. F. Schulz, "Regulation of intracellular signalling by the terminal membrane proteins of members of the *Gammaherpesvirinae*," *Journal of General Virology*, vol. 87, no. 5, pp. 1047–1074, 2006.

[102] U. Moens and M. Johannessen, "Mechanisms by which viral proteins of human DNA tumor viruses hijack the cell cycle," in *DNA Tumor Viruses*, H. E. Tao, Ed., pp. 1–48, Nova Science, New York, NY, USA, 2008.

[103] P. Sethupathy, M. Megraw, and A. G. Hatzigeorgiou, "A guide through present computational approaches for the identification of mammalian microRNA targets," *Nature Methods*, vol. 3, no. 11, pp. 881–886, 2006.

[104] X. Yang, Y. Chu, Y. Wang, Q. Guo, and S. Xiong, "Vaccination with IFN-inducible T cell α chemoattractant (ITAC) gene-modified tumor cell attenuates disseminated metastases of circulating tumor cells," *Vaccine*, vol. 24, no. 15, pp. 2966–2974, 2006.

[105] P. J. Hensbergen, P. G. Wijnands, M. W. J. Schreurs, R. J. Scheper, R. Willemze, and C. P. Tensen, "The CXCR3 targeting chemokine CXCL11 has potent antitumor activity in vivo involving attraction of $CD8^+$ T lymphocytes but not

inhibition of angiogenesis," *Journal of Immunotherapy*, vol. 28, no. 4, pp. 343–351, 2005.

[106] D. Ganem, "Kaposi's sarcoma-associated virus," in *Fields' Virology*, D. M. Knipe and P. M. Howeley, Eds., pp. 2847–2888, Lippincott Williams and Wilkins, Philadelphia, Pa, USA, 2007.

[107] V. Marshall, T. Parks, R. Bagni, et al., "Conservation of viraly encoded microRNAs in Kaposi sarcoma-associated herpesvirus in primary effusion lymphoma cell lines and in patients with Kaposi sarcoma or multicentric Castleman disease," *The Journal of Infectious Diseases*, vol. 195, no. 5, pp. 645–659, 2007.

[108] D. Ganem and J. Ziegelbauer, "MicroRNAs of Kaposi's sarcoma-associated herpes virus," *Seminars in Cancer Biology*, vol. 18, no. 6, pp. 437–440, 2008.

[109] J. M. Ziegelbauer, C. S. Sullivan, and D. Ganem, "Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs," *Nature Genetics*, vol. 41, no. 1, pp. 130–134, 2009.

[110] G. A. Calin and C. M. Croce, "MicroRNA signatures in human cancers," *Nature Reviews Cancer*, vol. 6, no. 11, pp. 857–866, 2006.

[111] S. Costinean, N. Zanesi, Y. Pekarsky, et al., "Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in Eμ-miR155 transgenic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 18, pp. 7024–7029, 2006.

[112] J. Sun, M. Brand, Y. Zenke, S. Tashiro, M. Groudine, and K. Igarashi, "Heme regulates the dynamic exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 6, pp. 1461–1466, 2004.

[113] E. Shaulian and M. Karin, "AP-1 as a regulator of cell life and death," *Nature Cell Biology*, vol. 4, no. 5, pp. E131–E136, 2002.

[114] A. Cimmino, G. A. Calin, M. Fabbri, et al., "*miR-15* and *miR-16* induce apoptosis by targeting BCL2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 39, pp. 13944–13949, 2005.

[115] M. S. Weinberg and K. V. Morris, "Are viral-encoded microRNAs mediating latent HIV-1 infection?" *DNA and Cell Biology*, vol. 25, no. 4, pp. 223–231, 2006.

[116] M. Barboric and B. M. Peterlin, "A new paradigm in eukaryotic biology: HIV Tat and the control of transcriptional elongation," *PLoS Biology*, vol. 3, no. 2, article e76, pp. 200–203, 2005.

[117] L. B. Ludwig, J. L. Ambrus Jr., K. A. Krawczyk, et al., "Human Immunodeficiency Virus-Type 1 LTR DNA contains an intrinsic gene producing antisense RNA and protein products," *Retrovirology*, vol. 3, article 80, pp. 1–20, 2006.

[118] S. Omoto and Y. R. Fujii, "Regulation of human immunodeficiency virus 1 transcription by nef microRNA," *Journal of General Virology*, vol. 86, no. 3, pp. 751–755, 2005.

[119] J. P. Couturier and R. S. Root-Bernstein, "HIV may produce inhibitory microRNAs (miRNAs) that block production of CD28, CD4 and some interleukins," *Journal of Theoretical Biology*, vol. 235, no. 2, pp. 169–184, 2005.

[120] C. L. Jopling, M. Yi, A. M. Lancaster, S. M. Lemon, and P. Sarnow, "Molecular biology: modulation of hepatitis C virus RNA abundance by a liver-specific microRNA," *Science*, vol. 309, no. 5740, pp. 1577–1581, 2005.

[121] G. Randall, M. Panis, J. D. Cooper, et al., "Cellular cofactors affecting hepatitis C virus infection and replication," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 31, pp. 12884–12889, 2007.

[122] J. Anderson, C. Schiffer, S. K. Lee, and R. Swanstrom, "Viral protease inhibitors," *Handbook of Experimental Pharmacology*, vol. 189, pp. 85–110, 2009.

[123] E. De Clercq and J. Neyts, "Antiviral agents acting as DNA or RNA chain terminators," *Handbook of Experimental Pharmacology*, vol. 189, pp. 53–84, 2009.

[124] P. Kumar, H.-S. Ban, S.-S. Kim, et al., "T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice," *Cell*, vol. 134, no. 4, pp. 577–586, 2008.

[125] C. Rauschhuber, H. Xu, F. H. Salazar, P. L. Marion, and A. Ehrhardt, "Exploring gene-deleted adenoviral vectors for delivery of short hairpin RNAs and reduction of hepatitis B virus infection in mice," *The Journal of Gene Medicine*, vol. 10, no. 8, pp. 878–889, 2008.

[126] F. Eckstein, "The versatility of oligonucleotides as potential therapeutics," *Expert Opinion on Biological Therapy*, vol. 7, no. 7, pp. 1021–1034, 2007.

[127] D. H. Le, "RNA-interference therapy for HBV infection enters Phase I clinical trial," *Expert Review of Anti-Infective Therapy*, vol. 6, no. 1, pp. 5–8, 2008.

[128] E. M. Westerhout, M. Ooms, M. Vink, A. T. Das, and B. Berkhout, "HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome," *Nucleic Acids Research*, vol. 33, no. 2, pp. 796–804, 2005.

[129] D. Boden, O. Pusch, and B. Ramratnam, "Overcoming HIV-1 resistance to RNA interference," *Frontiers in Bioscience*, vol. 12, pp. 3104–3116, 2007.

[130] S. He, Z. Yang, G. Skogerbo, et al., "The properties and functions of virus encoded microRNA, siRNA, and other small noncoding RNAs," *Critical Reviews in Microbiology*, vol. 34, no. 3-4, pp. 175–188, 2008.

[131] N. Blow, "Small RNAs: delivering the future," *Nature*, vol. 450, no. 7172, pp. 1117–1120, 2007.

[132] M. Scherr, L. Venturini, K. Battmer, et al., "Lentivirus-mediated antagomir expression for specific inhibition of miRNA function," *Nucleic Acids Research*, vol. 35, no. 22, article e149, pp. 1–9, 2007.

[133] E. R. Rayburn and R. Zhang, "Antisense, RNAi, and gene silencing strategies for therapy: mission possible or impossible?" *Drug Discovery Today*, vol. 13, no. 11-12, pp. 513–521, 2008.

[134] L. L. Snyder, J. M. Esser, C. J. Pachuk, and L. F. Steel, "Vector design for liver-specific expression of multiple interfering RNAs that target hepatitis B virus transcripts," *Antiviral Research*, vol. 80, no. 1, pp. 36–44, 2008.

[135] M. Dimitrova, C. Affolter, F. Meyer, et al., "Sustained delivery of siRNAs targeting viral infection by cell-degradable multilayered polyelectrolyte films," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 42, pp. 16320–16325, 2008.

[136] Y.-J. Zhang, K.-Y. Wang, D. A. Stein, et al., "Inhibition of replication and transcription activator and latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus by morpholino oligomers," *Antiviral Research*, vol. 73, no. 1, pp. 12–23, 2007.

[137] I. Lebars, T. Richard, C. Di Primo, and J.-J. Toulmé, "LNA derivatives of a kissing aptamer targeted to the trans-activating responsive RNA element of HIV-1," *Blood Cells, Molecules, and Diseases*, vol. 38, no. 3, pp. 204–209, 2007.

[138] J. S. Eisen and J. C. Smith, "Controlling morpholino experiments: don't stop making antisense," *Development*, vol. 135, no. 10, pp. 1735–1743, 2008.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 924565, 12 pages
doi:10.1155/2009/924565

Methodology Report

Gene Expression Analysis of an *EGFR* Indirectly Related Pathway Identified *PTEN* and *MMP9* as Reliable Diagnostic Markers for Human Glial Tumor Specimens

Sergio Comincini,[1] Mayra Paolillo,[2] Giulia Barbieri,[1] Silvia Palumbo,[1] Elena Sbalchiero,[1] Alberto Azzalin,[1] Marika A. Russo,[2] and Sergio Schinelli[2]

[1] *Dipartimento di Genetica e Microbiologia, Università di Pavia, Via Ferrata 1, 27100 Pavia, Italy*
[2] *Dipartimento di Farmacologia Sperimentale ed Applicata, Università di Pavia, Viale Taramelli 14, 27100 Pavia, Italy*

Correspondence should be addressed to Sergio Comincini, sergio.c@ipvgen.unipv.it

Received 9 February 2009; Revised 23 April 2009; Accepted 18 May 2009

Recommended by Zhumur Ghosh

In this study the mRNA levels of five *EGFR* indirectly related genes, *EGFR*, *HB-EGF*, *ADAM17*, *PTEN*, and *MMP9*, have been assessed by Real-time PCR in a panel of 37 glioblastoma multiforme specimens and in 5 normal brain samples; as a result, in glioblastoma, *ADAM17* and *PTEN* expression was significantly lower than in normal brain samples, and, in particular, a statistically significant inverse correlation was found between *PTEN* and *MMP9* mRNA levels. To verify if this correlation was conserved in gliomas, *PTEN* and *MMP9* expression was further investigated in an additional panel of 16 anaplastic astrocytoma specimens and, in parallel, in different human normal and astrocytic tumor cell lines. In anaplastic astrocytomas *PTEN* expression was significantly higher than in glioblastoma multiforme, but no significant correlation was found between *PTEN* and *MMP9* expression. *PTEN* and *MMP9* mRNA levels were also employed to identify subgroups of specimens within the different glioma malignancy grades and to define a gene expression-based diagnostic classification scheme. In conclusion, this gene expression survey highlighted that the combined measurement of *PTEN* and *MMP9* transcripts might represent a novel reliable tool for the differential diagnosis of high-grade gliomas, and it also suggested a functional link involving these genes in glial tumors.

1. Introduction

Glioblastoma multiforme is the most malignant brain tumor among astrocytic gliomas with a typical prognosis of about 12 months in spite of current therapeutic approaches that include neurosurgery followed by combined chemotherapy and radiotherapy [1]. Recently, the development of massive screening genome technologies, such as gene expression profiling, has prompted new attempts to the classification of glioblastoma subgroups on molecular basis in order to identify new diagnostic or prognostic tools. At present the search for potential molecular markers among aberrant signal transduction pathways in glioblastoma is actively exploited for the optimization of existing therapies or the development of innovative drugs [2]. However, the accomplishment of this ambitious task is severely hindered by the extreme heterogeneity of glioblastoma tumor samples and by the subsequent variability of possibly identified molecular markers. One way to overcome this limit could be represented by the concomitant analysis of the mRNA expression of several selected genes, already known to be functionally involved in the cellular malignant transformation. This analysis could highlight differences in gene expression levels among high-grade gliomas, or at the same time it could reveal relationships within glioma subtypes between the genes analyzed in order to improve their reliability as prognostic or diagnostic markers.

The epidermal growth factor (EGF) receptor (EGFR or ErbB1) plays a pivotal role in cancer physiology because its activation, elicited by at least six different endogenous peptidergic EGF-like ligands, leads to the activation of intracellular signalling pathways that modulate cell

Table 1: Age, gender, mRNA expression values (in femptograms), and anatomical location of glioblasytoma multiforme samples.

Age	Sex	*EGFR*	*ADAM17*	*HB-EGF*	*PTEN*	*MMP9*	Location
68	M	2.78	0.31	0.69	1.85	6.39	Parietal
84	F	155.97	0.31	4.43	6.81	31.13	Parietal
23	M	95.25	3.54	19.01	5.15	0.04	Cerebellum
50	M	579.69	0.87	9.28	8.97	43.32	Frontal dx
71	F	7.11	0.08	0.82	2.27	0.21	Frontal dx
58	M	236.18	0.91	8.98	15.05	183.29	Parietal dx
66	M	12.85	0.13	1.38	0.41	0.04	Temporal sx
50	M	150.97	3.08	4.87	1.65	3.09	Temporal dx
38	F	60.10	2.23	9.23	7.01	30.41	Occipital dx
61	F	2.88	0.41	1.75	1.96	0.72	Frontal sx
68	F	4.87	0.63	8.71	0.82	0.82	Occipital dx
70	M	23.38	2.13	2.82	3.92	3.51	Frontal dx
59	M	425.51	1.28	4.32	4.84	8.04	Frontal sx
67	F	1268.24	2.99	13.92	12.68	32.58	Temporal dx
31	M	13.33	1.87	52.08	6.66	13.33	Frontal sx
68	F	0.77	0.59	12.85	0.62	1.04	Parietal dx
39	F	6.87	13.33	0.21	3.14	0.16	Frontoinsular
39	M	2.37	0.42	3.96	4.79	0.21	Parietal sx
45	F	274.74	0.66	7.14	1.25	2.52	Frontal dx
63	M	1284.37	13.33	0.21	3.12	10.62	Temporal dx
71	M	30.11	5.42	10.62	2.92	2.08	Frontal sx
60	M	73.96	17.71	7.54	3.33	4.37	Temporal sx
44	F	2562.92	6.87	18.96	3.33	8.33	Thalamus sx
73	F	38.75	13.12	7.53	3.33	1.04	Paratrigonal sx
47	F	16.88	4.79	7.29	3.13	1.04	Frontal sx
63	M	12.01	12.31	28.22	3.85	3.28	Occipital dx
77	F	239.85	8.72	22.56	2.92	6.31	Cortical anterior
55	F	22.36	2.51	5.38	1.54	0.05	Parietal dx
70	F	13.18	9.18	12.31	3.08	0.16	Parietal dx
60	F	8.92	7.95	16.56	1.54	0.23	Temporal sx
54	M	1071.43	15.49	16.56	2.56	1.64	Temporal sx
55	M	371.49	9.54	19.49	1.54	1.85	Temporal sx
55	F	27.54	9.23	5.13	1.54	0.15	Temporal sx
58	M	21.38	20.87	17.38	3.08	1.23	Temporal sx
53	F	8.1	7.23	9.74	2.05	0.15	Occipital sx
69	F	4.66	12.56	26.69	3.08	1.69	Frontal sx
70	F	29.49	5.23	7.08	7.23	0.61	Parietal dx

proliferation, metastasis, and angiogenesis [3]. About 40%–50% of glioblastoma cases are characterized by *EGFR* gene amplification or overexpression, together with the expression of the mutated and constitutively active EGFR isoform EGFRvIII [3]. Upregulation of the EGFR pathway could also result from an increased availability of EGFR endogenous agonists belonging to the family of EGF-like growth factors.

Heparin-binding epidermal growth factor (HB-EGF) acts as a potent proliferative agent in many different cell types via the activation of EGFR or the other EGF-like receptor ErbB4 [4]. HB-EGF is initially synthesized as the membrane-spanning protein proHB-EGF and then is proteolytically cleaved by "A *D*isintegrin *A*nd *M*etalloproteinase" (ADAM) family members that release the soluble form (sHB-EGF) in the extracellular space. The ADAM isoform responsible for this process appears to be cell type dependent, since in different experimental models ADAM 10, 12, and 17 have been involved in proHB-EGF shedding [3]. The overexpression of ADAM17, also named "tumor necrosis factor-alpha-converting enzyme" (TACE), seems to be involved in the malignant potential of cancer cells [5], and, notably, this metalloprotease modulates HB-EGF shedding and cell proliferation in U373-MG glioblastoma cell line [5].

In the clinical practice only 10%–20% of glioblastoma patients respond to EGFR kinase inhibitors, and this poor response has been ascribed to a combination of EGFR

Table 2: Age, gender, *PTEN*, and *MMP9* expression values (in femtograms), and anatomical location of anaplastic astrocytoma samples.

Age	Sex	*PTEN*	*MMP9*	Location
44	M	1.72	1.25	Temporal sx
68	F	7.66	0.57	Thalamus sx
67	F	2.25	37.06	Frontal dx
60	F	2.44	1.62	Frontal sx
50	M	92.46	0.79	Temporal dx
39	M	79.25	32.92	Parietal dx
64	F	33.82	43.21	Parietal dx
28	F	25.64	26.24	Temporal sx
55	F	12.47	0.32	Temporal sx
31	M	19.81	1.95	Temporal sx
52	F	50.12	4.37	Temporal sx
71	F	17.98	0.28	Frontal cortex
62	M	20.41	0.03	Total cortex
50	M	60.72	0.76	Occipital cortex
52	M	45.11	0.45	Total cortex
68	F	25.30	0.23	Frontal cortex

Table 3: Age, gender, mRNA expression values (in femtograms), and anatomical location of normal brain specimens.

Age	Sex	*EGFR*	*ADAM17*	*HB-EGF*	*PTEN*	*MMP9*	Location
71	F	32.58	10.74	14.84	17.98	0.28	Frontal cortex
62	M	16.70	12.76	10.41	20.41	0.03	Total cortex
50	M	57.94	18.35	30.41	60.72	0.76	Occipital cortex
52	M	25.74	13.87	28.55	45.11	0.45	Total cortex
68	F	46.98	17.20	7.60	25.30	0.23	Frontal cortex

Table 4: *MMP9* and *PTEN* absolute quantitative expression in different human normal (NHA) and glioma (PRT-HU2 and U138-MG) cell lines. Expression is indicated in femptograms.

Cell line	*MMP9*	*PTEN*
NHA	0.59	117.97
PRT-HU2	0.56	45.62
U138-MG	3.09	4.08

overexpression and loss or mutation of the Phosphatase and TEN sin homolog deleted from chromosome 10 (PTEN) tumor suppressor protein [6]. The PTEN phosphatase reduces the levels of the second messenger phosphatidylinositol 3,4,5-triphosphate (PI3K) and regulates the activity of the downstream PI-3K/AKT- and mammalian target of rapamacyn- (mTOR-) dependent pathways [7]. Notably, PTEN functional loss or mutation is present in 60%–70% of high-grade gliomas and is associated with malignant phenotypic changes such as migration capability, probably by modulation of FAK activity [8]. Moreover, since PTEN loss appears to accelerate the formation of high-grade gliomas [6], it could potentially represent a valid candidate gene to discriminate between high- and low-grade gliomas.

One typical feature of glioblastoma is its high ability to disseminate and spread to distant brain areas. Proteases expressed by glioma cells appear to play a significant role in these processes because selective matrix metalloproteinases like MMP2 or MMP9 degrade the extracellular environment in order to facilitate tumor cell growth and migration. Expression of these proteases appears to increase with glioma grade and in vitro studies showed that modifications of their expression levels resulted in altered migratory properties [9]. Although some previous reports have examined the expression of EGFR [3], PTEN [10], HB-EGF [9], ADAM17 [11], and MMP9 [12] in astrocytoma samples, at present the transcriptional expression of these five EGFR pathway-related genes has not yet been simultaneously investigated in glioma.

Therefore, in this study we have evaluated by quantitative Real-time PCR the expression of *ADAM17*, *EGFR*, *HB-EGF*, *PTEN*, and *MMP9* mRNAs in a panel of glioblastoma and anaplastic astrocytomas specimens and cell lines, and we have finally compared them to normal control samples to ascertain whether these expression profiles might provide additional tools in glioma diagnosis and in tumor subtypes identification.

2. Materials and Methods

2.1. Human Biopsy Samples. Biopsy samples, obtained from Azienda Ospedaliera Universitaria di Parma (Parma, Italy) after informed consent of the patients, were placed in ice-cold Trizol reagent (Invitrogen, Paisley, UK) and immediately processed for RNA extraction. Sections of samples were

Table 5: GeneBank accession numbers, PCR primer sequences, and products.

Gene	Accession number	PCR primer sequences (5′-3′)	PCR product (bp)
EGFR	NM_005228	AGGAAGAAGCTTGCTGGTAGC CTCTGGAAGACTTGTGGCTTG	88
ADAM17	NM_003183	CAAGTCATTTGAGGATCTCACG TCTTTGCTGTCAACACGATTCT	96
HB-EGF	NM_001945	GCCTAGGCGATTTTGTCTACC GCCCAACCTCTTCTGAGACTT	119
PTEN	NM_000314	CAGCAGTGGCTCTGTGTGTAA ATGGACATCTGATTGGGATGA	98
MMP9	NM_004994	AAAGCCTATTTCTGCCAGGAC GCACTGCAGGATGTCATAGGT	105

independently histologically and morphologically evaluated by different neuropathologists and classified as grade IV (glioblastoma multiforme) or grade III (anaplastic astrocytoma), according to WHO guidelines [13]. Clinical data of glioblastoma patients are reported in Table 1, and they included 19 females and 18 males (age range 23–84 years, mean 57.8 ± 13.3). The anaplastic astrocytoma patients (Table 2) included 7 males and 9 females (age 28–68, mean 50.7 ± 13.9). Total RNA samples extracted from human postmortem normal brain (NB) cortical regions, as reported in Table 3, were purchased from Ambion (Foster City, Calif, USA): these included 2 females and 3 males (age range 50–71 years, mean 60.6 ± 9.3).

2.2. Cell Lines. Normal human astrocytes (NHAs) were purchased from Cambrex (East Rutherford, NJ, USA) and cultivated in the specific astrocyte AGM medium (Cambrex) according to the manufacturer's specifications. Human glioma cell line U138-MG, derived from a glioblastoma multiforme patient and widely employed, was purchased from ATCC (Rockville, Md, USA); PRT-HU2 cells, previously described [14], were analogously derived from a glioblastoma multiforme patient. U138-MG and PRT-HU2 cells were cultivated in D-MEM medium supplemented with 10% FBS, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 1% L-glutamine (Invitrogen, Paisley, UK).

2.3. Real Time Quantitative PCR. Total RNA was extracted as previously described [14] and accurately quantified using spectrophotometric and fluorimetric (Quant-it RNA Assay, Invitrogen) approaches. The gene-specific primers were designed using the "Primer3 input" software (http://frodo.wi.mit.edu/primer3/), and their specificity was verified using the Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome/). GeneBank accession numbers of the five genes examined, their respective primer pairs sequences, and PCR products lengths were reported in Table 5. Quantitative Real-time PCR analysis was performed as previously described [14]. For the absolute quantification of specific cDNA, standard curves were derived using different concentrations of *EGFR*, *ADAM17*, *MMP9*, *HB-EGF*, and *PTEN* DNA-sequenced templates, prepared by reamplifying the PCR purified products obtained from Real-time PCR. The second derivative maximum method in the Light Cycler software was used to calculate the crossing point (Cp) value, and the concentrations of each specific cDNA were determined. All results were quantitative expressed in femtograms (fg) of cDNA, normalized to a total RNA input of 1 microgram.

2.4. Statistical and Bioinformatics Analysis. All results were expressed as mean ± standard deviation of each biopsy specimens, assayed in duplicate. The Instat v.3 software (GraphPad Software Inc., Mass, USA) was used for the statistical analysis of differences in gene expression between groups by one-way ANOVA and for the analysis of correlations among gene expression profiles, using the Pearson coefficient r. A P-value less than .05 was considered statistically significant. The dendrogram and the classification tree analysis were performed using the Orange data mining software (http://www.ailab.si/orange/). In particular, for the hierarchical clustering analysis, an Euclidean distance matrix was adopted. Statistical trends were obtained from the *PTEN* and *MMP9* average quantitative expression within normal control, anaplastic astrocytoma, and glioblastoma multiforme diagnostic classes, using the Excel software with an exponential setting (Microsoft Word Package 2003, Redmond, Wash, USA).

3. Results

The mRNA expression of the investigated genes of glioblastoma multiforme and normal brain specimens were analytically reported in Tables 1 and 2 and depicted in Figures 1 and 2. On the basis of their expression patterns in glioblastoma and using the mean values of normal samples as cut-off, the investigated genes were roughly classified in three different subgroups, the first including *ADAM17, HB-EGF*, and *PTEN*, whose average levels in glioblastoma were below the controls, the second comprising *MMP9,* whose majority of values in glioblastoma specimens were higher than in controls and, finally, the last one constituted by the *EGFR* gene, displaying the widest variation and data dispersion (Figure 1).

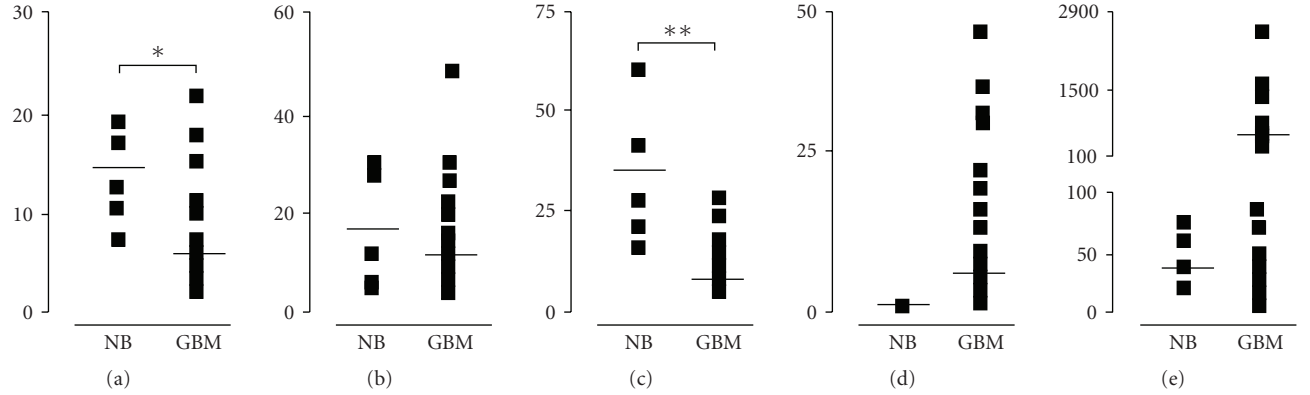


Figure 1: The mRNA expression levels of (a) *ADAM17*, (b) *HB-EGF*, (c) *PTEN*, (d) *MMP9*, and (e) *EGFR* in 37 human glioblastoma samples (GBM) and in five normal brain cortex samples (NB). Horizontal lines represent the mean values. $^{*}P < .002$, $^{**}P < .0001$.

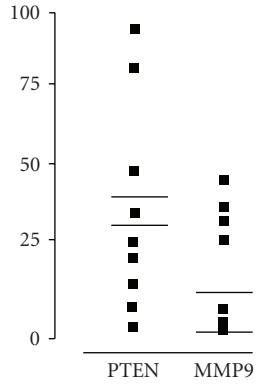


Figure 2: The mRNA expression levels of *PTEN* and *MMP9* in 16 human grade III astrocytoma samples (AA). Bold horizontal lines represent the mean values, and thin lines represent the mean normal brain cortex samples (NB) values.

The mRNA levels of *ADAM17* metallo-protease in glioblastoma (mean 5.88 fg ± 5.71) were significantly lower ($P < .002$) than in controls (14.51 fg ± 3.15). In particular, in 33 out of 37 cases (89.19%), *ADAM17* expression was lower than in control samples. For *HB-EGF* gene, although 31 out of 37 (83.78%) glioblastoma samples displayed mRNA levels lower than controls, statistical analysis revealed that the difference between controls (18.36 fg ± 10.52) and glioblastoma specimens (10.96 fg±10.13) was not statistically significant ($P < .434$).

PTEN expression pattern showed statistically lower mRNA levels in all glioblastoma samples compared to controls. Notably, the highest *PTEN* expression level among all glioblastoma had a quantitative expression (15.05 fg) that was less than 50% of the mean controls values (33.91 fg), with a control normalized ratio ranging from 2.25 to 82.72 fg. As expected, there was a very high statistically significant difference ($P < .0001$) of *PTEN* mRNA expression between glioblastoma (3.86 fg ± 3.15) and controls (33.91 fg ± 18.44).

MMP9 was overexpressed in the majority of glioblastoma specimens (72.22%) and, furthermore, in a subgroup of 24 cases (64.86%) the expression level was at least twofold higher than in controls, with a control normalized ratio within this subgroup ranging from 1.74 to 123.71 fg. However, the mean expression level of *MMP9* mRNA in glioblastoma (5.11 fg ± 8.78) was not significantly different ($P = .238$) from control samples (0.35 fg ± 0.27).

EGFR mRNA transcript levels, due to their amplitude in expression, were arbitrarily divided into two glioblastoma subgroups. The former, ranging from 0.77 to 95.25 fg, included 25 samples (67.63%); the latter, had *EGRF* absolute quantitative values ranging from 150.97 to 2562.92 fg. All control samples showed *EGFR* expression values below 100 fg, with a mean value of 35.98 fg ± 16.51. It was evident that for *EGFR* there was no statistically significant difference ($P < .371$) between glioblastoma (247.60 fg ± 517.45) and control samples (35.98 fg ± 16.52).

Within glioblastoma samples, a highly statistically significant negative correlation ($P < .0001$; Person coefficient $r = -0.776$) was related to the expression of *PTEN* and *MMP9*; in a different manner, a statistically significant positive correlation ($P < .05$; Pearson coefficient, $r = 0.9221$) was scored for the same genes within the control samples. The inverse correlation found between *PTEN* and *MMP9* mRNA expression in glioblastoma compared to control samples, prompted us to investigate whether this correlation was also detectable in other glioma grades of malignancy. Therefore, *PTEN* and *MMP9* expression, reported in Table 2 and illustrated in Figure 2, was investigated in 16 histological confirmed anaplastic astrocytoma specimens, previously classified as WHO grade III. In these samples, *PTEN* mRNA normalized quantitation (29.72 fg ± 31.62) was not significantly different from control ($P = .792$), but this value was significantly higher compared to glioblastoma ($P < .0001$). *MMP9* expression (13.60 fg ± 17.28) was neither significantly different from the control (0.35 fg ± 0.27, $P = .113$) nor from glioblastoma specimens ($P = .103$). Notably, no statistically significant correlation ($P = .709$) was found between the mRNA levels of these two genes in anaplastic astrocytoma samples. Differently from glioblastoma, no inverse correlation in *PTEN* and *MMP9* expression was found comparing anaplastic astrocytoma and control samples (Pearson coefficient, $r = 0.127$).

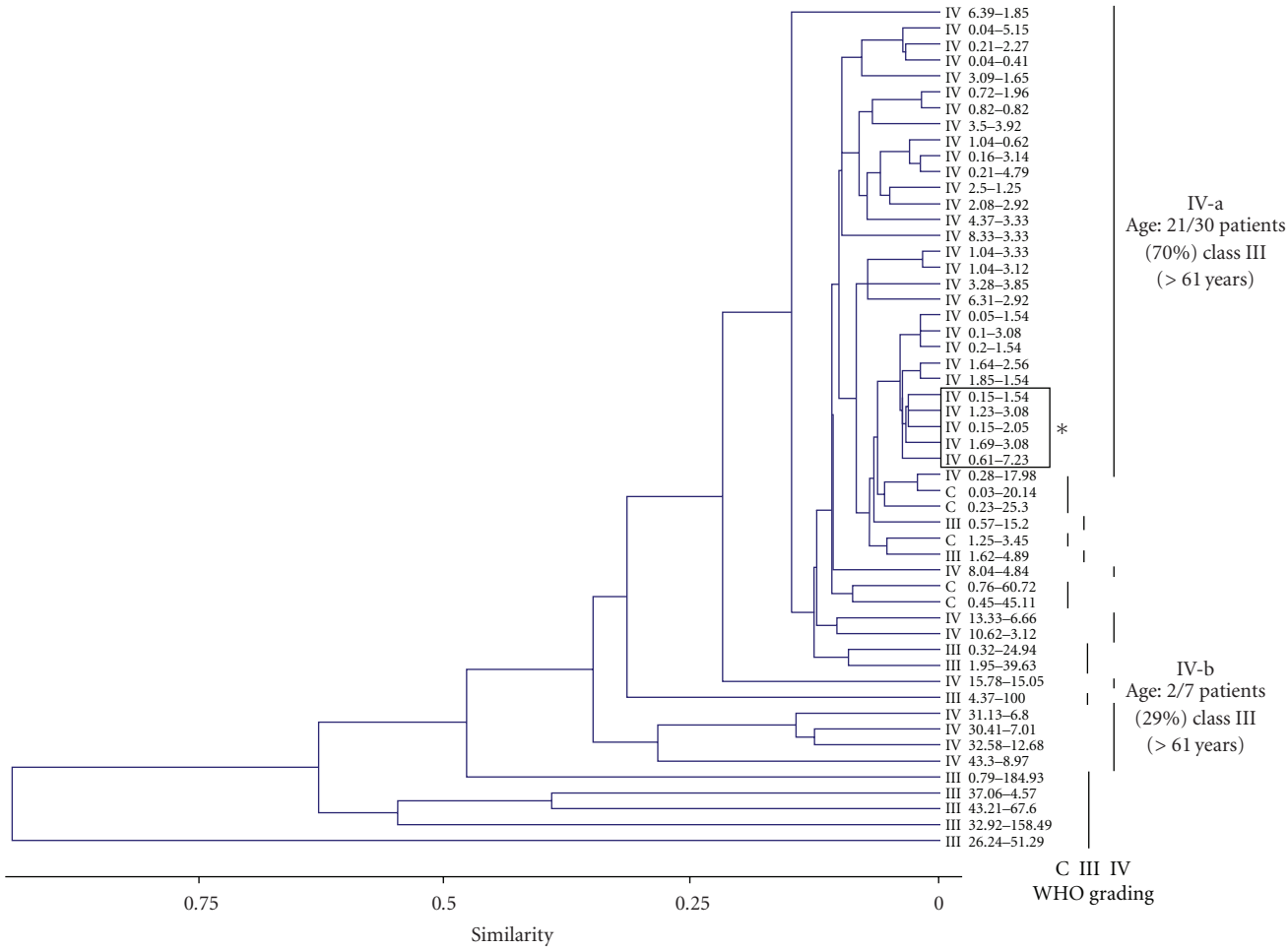


Figure 3: Dendrogram comparing *PTEN* and *MMP9* absolute quantitative expression. Absolute quantifications are expressed in femtograms, as reported in Materials and Methods. WHO grades of malignancy (III, IV) and healthy brain control (C) specimens are indicated. Glioblastoma multiforme subgroups IV-a, including the majority of glioblastoma specimens, and IV-b, described in Results, are highlighted. The box (∗) indicates subgrouping of patients sharing similar tumor anatomical localization (i.e., temporal). Subgroups IV-a and IV-b have a different and significant distribution of age-class III patients (>61 years, $P < .01$ Anova-one way).

Then, *PTEN* and *MMP9* mRNA levels were comparatively examined in two different human established glioblastoma cell lines (PRT-HU2 and U138-MG) and in a primary culture of embryonic normal human astrocytes (NHA), as shown in Table 4. In these cells, *PTEN* mRNA levels were much variable comparing to *MMP9* expression; similarly to the above examined control samples, normal astrocytes exhibited the highest *PTEN* expression.

We next exploited if *PTEN* and *MMP9* expression might be more tightly related to the glioma tumor progression. To this purpose, different bioinformatics analyses were performed using the experimental data set reported in Tables 1, 2, 3, and 4 and considering the WHO grading of the specimens. We therefore performed hierarchical clustering, a standard unsupervised learning method [10] of the tumor specimens. The WHO grading was simultaneously compared to *PTEN* and *MMP9* expression values, to identify homogeneous clusters. As reported in the dendrogram of Figure 3, different groups of samples were created, according to the above mentioned criteria. Using Euclidean distances, WHO grades IV and III and normal control samples were roughly classified into different clusters. Of note, a major subset of the glioblastoma specimens was identified (Figure 3, subgroup IV-a), showing the lowest and almost similar levels of both *PTEN* and *MMP9* transcripts; the remaining glioblastoma samples clustered within subgroup IV-b. The association of tumor anatomical localization and age of the patients with the levels of *PTEN* and *MMP9* expression was also investigated. According to the clinical data (Tables 1–3), ages of the patients were divided into three classes (i.e., I, 20–40; II, 41–60; III, >61 years): as reported in the dendrogram, the age-class III patients exhibited a statistically significant difference in distribution between subgroups IV-a and IV-b ($P < .01$, Anova one-way). Differently, no clear associations between levels of *PTEN* and *MMP9* expression and tumor localization were highlighted, with the exception of a single cluster of four class-III patients with a temporal tumor localization within subgroup IV-a.

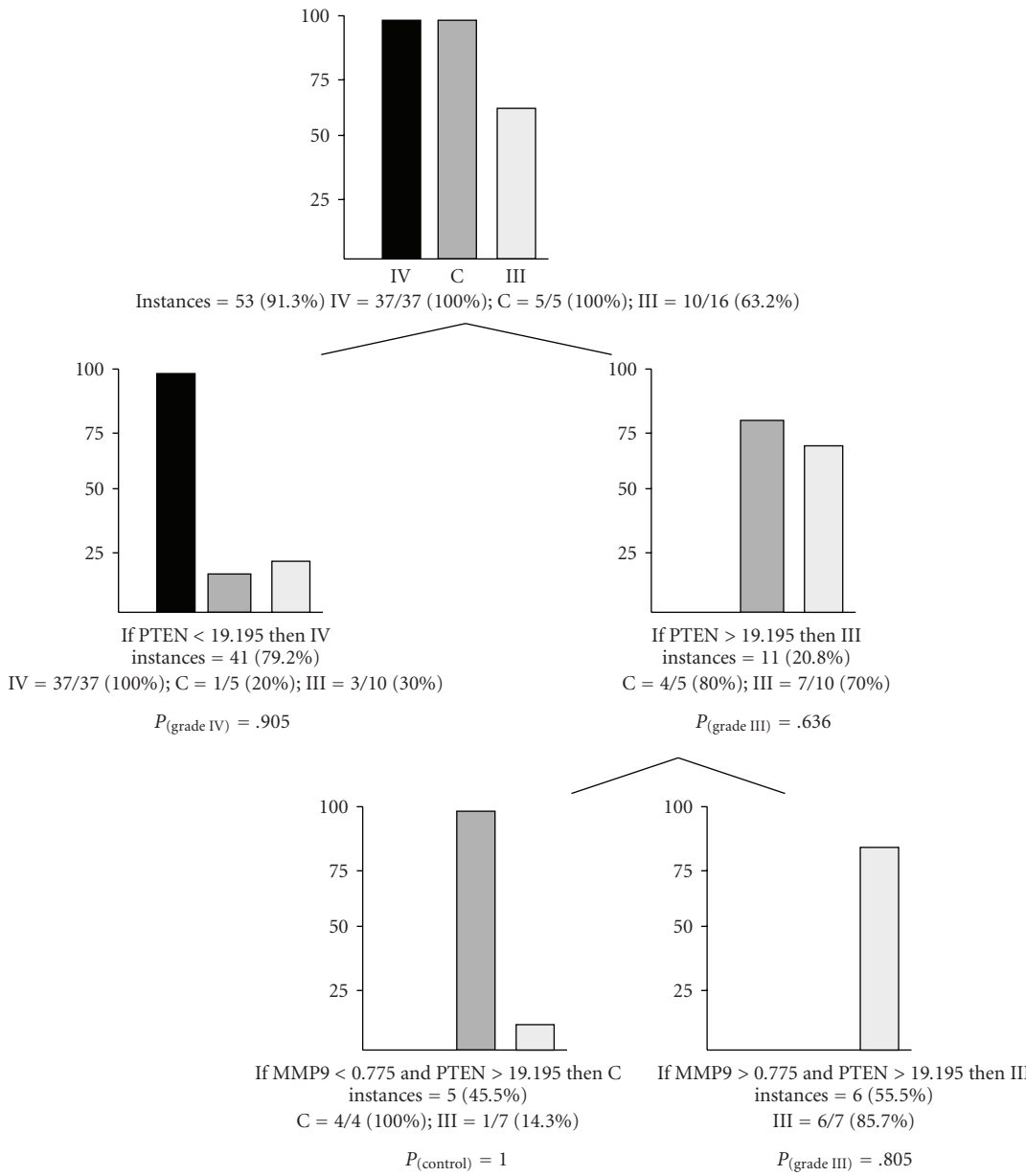


Figure 4: Classification tree derived from the combined measurement of *PTEN* and *MMP9* quantitative expression. At each root, diagnostic classes are divided following the absolute quantification and expressed in femtograms. Probabilities values (P) for the different classes are reported.

Then, a classification tree was produced, evaluating the performance of *PTEN* and *MMP9* expression in predicting roots characterized by different WHO malignant grades; as reported in Figure 4, 53 out of 58 total samples, corresponding to 53 instances (i.e., 91.3%) fitted with the classification tree; in detail, all grade IV samples had *PTEN* normalized expression values below a quantity of 19.195 fg (37/37 instances, $P = .905$), while the large majority of control (80% of the total C instances) and anaplastic astrocytomas samples (70%, $P = .636$) were grouped with *PTEN* > 19.195 fg; in this subgroup, to further differentiate control and anaplastic astrocytomas, *MMP9* < 0.775 fg clustered all control instances ($P = 1.000$).

Nomogram analysis, performed within the same data set and reported in Figure 5 confirmed that *PTEN* and *MMP9* expression analysis might be particularly helpful in the identification of anaplastic astrocytoma specimens having a 95% of probability ($P_{(\text{grade III})} = .95$) to correctly classify these samples, in correspondence of *MMP9*-normalized expression value of 19.195 fg, independently of *PTEN* expression level. Nomograms analysis with control and glioblastoma as target diagnostic classes showed less statistical significant probabilities ($P_{(\text{control})} = .60$ and $P_{(\text{grade IV})} = .55$).

To further elucidated if *PTEN* and *MMP9* expression had an expression trend that reflected the malignant grade of the specimens (i.e., normal, anaplastic astrocytomas,

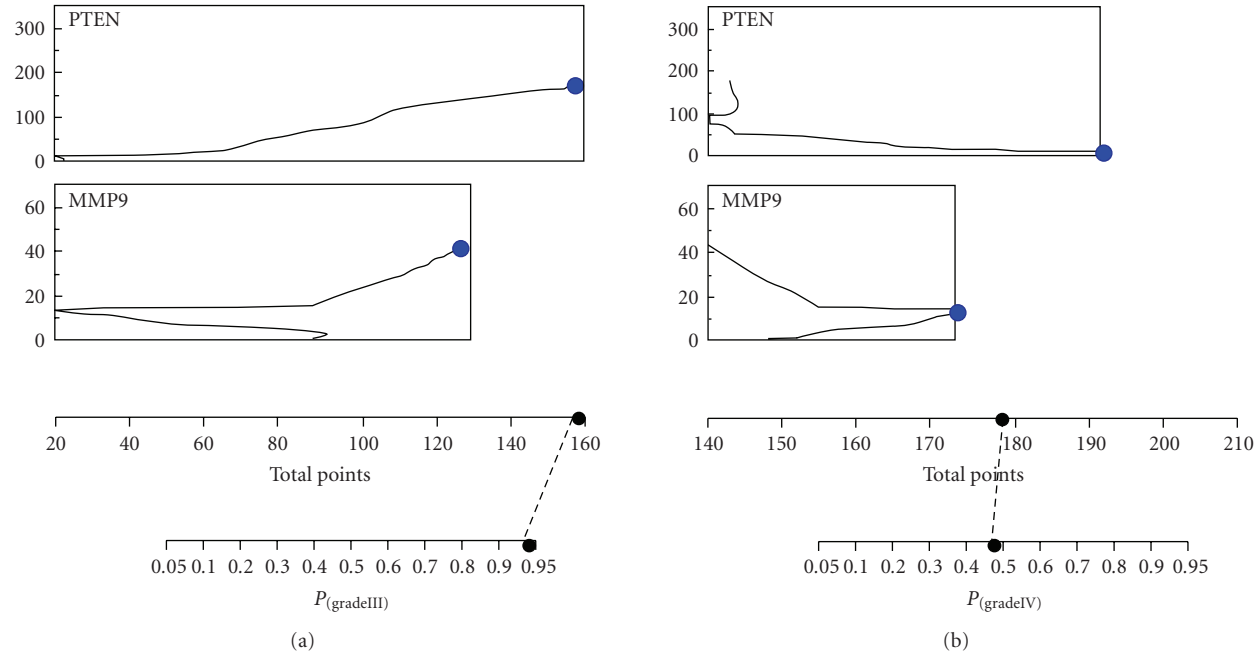


Figure 5: Nomogram analysis of *PTEN* and *MMP9* expression within the different WHO grades of malignancy (III, IV) of the astrocytomas specimens. Points and total points axes indicate the points attributed to each variable value and the sum of the points for each variable, respectively. *P* axes indicate the predicted probability that relates *PTEN* or *MMP9* quantitative expression to each WHO tumor malignancy grade.

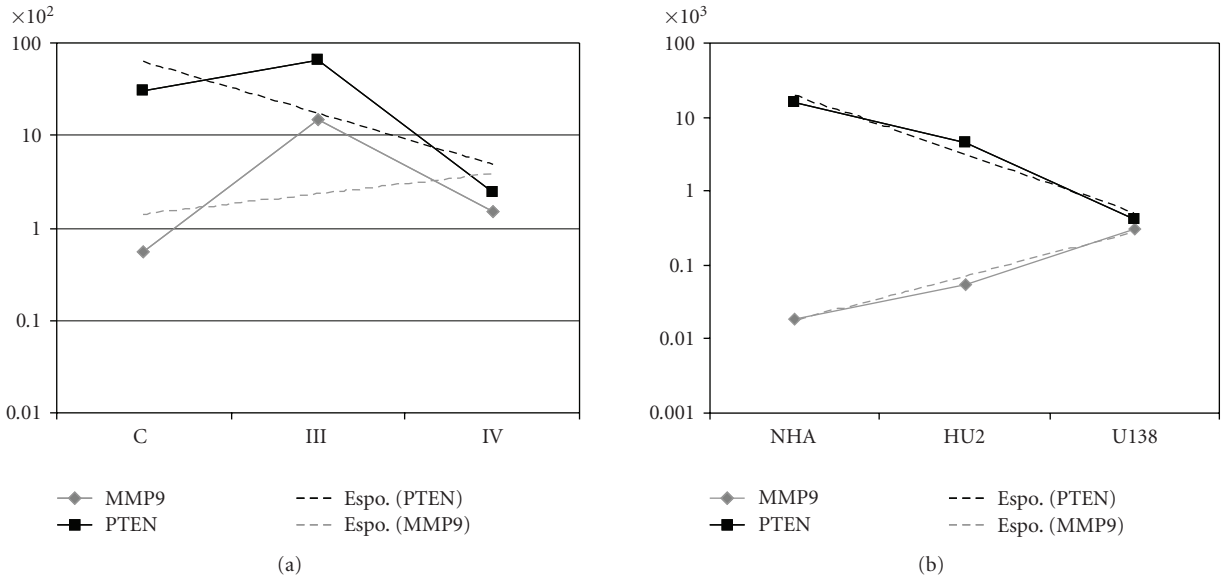


Figure 6: Statistical trends of *PTEN* (black dotted line) and *MMP9* (grey dotted line) expression in control (C), anaplastic astrocytomas (III) and glioblastoma multiforme (IV) specimens (Panel A), and in normal human astrocytes (NHA), glioma PRT-HU2 (HU2), and U138-MG (U138) cells (Panel B). Trends are calculated using average quantitative expression of *PTEN* and *MMP9*, and adopting exponential (Espo) models of prediction as follows: *PTEN* (C, III, IV average expression samples), $y = 2148e^{-1.29x}$; *MMP9* (C, III, IV), $y = 83716e^{0.50x}$; *PTEN* (NHA, HU2, U138), $y = 118596e^{-1.82x}$; *MMP9* (NHA, HU2, U138), $y = 42439e^{1.40x}$.

and glioblastoma multiforme), a statistical trend analysis was performed (Figure 6); the analysis reported in Panel A showed that *PTEN* and *MMP9* differences in their normalized expression clearly decreased following the malignant glioma grading, with the lowest values corresponding to glioblastoma multiforme specimens; similarly, the trend analysis, reported in Figure 6 Panel B, highlighted that, within the investigated cell lines, normal human astrocytes exhibited the largest differences in *PTEN* and *MMP9* expression levels, while, on the contrary, U138-MG cells,

isolated from a glioblastoma multiforme patient, showed nearly coincident *PTEN* and *MMP9* expression values; the glioblastoma derived PRT-HU2 cell line showed values of *PTEN* and *MMP9* expression trends, intermediate between NHA and U138-MG.

4. Discussion

The new challenge in cancer biology is to move from one purely morphological classification of cancer to one that is based on the integration of histological and molecular criteria [15]. Among several cancer specific investigated genes, the epidermal growth factor receptor (*EGFR*) plays a pivotal pathological role through the activation of downstream intracellular signalling pathways that can directly modulate cell proliferation, metastasis, and angiogenesis [8]. Glial tumors, in particular, due to processes of gene-amplification or mutation, showed altered *EGFR*-related functional pathways [3]. Within this context, in order to find clinically relevant correlations between gene expression and tumor malignant progression, a cohort of glioma specimens was analyzed for the expression of several genes, that is, *ADAM-17*, *PTEN*, *MMP9*, *EGFR*, and *HB-EGF*, indirectly involved in the *EGFR*-dependent signaling pathway, In fact, the concurrent measurement of the transcript levels of the different genes could potentially represent a useful tool to identify a dysregulation of receptor activation or of downstream signaling pathways or might also suggest functional links between these genes in pathological conditions [16].

Comparing a cohort of glioblastoma specimens and controls, only two genes, *ADAM-17* and *PTEN*, had expression levels that significantly changed between the two WHO classes; on the contrary, considering the expression of each transcript separately, we did not elucidate any association between clinical status and *EGFR*, *HB-EGF*, and *MMP9* expression profiles. In particular, the wide range of variation for the *EGFR* gene, found in glioblastoma specimens, was in partial agreement with previous studies carried out in glial tumor samples [8]. In general, nearly 50% of glioblastoma multiforme cases express amplified *EGFR*, and about 40% of them also express the constitutively activated mutated EGFRvIII isoform [11]. Since the primers we used in our experiments did not discriminate between *EGFR* and *EGFRvIII*, it is very likely that the *EGFR* mRNA levels found in our samples reflected the combined contribution of both the transcripts.

Since chemotherapic treatments enhance the EGFR-mediated proliferative responses via an increased *HB-EGF* expression and shedding [17], previous studies have suggested a prominent role for *HB-EGF* in tumorigenic processes. In fact, in glioma cell lines, the inducible expression of *EGFRvIII* can enhance *HB-EGF* expression and can activate *EGFR*-dependent pathways via a positive feedback autocrine loop [18]. The only previous published study, based on a semiquantitative assessment by Northern blot analysis, found an increased expression of *HB-EGF* in glioblastoma compared to control samples [9]. On the opposite, our data seemed to suggest that *HB-EGF* is not upregulated in glioblastoma; however, our results agreed with microarray gene expression profiles studies that showed no significant differences in *HB-EGF* expression between glioblastoma and control samples [19].

Recent mounting evidence showed that the expression of *MMP9* might play a critical role in brain neoplastic tissue invasion, metastasis, and angiogenesis [12]. Even if not completely statistically supported, our findings were in good agreement with a previous work showing an upregulation of *MMP9* mRNA levels in glioblastoma compared to controls and suggesting a close relationship between *MMP9* expression and tumor malignant progression [12].

Although *ADAM17* expression has been reported in normal human brain tissue and in cell lines [5], its expression at mRNA level has been poorly investigated in brain tumors. Functional studies in U373-MG glioma cells have demonstrated that cannabinoids induced cell proliferation through a two-step mechanism involving *ADAM17*-mediated shedding of *proHB-EGF* and subsequent *EGFR* stimulation [5]. Our finding that *ADAM17* mRNA levels in glioblastoma are statistically lower compared to controls is in contrast with a previous work that reported an increased expression of *ADAM17* in glioblastoma specimens [20]. This might reflect differences in tumor sampling or a consequence of glioblastoma multiforme cellular and molecular heterogeneity.

Our finding showing that glioblastoma expressed statistically significant lower *PTEN* mRNA levels compared to control samples confirmed previous reports [21], showing that *PTEN* expression variations were detectable only in a low fraction of anaplastic astrocytoma and were almost absent in low-grade brain tumors and controls. Taken together, these observations strengthen the hypothesis that an impairment of *PTEN* expression, together with a consequent aberrant activity of the PI3K-dependent pathway, might represent a typical hallmark of glioblastoma multiforme. A functional confirmation of this hypothesis was that, in a mouse astrocytoma model with genetic inactivation of the *Nf1* and *p53* tumor suppressor genes, the loss of *PTEN* heterozygosity and the Akt activation contributed to the brain tumor malignant progression [6].

Differently from the above mentioned investigated genes, the analysis of *PTEN* and *MMP9* expression, using a combination of unsupervised and supervised algorithms, provided interesting results: firstly, as reported in the dendrogram analysis, the expression profiling derived novel subsets of astrocytomas. This hierarchically clustering analysis clarified that tumor classification based even on a quantization of two genes could generate a patient stratification, clinically relevant and more informative than a single conventional histological classification. The *PTEN* and *MMP9* expression-generated subgroups produced also a different distribution of the patients according to their age: in particular subgroup IV-a, differently from IV-b, was enriched in age-class III patients (i.e., >61 years); this result might suggest that in glioblastoma multiforme tumor specimens *PTEN* and *MMP9* expression levels might be partly related with the elderly of the patients. On the contrary, in anaplastic astrocytoma and in control patients no association between age-related classes and *PTEN*/*MMP9* expression levels was evidenced. Furthermore, the originated dendrogram does

not reflect a classification of samples according to their anatomical tumor localization. However, in the light of the development of new pharmacological treatments, the identification of patient subsets with specific molecular signatures within tumor malignancy grades is becoming more and more relevant [22]. A finer analysis of *PTEN* and *MMP9* expression was also employed to derive a parsimonious classification tree of the investigated samples into their tumor malignancy grades. Specific *PTEN* and *MMP9* expression values significantly addressed the specimens into a specific diagnostic class, that is, glioblastoma or anaplastic astrocytomas. However, the significance and the sensitivity of this classification might be further refined with the increasing of specimens and through the identification of novel tumor-diagnostic markers. Basing on the average expression of *PTEN* and *MMP9*, the observed statistical trends clearly differentiate the control, anaplastic astrocytomas, and glioblastoma multiforme diagnostic classes; a similar expression trend for *PTEN* and *MMP9* genes was documented comparing normal versus astrocytic tumor cell lines. These results, in particular, reinforced the concept that anaplastic astrocytomas were intermediate-grade tumors, showing detectable mitotic activity, absent in low-grade astrocytomas, but not necrosis and prominent vascular proliferation, characteristic of glioblastoma multiforme [23, 24].

An additional interesting finding emerging from our study was the significant negative correlation between *PTEN* and *MMP9* mRNA expression in glioblastoma multiforme. Notably, not only was this negative correlation absent in anaplastic astrocytoma samples, but it was reversed in control samples. It was evident that differences in *PTEN* gene expression mainly account for these correlations because its levels in glioblastoma were significant higher compared to anaplastic astrocytoma samples, whilst no statistical difference was found for *MMP9* mRNA levels. The positive correlation between *PTEN* and *MMP9* in controls is derived essentially from an overexpression of *PTEN* rather than a low expression of *MMP9* compared to glioblastoma. The functional significance of these correlations is currently unknown, and future functional studies aimed at elucidating possible interplays of these genes in glioblastoma are clearly warranted. The negative correlation in glioblastoma between *MMP9* and *PTEN* could imply a functional interplays between these two genes, as already documented. It has been reported that *PTEN* modulates the expression and secretion of MMP2 and MMP9, thereby modifying tumor cell invasiveness [9, 25]. Notably, recent reports have clearly demonstrated that, in glioblastoma, *PTEN* may regulate migration via a PI3K-independent pathway [26]. In this model, the lack or functional loss of *PTEN* not only potentiates the migration induced by EGFR- and beta-integrin-dependent pathways but also enhances cell migration via a still largely unclear mechanism. On this regard, a recent report suggested that integrins could be a converging point in the mechanism supporting tumor invasion and migration of cancer cells with *PTEN* loss and *MMP9* overexpression.

The intrinsic genetic heterogeneity and redundant overlapping aberrant signalling transduction pathways underlie the failure of monotherapies in glioblastoma [8]. Therefore a sensitive and reliable method to measure gene expression, such as Real-time PCR, may greatly ameliorate diagnostic tools and eventually address the pharmacological approach using multitarget kinase inhibitors or combination of therapies based on multiple single-targeted receptor or intracellular kinase inhibitors. Some researchers have proposed that the combination of *PTEN* loss and *EGFR* hyperfunctionality could be predictive of the ineffectiveness of therapies with *EGFR* inhibitors [4] because these two pathways might synergize to enhance glioblastoma malignancy. This hypothesis has been elegantly supported by the recent observation that the pharmacological inhibition of PI3K-alpha and mTOR augments the antiproliferative activity of the EGFR inhibitor erlotinib in glioblastoma cell lines [7]. The inverse correlation between *PTEN* and *MMP9* expression reported here raised the issue whether the concomitant hyperactivation of the PI3K-alpha and MMP9-dependent pathways might be instrumental in devising or refining combined pharmacological therapies in glioblastoma. The modest efficacy of mTOR inhibitors alone in clinical trials was greatly enhanced when these compounds were administered in combination with the EGFR inhibitor Gefitinib [27]. Although monotherapy regimens with MMPs inhibitors in clinical trials have been quite disappointing, the relevance of MMPs as valid target has been reevaluated by the recent finding that the combined use of MMPs, COX2, and EGFR inhibitors reduced human breast cancer tumor growth [11]. We therefore speculate that the pivotal role of MMPs in glioma invasion and angiogenesis deserves future in vitro and in vivo experiments using MMP inhibitors in combination with PI3K inhibitors alone or with these latter compounds plus EGFR inhibitors.

The analyses of gene expression at transcriptional level in biopsy tissue samples are instrumental in delineating abnormal gene expression signature of brain tumors, but it should be mentioned that these studies suffer some pitfalls and limitations: in particular, the use of supervised approaches, based on the assumption that the grouping (i.e., the histological tumor diagnoses) is correct, may not be a valid assumption for all the clinical cases examined; additionally, the intrinsic heterogeneity of glioblastoma, together with the presence of nontumor cells in the samples, probably accounts for the variability found in transcripts levels and may represent a critical factor and a limitation in the interpretation of our results. From a technical point of view, a main difference of our contribution, compared to other reports, deals with the criteria adopted to express transcript levels in the investigated specimens. The majority of clinical gene expression profile studies performed by Real-time PCR normalized data using an internal housekeeping gene as a reference, but great caution in choosing this normalization method is necessary especially when analyzing tumor biopsy samples [28]. The tumorigenic process itself, via genomic mutations or amplifications, could induce modifications of housekeeping genes levels [29], and hence the choice of unreliable housekeeping genes may lead to interpretation errors and bias in experimental results [30]. Our attempts to use *GAPDH, ACTB*, and *HPRT* as reference genes were unsuccessful due to the great variations among

all the samples (data not shown); therefore, we decided to express gene expression as absolute amount of femtograms (fg) of transcripts, normalized to the total amount of RNA employed, through accurate quantification using the combination of spectrofotometric and fluorimetric approaches.

In conclusion, the combined analysis of the transcripts of *PTEN* and *MMP9* genes in biopsy specimens could represent a reliable diagnostic and prognostic marker of human glial tumor. Further epidemiological and functional in vitro studies are required to establish the reliability of *PTEN* and *MMP9* genes as possible valid molecular targets in the pharmacological strategies aimed at controlling human glioma malignant progression.

Acknowledgments

This study was supported by grants from the Italian MIUR, "Progetti di Ricerca di Rilevante Interesse Nazionale (2005)." A. A. is granted by Fondo Sociale Europeo (F.S.E.). The authors are particularly grateful to Dr. Eugenio Benericetti (Azienda Ospedaliera di Parma, Italy) for providing WHO graded glioma specimens. Orange Software is released under General Programming License (GPL). The authors are therefore particularly grateful to Orange Program's authors, Demsar J, Zupan B, Leban G: (2004) Orange: From Experimental Machine Learning to Interactive Data Mining, White Paper (http://www.ailab.si/orange/), Faculty of Computer and Information Science, University of Ljubljana (Slovenia).

References

[1] D. N. Louis, "Molecular pathology of malignant gliomas," *Annual Review of Pathology*, vol. 1, pp. 97–117, 2006.

[2] H. Ohgaki and P. Kleihues, "Genetic pathways to primary and secondary glioblastoma," *American Journal of Pathology*, vol. 170, no. 5, pp. 1445–1453, 2007.

[3] S. Higashiyama and D. Nanba, "ADAM-mediated ectodomain shedding of HB-EGF in receptor cross-talk," *Biochimica et Biophysica Acta*, vol. 1751, no. 1, pp. 110–117, 2005.

[4] S. Miyamoto, H. Yagi, F. Yotsumoto, T. Kawarabayashi, and E. Mekada, "Heparin-binding epidermal growth factor-like growth factor as a novel targeting molecule for cancer therapy," *Cancer Science*, vol. 97, no. 5, pp. 341–347, 2006.

[5] S. Hart, O. M. Fischer, and A. Ullrich, "Cannabinoids induce cancer cell proliferation via tumor necrosis factor α-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor," *Cancer Research*, vol. 64, no. 6, pp. 1943–1950, 2004.

[6] C.-H. Kwon, D. Zhao, J. Chen, et al., "Pten haploinsufficiency accelerates formation of high-grade astrocytomas," *Cancer Research*, vol. 68, no. 9, pp. 3286–3294, 2008.

[7] Q.-W. Fan, C. K. Cheng, T. P. Nicolaides, et al., "A dual phosphoinositide-3-kinase α/mTOR inhibitor cooperates with blockade of epidermal growth factor receptor in PTEN-mutant glioma," *Cancer Research*, vol. 67, no. 17, pp. 7960–7965, 2007.

[8] J. B. Johnston, S. Navaratnam, M. W. Pitz, et al., "Targeting the EGFR pathway for cancer therapy," *Current Medicinal Chemistry*, vol. 13, no. 29, pp. 3483–3492, 2006.

[9] M.-S. Kim, M.-J. Park, E.-J. Moon, S.-J. Kim, C.-H. Lee, and H. Yoo, "Hyaluronic acid induces osteopontin via the phosphatidylinositol 3-kinase/Akt pathway to enhance the motility of human glioma cells," *Cancer Research*, vol. 65, no. 3, pp. 686–691, 2005.

[10] T. Hastie, R. Tibshirani, D. Botstein, and P. Brown, "Supervised harvesting of expression trees," *Genome Biology*, vol. 2, no. 1, article research0003.1-0003.12, 2001.

[11] I. K. Mellinghoff, M. Y. Wang, I. Vivanco, et al., "Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors," *The New England Journal of Medicine*, vol. 353, no. 19, pp. 2012–2024, 2005.

[12] K. Komatsu, Y. Nakanishi, N. Nemoto, T. Hori, T. Sawada, and M. Kobayashi, "Expression and quantitative analysis of matrix metalloproteinase-2 and -9 in human gliomas," *Brain Tumor Pathology*, vol. 21, no. 3, pp. 105–112, 2004.

[13] P. Kleihues and W. K. Cavenee, *World Health Organization Classification of Tumors of the Nervous System*, Lyon, France, 2000.

[14] M. Paolillo, A. Barbieri, P. Zanassi, and S. Schinelli, "Expression of endothelins and their receptors in glioblastoma cell lines," *Journal of Neuro-Oncology*, vol. 79, no. 1, pp. 1–7, 2006.

[15] D. N. Louis, E. C. Holland, and J. G. Cairncross, "Glioma classification: a molecular reappraisal," *American Journal of Pathology*, vol. 159, no. 3, pp. 779–786, 2001.

[16] Y. Liang, M. Diehn, N. Watson, et al., "Gene expression profiling reveals molecularly and clinically distinct subtypes of glioblastoma multiforme," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 16, pp. 5814–5819, 2005.

[17] F. Wang, R. Liu, S. W. Lee, C. M. Sloss, J. Couget, and J. C. Cusack, "Heparin-binding EGF-like growth factor is an early response gene to chemotherapy and contributes to chemotherapy resistance," *Oncogene*, vol. 26, no. 14, pp. 2006–2016, 2007.

[18] D. B. Ramnarain, S. Park, D. Y. Lee, et al., "Differential gene expression analysis reveals generation of an autocrine loop by a mutant epidermal growth factor receptor in glioma cells," *Cancer Research*, vol. 66, no. 2, pp. 867–874, 2006.

[19] D. S. Rickman, M. P. Bobek, D. E. Misek, et al., "Distinctive molecular profiles of high-grade and low-grade gliomas based on oligonucleotide microarray analysis," *Cancer Research*, vol. 61, no. 18, pp. 6885–6891, 2001.

[20] D. Wildeboer, S. Naus, Q.-X. A. Sang, J. W. Bartsch, and A. Pagenstecher, "Metalloproteinase disintegrins ADAM8 and ADAM19 are highly regulated in human primary brain tumors and their expression levels and activities are associated with invasiveness," *Journal of Neuropathology and Experimental Neurology*, vol. 65, no. 5, pp. 516–527, 2006.

[21] C. B. Knobbe, A. Merlo, and G. Reifenberger, "Pten signalling in gliomas," *Neuro-Oncology*, vol. 4, no. 3, pp. 196–211, 2002.

[22] G. N. Fuller, K. R. Hess, C. H. Rhee, et al., "Molecular classification of human diffuse gliomas by multidimensional scaling analysis of gene expression profiles parallels morphology-based classification, correlates with survival, and reveals clinically-relevant novel glioma subsets," *Brain Pathology*, vol. 12, no. 1, pp. 108–116, 2002.

[23] K. Ichimura, H. Ohgaki, P. Kleihues, and V. P. Collins, "Molecular pathogenesis of astrocytic tumours," *Journal of Neuro-Oncology*, vol. 70, no. 2, pp. 137–160, 2004.

[24] B. K. Ahmed Rasheed, R. N. Wiltshire, S. H. Bigner, and D. D. Bigner, "Molecular pathogenesis of malignant gliomas," *Current Opinion in Oncology*, vol. 11, no. 3, pp. 162–167, 1999.

[25] K. Furukawa, Y. Kumon, H. Harada, et al., "PTEN gene transfer suppresses the invasive potential of human malignant

gliomas by regulating cell invasion-related molecules," *International Journal of Oncology*, vol. 29, no. 1, pp. 73–81, 2006.

[26] M. Raftopoulou, S. Etienne-Manneville, A. Self, S. Nicholls, and A. Hall, "Regulation of cell migration by the C2 domain of the tumor suppressor PTEN," *Science*, vol. 303, no. 5661, pp. 1179–1181, 2004.

[27] S. Sathornsumetee, D. A. Reardon, A. Desjardins, J. A. Quinn, J. J. Vredenburgh, and J. N. Rich, "Molecularly targeted therapy for malignant glioma," *Cancer*, vol. 110, no. 1, pp. 12–24, 2007.

[28] C. Tricarico, P. Pinzani, S. Bianchi, et al., "Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies," *Analytical Biochemistry*, vol. 309, no. 2, pp. 293–300, 2002.

[29] S. Waxman and E. Wurmbach, "De-regulation of common housekeeping genes in hepatocellular carcinoma," *BMC Genomics*, vol. 8, pp. 243–250, 2007.

[30] J. L. Aerts, M. I. Gonzales, and S. L. Topalian, "Selection of appropriate control genes to assess expression of tumor antigens using real-time RT-PCR," *BioTechniques*, vol. 36, no. 1, pp. 84–91, 2004.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 594738, 9 pages
doi:10.1155/2009/594738

Review Article

An Evolutionary Perspective of Animal MicroRNAs and Their Targets

Noam Shomron,[1] David Golan,[1] and Eran Hornstein[2]

[1] *Department of Cell & Developmental Biology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel*
[2] *Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel*

Correspondence should be addressed to Noam Shomron, nshomron@post.tau.ac.il

Received 27 March 2009; Accepted 17 June 2009

Recommended by Bibekanand Mallick

MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression through translational inhibition or mRNA degradation by binding to sequences on the target mRNA. miRNA regulation appears to be the most abundant mode of posttranscriptional regulation affecting ~50% of the transcriptome. miRNA genes are often clustered and/or located in introns, and each targets a variable and often large number of mRNAs. Here we discuss the genomic architecture of animal miRNA genes and their evolving interaction with their target mRNAs.

1. Introduction

MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression by binding to sequences on the target mRNA (reviewed by [1–7]. Gene silencing initiates when the miRNA, located within an RNA-Induced Silencing Complex (RISC), directs binding to complementary sequences on the mRNA's 3′ untranslated region (UTR). The miRNA-mRNA recognition binding sequences are short, usually 6–8 nt [8–11]. Inhibition of gene expression takes place via facilitated mRNA degradation, mRNA cleavage, or interference with translation.

2. Generation of miRNA Genes

2.1. miRNA Gene Origins. During animal evolution there were distinct, characterized phases of large scale genome duplications [12–14]. miRNA origin, as well, is traced back to genomic episodes dominated by large duplication events which coincide with the advent of bilaterians, vertebrates, and (placental) mammals [15]. The current wealth of miRNA genes results, additionally, from specific duplication events of miRNA clusters [16, 17] and from mechanisms such as the integration of repetitive genetic elements [18].

2.2. The Gatekeeper of the miRNA Biogenesis. The transcription of miRNA genes is controlled by enhancer-promoter elements comparable with those of protein-coding genes [19]. Additional regulation of miRNA expression is obtained through posttranscriptional processing [20], RNA A-to-I editing [21, 22], selective export into the cytoplasm [23, 24], and subcellular localization [25] (Figure 1 (see [2, 26–43]) also see review by [44]).

While several mechanisms control miRNA's expression along its biogenesis pathway, it seems that the rate limiting step in acquiring a novel miRNA is the recognition of the RNA secondary structure by Drosha. This stems from the fact that mammals express only several hundred miRNAs from myriad amounts of expressed RNA secondary structures [16, 45–47]. Thus, processing of miRNA precursor by the microprocessor is probably the gatekeeper of the miRNA biogenesis pathway, which allows for only a portion of the transcribed RNA hairpins to be further processed down the miRNA biosynthetic pathway. Analysis of miR-220 recent evolution provides an intriguing example for a gene that apparently did not encode for a miRNA but became competent for Drosha-dependent microprocessing. miR-220, which contains sequences of a tubulin gene, was probably originally processed from an antisense strand (see [48, 49]) of tubulin,

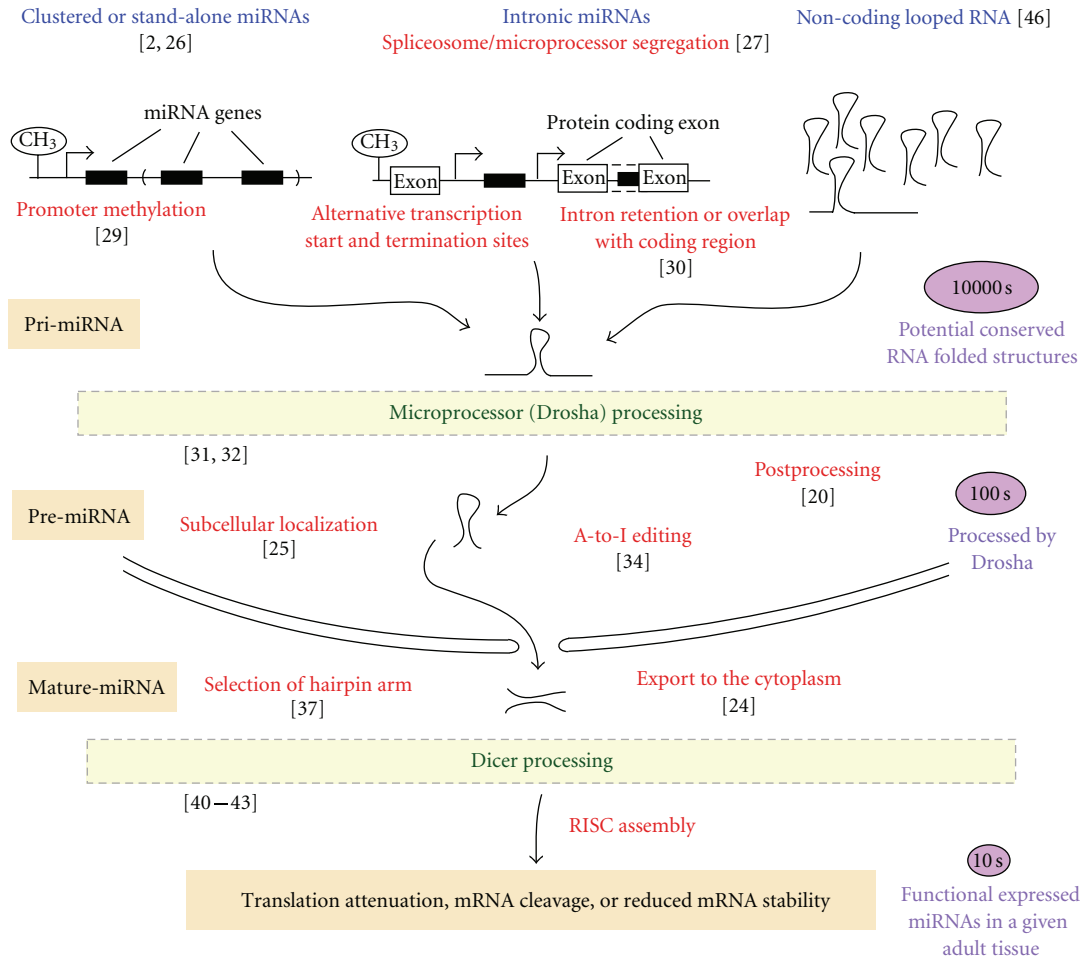


Figure 1: Several layers of regulation control canonical miRNA gene biogenesis: transcription activation, splicing, recognition by Drosha, postprocessing, RNA editing, subcellular localization, nuclear export, and hairpin arm selection. Selected examples are referenced within.

which folds back into a proper stem-loop structure in human but not in other vertebrates [15]. Comparative studies of the tubulin antisense strand sequence may shed light on the reasons for which human Drosha enables microprocessing while in other species it is skipped. Though canonical miRNA bioprocessing is Drosha-dependent, a novel splicing-dependent [33] mechanism was suggested recently to bypass initial steps of microprocessing [33, 50, 51]. Despite the functional robustness of miRNA secondary structures in light of accumulating mutations it is still not clear what the precise requirements for passing the gatekeeper of miRNA biogenesis are.

3. The Genomic Architecture of miRNA Genes and Their Expression

Two characteristics of miRNA genes stand out in regard to genomic organization of protein-coding genes. First, miRNA genes are often found in clusters (30%–42%) [52–54]. Additionally, miRNA genes are often embedded within introns (25% or more) [27, 55–59].

3.1. Chromosomal Organization of miRNA Genes. In accordance with genomic duplication events that accompany evolution of species, we see a correlation between the number of miRNA genes and chromosome length. miRNA gene number per chromosome also correlates with the protein-coding gene density (Figure 2(a) and 2(b)). This indicates that integration and/or maintenance of miRNA genes roughly follows protein-coding genes.

However, *Homo Sapiens* chromosomes 14, 19, and X are exceptionally enriched for miRNA genes. Chromosomes 14 and 19 both possess a single miRNA cluster, accounting for 93% and 80% of the total number of miRNA genes on each chromosome, respectively [16, 61]. The cluster on chromosome 14 is located in the human imprinted domain (14 q32) where only maternally inherited miRNAs are expressed [62]. Chromosome 19 hosts the primate-specific "500" cluster [16], a recently emerging, placental-specific cluster [16]. The *X* chromosome, on the other hand, does not have one large cluster, but it exhibits rapid emergence of smaller miRNA clusters due to frequent tandem duplications and nucleotide substitutions [17]. We note that despite the parallel evolution

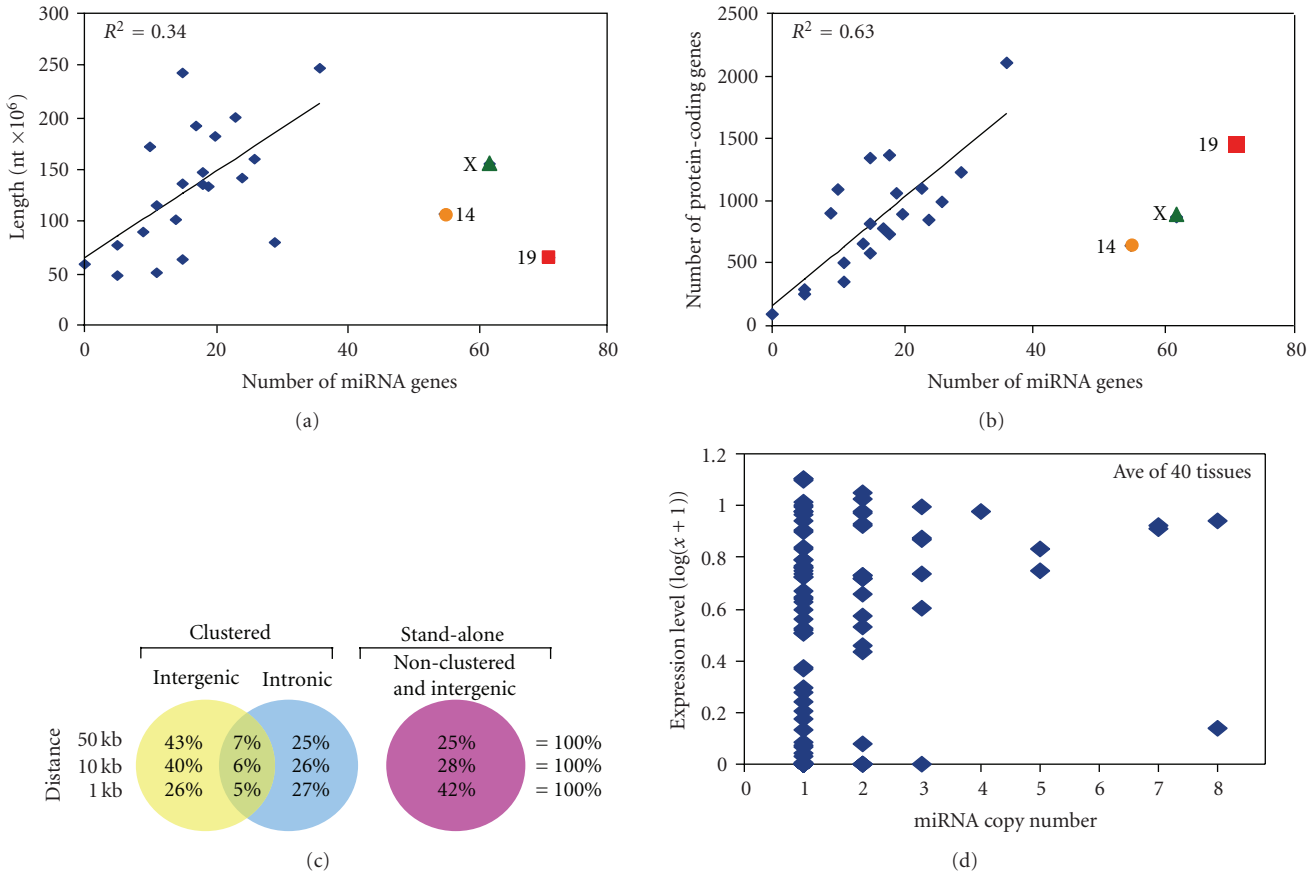


Figure 2: Genomic organization of miRNA genes and their expression. The number of miRNA genes correlates with chromosome length (a) and the number of protein-coding genes (b). Outliers chromosomes 14, 19, and X are indicated. When removing these three chromosomes the number of miRNA genes aligns well on the regression line (R^2 indicated). (c) Proportion of miRNA genes hosted in Introns—Intronic (based on Refseq genes), Clustered on the same genomic strand, or Stand-alone miRNAs. Overlapping intronic and clustered miRNAs are also indicated. Each row refers to 50 kb, 10 kb, and 1 kb distance between paired genes on the same strand. It was shown that clusters of size 3 kb give a large proportion of clusters (27%) with little change when increasing pairwise distance to 10 kb [54]. Diagrams are based on data from Refseq. (d) Human miRNA copy number was plotted against the average miRNA expression level of 40 Human tissues [60]. A similar plot of another dataset [55] gave comparable results (data not shown).

of miRNAs in animals and plants, miRNA clusters were observed in both kingdoms [63].

3.2. Clusters of miRNA Genes. Plausibly, employment of an already existing functional promoter by new miRNA genes is an efficient way to express new miRNAs, eliminating the need for *de novo* establishment of promoter-enhancer sequences upstream of the miRNA gene (such as in [64–66]). This may be the rational underlying miRNA aggregation into polycistronic miRNA clusters and for their genomic preference for introns of transcribed genes (see Figure 2(c)). The consequence on the genomic level is that many miRNAs within up to 50 kb DNA fragment tend to be coexpressed [54, 55]. Amplification of an ancestral miRNA inside a cluster [54, 56] could contribute to the effective dosage of a given expressed miRNA homolog. However, at lower copy number gene dose does not seem to be a powerful predictor of expression levels (Figure 2(d)). The most likely interpretation is that the magnitude of promoter activity probably dominates regulation of miRNA expression. The correlation between miRNA gene copy number and expression level that was noted in some cases [67] may nonetheless suggest that when miRNA copy number is high (>3; Figure 2(d)), it may also serve to impact the expression level.

3.3. Intronic miRNA Genes. At least 25% of miRNA genes are hosted in introns of both protein-coding and noncoding RNAs (Figure 2(c) also see [27, 56, 59]). This is a striking feature of noncoding RNAs (reviewed by [68]), plausibly implying that some noncoding RNAs have developed a functional relationship with their host genes [38, 69]. The use of the same promoter-enhancer system enables coupling of miRNA expression with its host gene, therefore not surprisingly frequently seen [27, 55, 70]. When derived from the same primary transcript, it appears that pri-miRNA maturation by the microprocessor and pre-mRNA editing by the Spliceosome can either coexist independently or interconnect. While some studies imply that these processes

hardly interact [59], others have shown strong interactions initiating at transcription [71–73]. Overall, given the tight proximity of these cellular events in time and space it is hard to imagine how these functional complexes avoid each other. Further analysis would be required to determine the extent of this interaction and whether this is true for all given transcripts [74].

3.4. Functional Expression of miRNAs and Their Host Genes. While mRNA/miRNA derived from the same transcript may simply reflect an efficient use of a promoter-enhancer cassette [59], in a subset of cases a coordinated expression of an miRNA-protein pair from the same genomic locus may reflect a genetic interaction. For example, platelets contain two cAMP phosphodiesterases (PDEs)—PDE2A and PDE3A—each regulating a specific intracellular pool of cAMP [75]. miR-139 that is hosted in an intron of the PDE2A targets PDE3A (TargetScanS, see [76]), implying that the miRNA expression from PDE2A regulates the balance between the two isoforms. Similarly, miR-208 is encoded by an intron of the cardiac-specific alpha myosin heavy chain (MHC) gene, a major cardiac contractile protein. Alpha MHC responses to stress and hypothyroidism [35, 77] partially by coexpressing miR-208. The miRNA targets and downregulates beta MHC expression [70]. Thus, the precision in regulating an miRNA and a gene product may be hardwired into the genomic organization, to promise proper balance in their opposing or collaborating functions.

4. Generation of miRNA Targets and Their Interaction with miRNAs

4.1. Reciprocal Evolutionary Interaction between miRNAs and Their Targets. Our current understanding of miRNA binding sites suggests that a stretch of 6 nucleotide "seed" region, matching between the 5′ end of the miRNA and the mRNA 3′ UTR, may suffice for regulation by miRNAs [9, 10, 76, 78]. Because changes in *cis* sequences often dominates rewiring of genetic networks, [79] it is likely that the 3′ UTR of mRNA targets change their repertoire of seed matches faster than the highly conserved transacting miRNAs. This can be intuitively explained merely because the large number of targets affected by mutations in any given miRNA gene acts as a stabilizing element on the miRNA itself. So given a virtually fixed population of miRNAs, targets gain and lose binding sites in a way that supports their controlled miRNA expression. This can be viewed as an evolutionary reciprocal interaction between the miRNA and its accumulating targets. After miRNA emergence, once a critical number of targets are functionally regulated by the miRNA, stabilization of its primary sequence is gained [80], while at the same time, stabilizing selection decreases variation in target seed match [28, 81, 82].

The target set size is also dramatically affected by the nucleotide composition of the new miRNA, and, as mentioned above, this characteristic affects the average selective pressure on the miRNA itself [78]. Given a set of 17 000 3′ UTRs ("Known Genes" in the UCSC genome browser database), some ~2000 UTRs would randomly have a single binding site for a heptamer seed composed of A/U residues. This number falls to only ~200 seed matches with a G/C-only seed content and is somewhere in between (~800) for a mixed nucleotide composition (equal number of A/U and G/C). Once emerged, the set of targets affected by a novel miRNA is subject to selective pressure which molds the transcriptome such that binding sites would either be acquired or lost. In fact, selective loss of seed matches, to a level below the randomly predicted baseline, dubbed "anti-targets" [9, 83], provides strong support for the evolutionary power underlying the structure of miRNA binding sites (also see [84]).

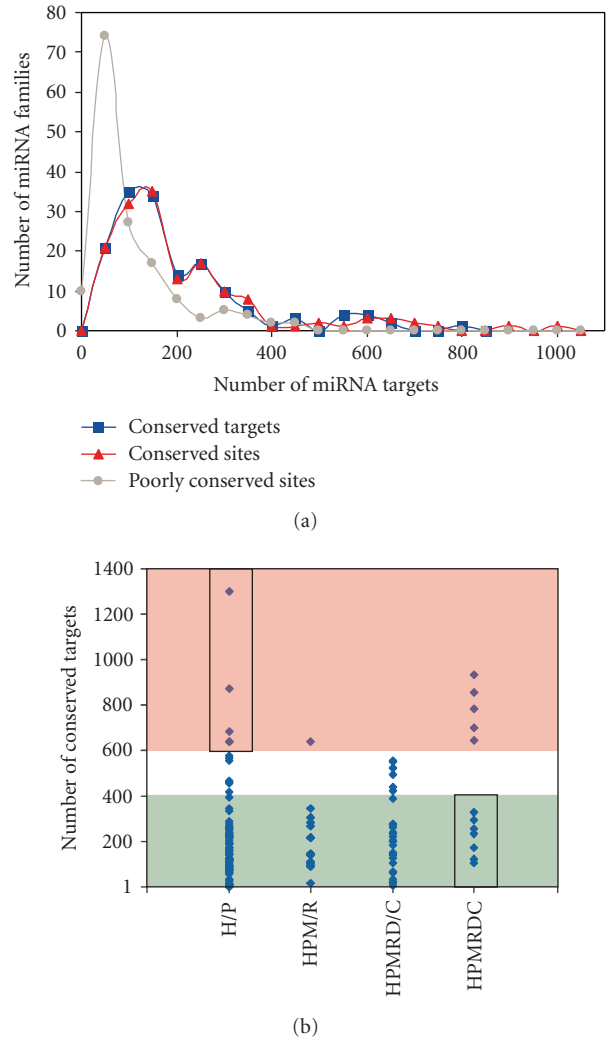


Figure 3: The number of predicted conserved miRNA target sites. (a) Predicted number of conserved targets, conserved target sites and poorly conserved sites of human miRNAs (based on TargetScanS). (b) The number of predicted conserved miRNA targets was divided according to the conservation level of the miRNA itself (H, Human; P, Chimp; M, Mouse; R, Rat; D, Dog; C, Chicken; based on TargetScanS). Shaded in red/green are the regions with the largest/least number of targets (resp.). Extreme numbers of targets are boxed and are discussed in the text.

The reciprocal interaction between miRNAs and their targets gets an additional perception when looking at this relationship in viral miRNAs. Several viruses express miRNAs for controlling specific cellular genes or pathways. For this purpose, most cellular mRNA targets of viral miRNAs identified to date play a role in either regulation of apoptosis or host antiviral immune response. miRNAs are suitable for a viral genome expression as they are short and compact. In addition, they can be generated more readily than proteins against new target genes and do not elicit any antigenic response. Their evolutionary flexibility is based on the high mutation rates of the viruses. This leads to modifications in the miRNA genes themselves, and thus even the largest virus family containing miRNAs (herpesvirus) shows little conservation between their miRNAs. It also indicates that it is unlikely that host miRNA targets viral mRNAs as these would mutate away from disruptive regulation (also see [85, 86]) .

4.2. The Large Variation in miRNA Target Sites. Conserved complementarities to a minimal hexamer region (matching nt 2–7 of the miRNA) [8] indicates that once a seed match emerges, it becomes functional. If the binding is preferentially beneficial, it might serve as a favorable and directional intermediate species. Within Tetrapods, the average number of predicted conserved sites per miRNA is at the range of 200 (Figure 3(a), TargetScanS, plotted for Human miRNAs). However, the number of targets is skewed to the higher values, while the upper and lower 10-percentiles regulate more than 450 or less than 50 genes, respectively (also see [87, 88]). Comparative genomics suggests that ancient miRNAs have on average twofold more targets than newly generated ones (compare 453 to 194, resp.). Some discrepancies result from misestimating miRNA antiquity or overlapping miRNA functional sites. Specifically, the age of some miRNA genes might have been misestimated, as cross-species orthologues searches are not exhausted yet. miR-761, for example, identified only in mouse [57] is in fact conserved in six other mammals (including human and opossum; see [89] also see miRviewer at http://people.csail.mit.edu/akiezun/miRviewer). Alternatively, overlapping functional sites shared by miRNAs and other regulatory factors may bias the distribution of targets. For example, pre-existing "scaffolds" of other regulatory systems could serve as anchors for miRNA binding. In the case of miR-16, a component of the AU-rich mediated deregulation of mRNA stability [90], the miRNA is a late addition onto a mechanism that was probably functional in the common ancestor of yeast [91], before the innovation of miRNAs. In this train of thought, some transcriptional termination or pause sites [92, 93] overlap with miRNA seed-matches (miR-525 and miR-488). In human, *Alu* transposable elements exhibit complementarities in some of their regions to almost 30 human miRNAs [94]. In other instances, the attempt to avoid specific protein binding domains in the 3′ UTRs may expel miRNA binding sites. For example, 3′ UTRs may avoid miR-518a seed (which has only 26 predicted conserved targets) because it perfectly matches the proline and acidic rich (PAR) protein binding sequence [95]. Other miRNA interference events may involve binding to promoters *via* antisense transcription, which is estimated to be as common as 15% in the human genome [96]. Overlapping sequences as such might coincide with promiscuous promoter-associated functions of small RNAs [36] or increase in transcription [97]. Plausibly a selective pressure to avoid the binding of the aryl hydrocarbon receptor (AhR) [98] onto miR-521 sites (AhR and miR-521 share the same sequence) may explain how miRNAs of similar antiquity and A/U content (compare to miR-520 h) dramatically vary in their predicted numbers of conserved targets (compare 8 to >400, resp.; both miRNAs are part of the same primary transcript, BF773110). It is noteworthy that the low number of miR-521 targets cannot be explained by a conflict of expression in a broad set of tissues since miR-521 is expressed only in placenta.

4.3. Unique Features of miRNAs with Most Number of Targets. In order to further explore the characteristics of miRNAs with extreme number of targets we compared the group of miRNAs with the largest number of targets to that with the least number of targets (Figure 3(b), shaded red and green, resp.). We found some correlation between miRNA conservation and its potential number of predicted targets. This correlation is emphasized in the conserved target sets where human-to-mouse conserved miRNAs have on average 197 predicted conserved targets; human-to-dog conserved miRNAs have 245, and human-to-chicken conserved miRNAs 453. miRNAs with the largest number of targets tend to be expressed mostly from one arm of the pre-miRNA hairpin (they do not exhibit both 5′ and 3′ arm expression) and are often expressed at higher levels and in a broader set of tissues compared to miRNAs with the least number of targets (also see [99]).

miRNAs with the largest number of targets are A/U-rich. The average A/U percentage within the seed of the top 20 miRNAs with the largest number of targets is 57%, compared to 41% for those with the least number of targets. This may be required for weaker secondary structures in the target mRNA and for ongoing accessibility [11]. Consistently, a general mutational trend (in the human genome) from G-to-A and C-to-T is more abundant than the reverse direction [100]. Analysis of human Single Nucleotide Polymorphisms (SNPs) on a representative chromosome (chromosome 1; 661 SNPs) confirms that the majority of polymorphisms generating new potential miRNA binding sites are G-to-A and C-to-T substitutions (occurring 1.7-fold more than the reverse direction). Interestingly, the two most pronounced examples of target polymorphic changes are G-to-A mutations [39, 101].

In summary, miRNA gene integration and maintenance roughly follow protein-coding genes. After emergence, the miRNA gene sequence is refined through an evolutionary reciprocal interaction with its accumulating targets, and these later stabilize the miRNA when reaching a large enough number of functional targets. Finally, overlapping functional sites shared by miRNAs and other regulatory factors may facilitate or inhibit miRNA target formation and thus influence miRNA target set size.

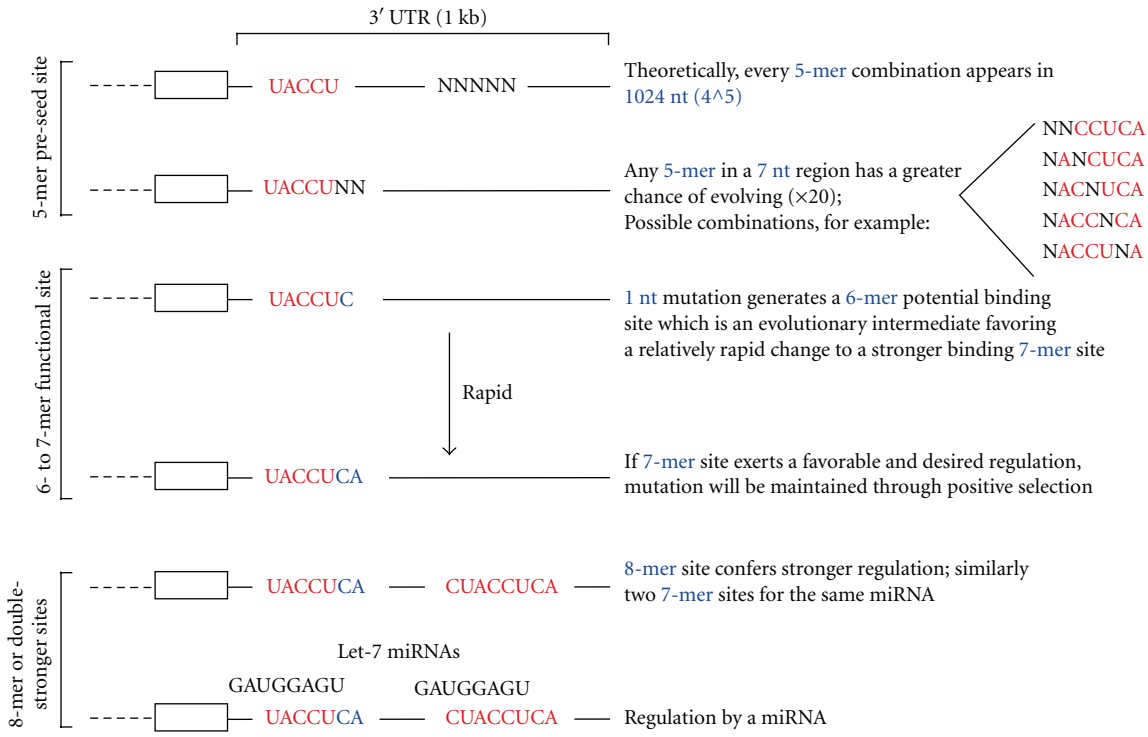


Figure 4: A possible scenario for acquiring a functional miRNA binding site.

5. A Timescale for miRNA Target-Site Evolution

It would take several million years for a specific 7-mer binding site to evolve from a complete null binding sequence [102]. However, miRNA binding sites evolve from existing sequences, and based on these partial binding sequences, ("almost-binding" sites or "pre-seed" sites), a corrected estimated time for a miRNA binding site to emerge is 0.2 million years (Durrett R., personal communications). For example, a 5 nt pre-seed site will appear every 1024 nt (4^5) or even 20 times more often since the position of the 5 nt within the 7 nt is not restricted and may also include inserts. Thus, a 1 kb 3′ UTR will contain several potential pre-seed sequences. A human specific miRNA that is absent even from the chimp genome should be roughly 6 million years old (last estimated split between human and chimp). Given 0.2 million years required for a 7-mer binding site to evolve, around 30 perfect 7-mer binding sites are expected. For an miRNA that is traced back to mouse (split more than 100 million years ago from human), about 500 conserved targets per miRNA are reasonable. This simplified calculation might indicate that, given a spontaneous mutation rate, there should be a direct correlation between the age of an miRNA and the number of targets it possesses and also to the number of duplicated events of the same miRNA site on one transcript. Eventually, it is not enough for the mutation to occur—it should also be maintained in the population after exhibiting a strong selective pressure towards a favorable regulation which can only take place when an miRNA and its targets are spatially and temporally coexpressed [83, 103]. This calculation allows us to set the general time line of events for miRNA formation. Nevertheless there are many outstanding exceptions of small and large miRNA target repertoires (also see Figure 4).

Websites Used

Ensembl: http://www.ensembl.org
GenBank: http://www.ncbi.nlm.nih.gov
miRBase: http://microrna.sanger.ac.uk/
miRNAminer: http://groups.csail.mit.edu/pag/mirnaminer
miRviewer: http://people.csail.mit.edu/akiezun/miRviewer
Patrocles: http://www.patrocles.org/
TargetRank: http://hollywood.mit.edu/targetrank
TargetScanS: http://www.targetscan.org
UCSC genome browser: http://genome.ucsc.edu.

Acknowledgments

The authors thank the following people for commenting on their manuscript: Brad Friedman, Alex Stark, Iftach Nachman, Robin Friedman, Eric Wang, Rickard Sandberg, Etgar Levy-Nissenbaum, and the Shomron lab members. They thank Rick Durrett and Deena Schmidt for assistance in statistical calculations. Work at the NS lab is supported by the Israeli Ministry of Health and the Kunz-Lion Foundation. EH is the incumbent of the Helen and Milton A. Kimmelman Career Development Chair. Work at the EH lab is supported by grants from the JDRF, ISF, ISf-Legacy, GIF, the Benoziyo Center for Neurological Disease, the Estate of Flourence Blau and the Wolfson Family Charitable trust for miRNA.

References

[1] V. Ambros, "The functions of animal microRNAs," *Nature*, vol. 431, no. 7006, pp. 350–355, 2004.

[2] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[3] L. He and G. J. Hannon, "MicroRNAs: small RNAs with a big role in gene regulation," *Nature Reviews Genetics*, vol. 5, no. 7, pp. 522–531, 2004.

[4] T. Du and P. D. Zamore, "microPrimer: the biogenesis and function of microRNA," *Development*, vol. 132, no. 21, pp. 4645–4652, 2005.

[5] W. Filipowicz, L. Jaskiewicz, F. A. Kolb, and R. S. Pillai, "Post-transcriptional gene silencing by siRNAs and miRNAs," *Current Opinion in Structural Biology*, vol. 15, no. 3, pp. 331–341, 2005.

[6] R. W. Carthew, "Gene regulation by microRNAs," *Current Opinion in Genetics and Development*, vol. 16, no. 2, pp. 203–208, 2006.

[7] R. H. A. Plasterk, "Micro RNAs in animal development," *Cell*, vol. 124, no. 5, pp. 877–881, 2006.

[8] B. P. Lewis, I.-H. Shih, M. W. Jones-Rhoades, D. P. Bartel, and C. B. Burge, "Prediction of mammalian microRNA targets," *Cell*, vol. 115, no. 7, pp. 787–798, 2003.

[9] J. Brennecke, A. Stark, R. B. Russell, and S. M. Cohen, "Principles of microRNA-target recognition," *PLoS Biology*, vol. 3, no. 3, article e85, 2005.

[10] A. Krek, D. Grün, M. N. Poy, et al., "Combinatorial microRNA target predictions," *Nature Genetics*, vol. 37, no. 5, pp. 495–500, 2005.

[11] C. B. Nielsen, N. Shomron, R. Sandberg, E. Hornstein, J. Kitzman, and C. B. Burge, "Determinants of targeting by endogenous and exogenous microRNAs and siRNAs," *RNA*, vol. 13, no. 11, pp. 1894–1910, 2007.

[12] S. Ohno, *Evolution by Gene Duplication*, Springer, New York, NY, USA, 1970.

[13] P. W. H. Holland, J. Garcia-Fernandez, N. A. Williams, and A. Sidow, "Gene duplications and the origins of vertebrate development," *Development*, vol. 120, supplement, pp. 125–133, 1994.

[14] L.-G. Lundin, "Gene duplications in early metazoan evolution," *Seminars in Cell and Developmental Biology*, vol. 10, no. 5, pp. 523–530, 1999.

[15] J. Hertel, M. Lindemeyer, K. Missal, et al., "The expansion of the metazoan microRNA repertoire," *BMC Genomics*, vol. 7, article 25, 2006.

[16] I. Bentwich, A. Avniel, Y. Karov, et al., "Identification of hundreds of conserved and nonconserved human microRNAs," *Nature Genetics*, vol. 37, no. 7, pp. 766–770, 2005.

[17] R. Zhang, Y. Peng, W. Wang, and B. Su, "Rapid evolution of an X-linked microRNA cluster in primates," *Genome Research*, vol. 17, no. 5, pp. 612–617, 2007.

[18] J. Piriyapongsa, L. Mariño-Ramírez, and I. K. Jordan, "Origin and evolution of human microRNAs from transposable elements," *Genetics*, vol. 176, no. 2, pp. 1323–1337, 2007.

[19] F. Ozsolak, L. L. Poling, Z. Wang, et al., "Chromatin structure analyses identify miRNA promoters," *Genes & Development*, vol. 22, no. 22, pp. 3172–3183, 2008.

[20] J. M. Thomson, M. Newman, J. S. Parker, E. M. Morin-Kensicki, T. Wright, and S. M. Hammond, "Extensive post-transcriptional regulation of microRNAs and its implications for cancer," *Genes & Development*, vol. 20, no. 16, pp. 2202–2207, 2006.

[21] W. Yang, T. P. Chendrimada, Q. Wang, et al., "Modulation of microRNA processing and expression through RNA editing by ADAR deaminases," *Nature Structural and Molecular Biology*, vol. 13, no. 1, pp. 13–21, 2006.

[22] Y. Kawahara, B. Zinshteyn, P. Sethupathy, H. Iizasa, A. G. Hatzigeorgiou, and K. Nishikura, "Redirection of silencing targets by adenosine-to-inosine editing of miRNAs," *Science*, vol. 315, no. 5815, pp. 1137–1140, 2007.

[23] E. Lund, S. Güttinger, A. Calado, J. E. Dahlberg, and U. Kutay, "Nuclear export of microRNA precursors," *Science*, vol. 303, no. 5654, pp. 95–98, 2004.

[24] R. Yi, B. P. Doehle, Y. Qin, I. G. Macara, and B. R. Cullen, "Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs," *RNA*, vol. 11, no. 2, pp. 220–226, 2005.

[25] H.-W. Hwang, E. A. Wentzel, and J. T. Mendell, "A hexanucleotide element directs microRNA nuclear import," *Science*, vol. 315, no. 5808, pp. 97–100, 2007.

[26] A. Tanzer and P. F. Stadler, "Molecular evolution of a microRNA cluster," *Journal of Molecular Biology*, vol. 339, no. 2, pp. 327–335, 2004.

[27] A. Rodriguez, S. Griffiths-Jones, J. L. Ashurst, and A. Bradley, "Identification of mammalian microRNA host genes and transcription units," *Genome Research*, vol. 14, no. 10A, pp. 1902–1910, 2004.

[28] N. Iwai and H. Naraba, "Polymorphisms in human pre-miRNAs," *Biochemical and Biophysical Research Communications*, vol. 331, no. 4, pp. 1439–1444, 2005.

[29] B. Weber, C. Stresemann, B. Brueckner, and F. Lyko, "Methylation of human MicroRNA genes in normal and neoplastic cells," *Cell Cycle*, vol. 6, no. 9, pp. 1001–1005, 2007.

[30] S. Tan, J. Guo, Q. Huang, et al., "Retained introns increase putative microRNA targets within 3′ UTRs of human mRNA," *FEBS Letters*, vol. 581, no. 6, pp. 1081–1086, 2007.

[31] R. I. Gregory, K.-P. Yan, G. Amuthan, et al., "The Microprocessor complex mediates the genesis of microRNAs," *Nature*, vol. 432, no. 7014, pp. 235–240, 2004.

[32] A. M. Denli, B. B. J. Tops, R. H. A. Plasterk, R. F. Ketting, and G. J. Hannon, "Processing of primary microRNAs by the microprocessor complex," *Nature*, vol. 432, no. 7014, pp. 231–235, 2004.

[33] E. Berezikov, W.-J. Chung, J. Willis, E. Cuppen, and E. C. Lai, "Mammalian mirtron genes," *Molecular Cell*, vol. 28, no. 2, pp. 328–336, 2007.

[34] D. J. Luciano, H. Mirsky, N. J. Vendetti, and S. Maas, "RNA editing of a miRNA precursor," *RNA*, vol. 10, no. 8, pp. 1174–1177, 2004.

[35] R. Matsuoka, "Study of the vertebrate MHC multigene family during heart development," *Advances in Experimental Medicine and Biology*, vol. 538, pp. 17–30, 2003.

[36] B. A. Janowski, S. T. Younger, D. B. Hardy, R. Ram, K. E. Huffman, and D. R. Corey, "Activating gene expression in mammalian cells with promoter-targeted duplex RNAs," *Nature Chemical Biology*, vol. 3, no. 3, pp. 166–173, 2007.

[37] D. S. Schwarz, G. Hutvágner, T. Du, Z. Xu, N. Aronin, and P. D. Zamore, "Asymmetry in the assembly of the RNAi enzyme complex," *Cell*, vol. 115, no. 2, pp. 199–208, 2003.

[38] T. Hirose, M.-D. Shu, and J. A. Steitz, "Splicing-dependent and -independent modes of assembly for intron-encoded box C/D snoRNPs in mammalian cells," *Molecular Cell*, vol. 12, no. 1, pp. 113–123, 2003.

[39] A. Clop, F. Marcq, H. Takeda, et al., "A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep," *Nature Genetics*, vol. 38, no. 7, pp. 813–818, 2006.

[40] A. Grishok, A. E. Pasquinelli, D. Conte, et al., "Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing," *Cell*, vol. 106, no. 1, pp. 23–34, 2001.

[41] G. Hutvágner, J. McLachlan, A. E. Pasquinelli, E. Bálint, T. Tuschl, and P. D. Zamore, "A cellular function for the RNA-interference enzyme dicer in the maturation of the let-7 small temporal RNA," *Science*, vol. 293, no. 5531, pp. 834–838, 2001.

[42] R. C. Lee and V. Ambros, "An extensive class of small RNAs in Caenorhabditis elegans," *Science*, vol. 294, no. 5543, pp. 862–864, 2001.

[43] J. W. Pham, J. L. Pellino, Y. S. Lee, R. W. Carthew, and E. J. Sontheimer, "A Dicer-2-dependent 80S complex cleaves targeted mRNAs during RNAi in Drosophila," *Cell*, vol. 117, no. 1, pp. 83–94, 2004.

[44] J. Winter, S. Jung, S. Keller, R. I. Gregory, and S. Diederichs, "Many roads to maturity: microRNA biogenesis pathways and their regulation," *Nature Cell Biology*, vol. 11, no. 3, pp. 228–234, 2009.

[45] P. Bertone, V. Stolc, T. E. Royce, et al., "Global identification of human transcribed sequences with genome tiling arrays," *Science*, vol. 306, no. 5705, pp. 2242–2246, 2004.

[46] J. S. Pedersen, G. Bejerano, A. Siepel, et al., "Identification and classification of conserved RNA secondary structures in the human genome," *PLoS Computational Biology*, vol. 2, no. 4, article e33, 2006.

[47] P. Landgraf, M. Rusu, R. Sheridan, et al., "A mammalian microRNA expression atlas based on small RNA library sequencing," *Cell*, vol. 129, no. 7, pp. 1401–1414, 2007.

[48] R. Yelin, D. Dahary, R. Sorek, et al., "Widespread occurrence of antisense transcription in the human genome," *Nature Biotechnology*, vol. 21, no. 4, pp. 379–386, 2003.

[49] G. Lavorgna, D. Dahary, B. Lehner, R. Sorek, C. M. Sanderson, and G. Casari, "In search of antisense," *Trends in Biochemical Sciences*, vol. 29, no. 2, pp. 88–94, 2004.

[50] K. Okamura, J. W. Hagen, H. Duan, D. M. Tyler, and E. C. Lai, "The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila," *Cell*, vol. 130, no. 1, pp. 89–100, 2007.

[51] J. G. Ruby, C. H. Jan, and D. P. Bartel, "Intronic microRNA precursors that bypass Drosha processing," *Nature*, vol. 448, no. 7149, pp. 83–86, 2007.

[52] M. Lagos-Quintana, R. Rauhut, W. Lendeckel, and T. Tuschl, "Identification of novel genes coding for small expressed RNAs," *Science*, vol. 294, no. 5543, pp. 853–858, 2001.

[53] E. C. Lai, P. Tomancak, R. W. Williams, and G. M. Rubin, "Computational identification of Drosophila microRNA genes," *Genome Biology*, vol. 4, no. 7, article R42, 2003.

[54] Y. Altuvia, P. Landgraf, G. Lithwick, et al., "Clustering and conservation patterns of human microRNAs," *Nucleic Acids Research*, vol. 33, no. 8, pp. 2697–2706, 2005.

[55] S. Baskerville and D. P. Bartel, "Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes," *RNA*, vol. 11, no. 3, pp. 241–247, 2005.

[56] M. J. Weber, "New human and mouse microRNA genes found by homology search," *FEBS Journal*, vol. 272, no. 1, pp. 59–73, 2005.

[57] E. Berezikov, G. van Tetering, M. Verheul, et al., "Many novel mammalian microRNA candidates identified by extensive cloning and RAKE analysis," *Genome Research*, vol. 16, no. 10, pp. 1289–1298, 2006.

[58] S.-L. Lin, J. D. Miller, and S.-Y. Ying, "Intronic microRNA (miRNA)," *Journal of Biomedicine and Biotechnology*, vol. 2006, no. 4, p. 26818, 2006.

[59] Y.-K. Kim and V. N. Kim, "Processing of intronic microRNAs," *The EMBO Journal*, vol. 26, no. 3, pp. 775–783, 2007.

[60] Y. Liang, D. Ridzon, L. Wong, and C. Chen, "Characterization of microRNA expression profiles in normal human tissues," *BMC Genomics*, vol. 8, article 166, 2007.

[61] A. Sewer, N. Paul, P. Landgraf, et al., "Identification of clustered microRNAs using an ab initio prediction method," *BMC Bioinformatics*, vol. 6, article 267, 2005.

[62] H. Seitz, H. Royo, M.-L. Bortolin, S.-P. Lin, A. C. Ferguson-Smith, and J. Cavaillé, "A large imprinted microRNA gene cluster at the mouse Dlk1-Gtl2 domain," *Genome Research*, vol. 14, no. 9, pp. 1741–1748, 2004.

[63] B. Zhang, X. Pan, C. H. Cannon, G. P. Cobb, and T. A. Anderson, "Conservation and divergence of plant microRNA genes," *Plant Journal*, vol. 46, no. 2, pp. 243–259, 2006.

[64] U. Ohler, S. Yekta, L. P. Lim, D. P. Bartel, and C. B. Burge, "Patterns of flanking sequence conservation and a characteristic upstream motif for microRNA gene identification," *RNA*, vol. 10, no. 9, pp. 1309–1322, 2004.

[65] J. Lee, Z. Li, R. Brower-Sinning, and B. John, "Regulatory circuit of human microRNA biogenesis," *PLoS Computational Biology*, vol. 3, no. 4, article e67, 2007.

[66] X. Zhou, J. Ruan, G. Wang, and W. Zhang, "Characterization and identification of microRNA core promoters in four model species," *PLoS Computational Biology*, vol. 3, no. 3, article e37, 2007.

[67] B. Ason, D. K. Darnell, B. Wittbrodt, et al., "Differences in vertebrate microRNA expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 39, pp. 14385–14389, 2006.

[68] W. Filipowicz and V. Pogacic, "Biogenesis of small nucleolar ribonucleoproteins," *Current Opinion in Cell Biology*, vol. 14, no. 3, pp. 319–327, 2002.

[69] S. Vincenti, V. de Chiara, I. Bozzoni, and C. Presutti, "The position of yeast snoRNA-coding regions within host introns is essential for their biosynthesis and for efficient splicing of the host pre-mRNA," *RNA*, vol. 13, no. 1, pp. 138–150, 2007.

[70] E. van Rooij, L. B. Sutherland, X. Qi, J. A. Richardson, J. Hill, and E. N. Olson, "Control of stress-dependent cardiac growth and gene expression by a microRNA," *Science*, vol. 316, no. 5824, pp. 575–579, 2007.

[71] J. M. Pawlicki and J. A. Steitz, "Primary microRNA transcript retention at sites of transcription leads to enhanced microRNA production," *Journal of Cell Biology*, vol. 182, no. 1, pp. 61–76, 2008.

[72] J. M. Pawlicki and J. A. Steitz, "Subnuclear compartmentalization of transiently expressed polyadenylated pri-microRNAs: processing at transcription sites or accumulation in SC35 foci," *Cell Cycle*, vol. 8, no. 3, pp. 345–356, 2009.

[73] M. Morlando, M. Ballarino, N. Gromak, F. Pagano, I. Bozzoni, and N. J. Proudfoot, "Primary microRNA transcripts are processed co-transcriptionally," *Nature Structural and Molecular Biology*, vol. 15, no. 9, pp. 902–909, 2008.

[74] N. Shomron and C. Levy, "MicroRNA-biogenesis and pre-mRNA splicing crosstalk," *Journal of Biomedicine and Biotechnology*, vol. 2009, Article ID 594678, 6 pages, 2009.

[75] S. Butenas, R. F. Branda, C. Van't Veer, K. M. Cawthern, and K. G. Mann, "Platelets and phospholipids in tissue factor-initiated thrombin generation," *Thrombosis and Haemostasis*, vol. 86, no. 2, pp. 660–667, 2001.

[76] B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell*, vol. 120, no. 1, pp. 15–20, 2005.

[77] E. Morkin, "Control of cardiac myosin heavy chain gene expression," *Microscopy Research and Technique*, vol. 50, no. 6, pp. 522–531, 2000.

[78] D. Gaidatzis, E. van Nimwegen, J. Hausser, and M. Zavolan, "Inference of miRNA targets using evolutionary conservation and pathway analysis," *BMC Bioinformatics*, vol. 8, article 69, 2007.

[79] R. Revilla-i-Domingo and E. H. Davidson, "Developmental gene network analysis," *International Journal of Developmental Biology*, vol. 47, no. 7-8, pp. 695–703, 2003.

[80] L. F. Sempere, C. N. Cole, M. A. Mcpeek, and K. J. Peterson, "The phylogenetic distribution of metazoan microRNAs: insights into evolutionary complexity and constraint," *Journal of Experimental Zoology Part B*, vol. 306, no. 6, pp. 575–588, 2006.

[81] K. Chen and N. Rajewsky, "Natural selection on human microRNA binding sites inferred from SNP data," *Nature Genetics*, vol. 38, no. 12, pp. 1452–1456, 2006.

[82] M. A. Saunders, H. Liang, and W.-H. Li, "Human polymorphism at microRNAs and microRNA target sites," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 9, pp. 3300–3305, 2007.

[83] K. K.-H. Farh, A. Grimson, C. Jan, et al., "The widespread impact of mammalian microRNAs on mRNA repression and evolution," *Science*, vol. 310, no. 5755, pp. 1817–1821, 2005.

[84] R. C. Friedman, K. K.-H. Farh, C. B. Burge, and D. P. Bartel, "Most mammalian mRNAs are conserved targets of microRNAs," *Genome Research*, vol. 19, no. 1, pp. 92–105, 2009.

[85] J. L. Umbach and B. R. Cullen, "The role of RNAi and microRNAs in animal virus replication and antiviral immunity," *Genes & Development*, vol. 23, no. 10, pp. 1151–1164, 2009.

[86] B. R. Cullen, "Viral and cellular messenger RNA targets of viral microRNAs," *Nature*, vol. 457, no. 7228, pp. 421–425, 2009.

[87] B. John, A. J. Enright, A. Aravin, T. Tuschl, C. Sander, and D. S. Marks, "Human microRNA targets," *PLoS Biology*, vol. 2, no. 11, article e363, 2004.

[88] R. Shalgi, D. Lieber, M. Oren, and Y. Pilpel, "Global and local architecture of the mammalian microRNA-transcription factor regulatory network," *PLoS Computational Biology*, vol. 3, no. 7, article e131, 2007.

[89] S. Artzi, A. Kiezun, and N. Shomron, "miRNAminer: a tool for homologous microRNA gene search," *BMC Bioinformatics*, vol. 9, article 39, 2008.

[90] Q. Jing, S. Huang, S. Guth, et al., "Involvement of microRNA in AU-rich element-mediated mRNA instability," *Cell*, vol. 120, no. 5, pp. 623–634, 2005.

[91] C. A. Beelman and R. Parker, "Degradation of mRNA in eukaryotes," *Cell*, vol. 81, no. 2, pp. 179–183, 1995.

[92] R. Ashfield, P. Enriquez-Harris, and N. J. Proudfoot, "Transcriptional termination between the closely linked human complement genes C2 and factor B: common termination factor for C2 and c-myc?" *The EMBO Journal*, vol. 10, no. 13, pp. 4197–4207, 1991.

[93] M. Yonaha and N. J. Proudfoot, "Transcriptional termination and coupled polyadenylation in vitro," *The EMBO Journal*, vol. 19, no. 14, pp. 3770–3777, 2000.

[94] N. R. Smalheiser and V. I. Torvik, "Alu elements within human mRNAs are probable microRNA targets," *Trends in Genetics*, vol. 22, no. 10, pp. 532–536, 2006.

[95] K. Newcombe, T. Glassco, and C. Mueller, "Regulation of the DBP promoter by PAR proteins and in leukemic cells bearing an E2A/HLF translocation," *Biochemical and Biophysical Research Communications*, vol. 245, no. 2, pp. 633–639, 1998.

[96] M. Lapidot and Y. Pilpel, "Genome-wide natural antisense transcription: coupling its regulation to its different regulatory mechanisms," *EMBO Reports*, vol. 7, no. 12, pp. 1216–1222, 2006.

[97] L.-C. Li, S. T. Okino, H. Zhao, et al., "Small dsRNAs induce transcriptional activation in human cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 46, pp. 17337–17342, 2006.

[98] F. Saatcioglu, D. J. Perry, D. S. Pasco, and J. B. Fagan, "Multiple DNA-binding factors interact with overlapping specificities at the aryl hydrocarbon response element of the cytochrome P450IA1 gene," *Molecular and Cellular Biology*, vol. 10, no. 12, pp. 6408–6416, 1990.

[99] K. Chen and N. Rajewsky, "The evolution of gene regulation by transcription factors and microRNAs," *Nature Reviews Genetics*, vol. 8, no. 2, pp. 93–103, 2007.

[100] E. S. Lander, L. M. Linton, B. Birren, et al., "Initial sequencing and analysis of the human genome," *Nature*, vol. 409, no. 6822, pp. 860–921, 2001.

[101] J. F. Abelson, K. Y. Kwan, B. J. O'Roak, et al., "Sequence variants in *SLITRK1* are associated with Tourette's syndrome," *Science*, vol. 310, no. 5746, pp. 317–320, 2005.

[102] R. Durrett and D. Schmidt, "Waiting for regulatory sequences to appear," *Annals of Applied Probability*, vol. 17, no. 1, pp. 1–32, 2007.

[103] A. Stark, J. Brennecke, N. Bushati, R. B. Russell, and S. M. Cohen, "Animal microRNAs confer robustness to gene expression and have a significant impact on 3′ UTR evolution," *Cell*, vol. 123, no. 6, pp. 1133–1146, 2005.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 363145, 11 pages
doi:10.1155/2009/363145

Research Article

Interaction Map and Selection of microRNA Targets in Parkinson's Disease-Related Genes

Shinde Santosh P.,[1] Neelima Arora,[1] Pranjal Sarma,[2] Manika Pal-Bhadra,[2] and Utpal Bhadra[1]

[1] *Functional Genomics and Gene Silencing Group, Centre for Cellular and Molecular Biology, Hyderabad 500007, AP, India*
[2] *Centre for Chemical Biology, Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500007, AP, India*

Correspondence should be addressed to Utpal Bhadra, utpal@ccmb.res.in

Received 31 March 2009; Revised 31 July 2009; Accepted 11 August 2009

Recommended by Zhumur Ghosh

Parkinson's disease (PD) is a complex multigenic neurodisorder frequently occurring in elderly persons. To investigate noncoding tiny microRNA mediated gene regulation, miRanda (version 1.0b) was used to predict human miRNA target sites on selected 29 genes related to PD. To verify output generated from miRanda, a similar analysis was performed only for microRNA target sites in 3′UTR using TargetScan (version 5.1). Data extracted by miRanda elucidates the mode of microRNA action based on the location of target sites in the Parkinson genes. Sites prone to action of multiple miRNAs were identified as "hot spots." Important properties of each miRNA including multiplicity and cooperativity appear to contribute towards a complex interplay between miRNAs and their targets. Two sets of predicted results were explored for the occurrence of target sites of 112 miRNAs expressed in midbrain. Overall, convergence of results predicted by two algorithms revealed that 48 target sites for midbrain-specific miRNA occur in close proximity in 9 genes. This study will pave a way for selection of potential miRNA candidates for Parkinson's disease-related genes for quick therapeutic applications and diagnosis.

1. Introduction

Mysteries underlying the neurological disorders are as complex and bewildering as human mind itself. Aging related disorders such as Alzheimer and Parkinson's diseases are major culprits behind poor memory in elderly persons. Parkinson's disease has emerged as the second most common neurodegenerative disorder afflicting about 4 million people across the globe [1]. Steep rise in PD cases in aged population is quite distressing [2]. Though sporadic cases are more common yet in a significant fraction of western population, it can be attributed to delicate and fine tuning of gene regulation profile related to disorders [3]. PD is manifested as an outcome of interaction of copious genetic and environmental factors [4].

Parkinson's disease is characterized by motor impairments such as tremor of a limb usually restricted to one side of body. Concomitant symptoms including rigidity or stiffness of the limbs and trunk, akinesia, and impaired balance and postural instability [5–8] are often accompanied with depression to complete the clinical picture of PD. Onset of symptoms is the result of loss of neurons substantia nigra pars compacta causing a considerable decline in levels of Dopamine, a neurotransmitter. The hallmarks essential for PD diagnosis is the occurrence of eosinophilic proteinaceous inclusions, Lewy bodies in extant dopaminergic neurons. PD is incurable and various drugs prescribed for PD treatment offer merely symptomatic relief and contribute little to the halt of disease progression.

Since its description in 1817, very little was known about its etiology until recent days. The discovery of a series of genes involved in rare familial PD has instilled immense exhilaration and provided much needed impetus to research in this arena. There is mounting evidence that several genes like a-synuclein, Parkin, PTEN induced putative kinase 1 (PINK1), DJ-1, leucine-rich repeat kinase 2 (LRRK2), and ATP13A2 are misregulated in PD [9]. But whether these genes contribute in a common regulatory pathway or

multiple parallel subpathways converging to same sequence in molecular pathogenesis of PD is yet to be resolved. microRNAs, generally known as negative regulators of gene expression, have attracted a lot of attention in recent times for their possible role on fine tuning of disease related genes. miRNAs are known to regulate approximately 30% of genes in human genome [10]. There is escalating evidence regarding the involvement of the abundant and endogenous 21–23 nt long RNA in various neurodegenerative disorders. Elucidation of precise biological function of these miRNAs has been the subject of many studies. miRNAs are involved in cell differentiation, development, apoptosis [11], stress resistance [12], tumor formation [13], and more importantly in neurodegenerative disorders [11, 14–16]. The establishment of role of miR-133b in mammalian midbrain dopaminergic neurons (DNs) has spurred a new interest in studies of the prospective function of these miRNAs in Parkinson's disease [17]. It appears that level of several miRNAs (miR-10a, miR-10b, miR-212, miR-132, and 495) modulates genes related to PD considerably [18]. Earlier studies provide some evidences about the involvement of miRNAs in Parkinson disease [19] but do not offer a full comprehensive view of microRNA dependent regulation of PD genes. Availability of simple, rapid, and accurate computer-based methods and development of efficient algorithms for micro RNA prediction have generated a great deal of interest [20, 21]. Here, our analysis reveals a complex interplay between microRNA and Parkinson genes for understanding the mechanism of PD pathogenesis. In practice, conventional biochemical miRNA profile is often encountered with several problems including transient, and low level of microRNA expression, tissue specificity and complex interaction with targets [20, 22]. Computational prediction of miRNA target sites can readily predict the role of miRNAs in the regulatory pathways.

The multifarious relationships shared by genes related to PD pathway, regulation of various miRNAs by other miRNAs in response to indefinite cues impose the need of an interaction map. This study aims at developing a complex interaction map between genes and microRNAs in the PD pathways, which will provide readymade clues for selection of miRNA using a comprehensive view of overall interplay between genes and microRNAs. This interaction map unravels some unexpected complexity in searching of microRNA target selection.

2. Methods

In the current study, we emphasized on identification of fraction of miRNA specific to the PD genetic pathway and propose an interaction network between these genes and targeted miRNA. Figure 1 represents stepwise workflow undertaken for the study.

2.1. Selection of Genes. Parkinson's disease pathway (Figure 2) in Kyoto Encyclopedia of genes and Genomes (KEGG) available at http://www.genome.jp/kegg/ consists of 27 genes. Among them, genetic studies have identified few candidates such as parkin (PARK5, PARK6, PARK7, PARK8), alpha-syncline, NR4A2, synphilin-1, GBA, SNCA which, once mutated, can result Parkinson's disease like symptoms. Here, 2 more genes were also added to the PD network based on literature survey.

Genes selected are CASP3, CASP9, COX6B2, CYCS, GPR37, HTRA2, APAF1, UQCRFSL1, LOC100133737, LRRK2, NDUFS7, PARK2, PARK7, PTEN induced putative kinase1 (PINK1), SDHA, SEPT5, SLC6A3, SLC18A1, SLC25A4, SNCA, SNCAIP, TH, UBE1, UBE2J2, UBE2L3, ubiquitinB, UCHL1, GBA [23–25], NR4A2 [26]. Out of the 29 genes selected for this study, PINK1, PARK7, UBE2J2, GBA, and CASP9 are located on chromosome 1 while UCHL1, SNCA, CASP3, SLC25A4 are located on chromosome 4. Genes SDHA, SLC6A3, SNCAIP are present on chromosome 5. The position and location of each gene on the chromosomes is summarized (see Table S1 in Supplementary Material available online at doi:10.1155/2009/363145). Sequences for the selected genes were collected in Fasta format from NCBI.

Total 866 human miRNA were downloaded in Fasta format from miRBASE (http://microrna.sanger.ac.uk/sequences/).

For prediction of miRNA targets, miRanda (version 1.0b) employs dynamic programming based on sequence complementarity, allotting higher weights to matches at the 5′ end of the mature miRNA while considering the free energy of the RNA-RNA duplex (calculated using RNAFold) [27] and the extent of conservation of the miRNA target across related genomes. miRanda [28, 29] software version 1.0b available at http://www.microrna.org/miranda was employed to predict target sites for these miRNAs. Cut-off values for prediction of target sites selected in analysis were Gap Open Penalty: 2.0, Gap Extend: 8.00 match score (S) $\geq$150.00, duplex free energy (ΔG) = -25.00 kcal/mol, Scaling Parameter (w) = 3.00. The selected gene sequences and human miRNA sequences were used as reference and query sequences, respectively, as input to miRanda. Conservation criterion was not used as it is reported that the nonconserved target sites may also play a part in repression.

Output generated from miRanda was used for target site identification and subsequent analysis. Top 10 microRNAs were selected based on the highest threshold match scores. Multiplicity and cooperativity were determined for all the miRNAs. Positions of the target sites for these miRNAs on 29 genes were explored and sites prone to multiple miRNAs were identified. After assembling the data for all genes, top 10 miRNAs were selected based on high multiplicity and ClustalW provided in miRBase was employed to investigate the conservation pattern in these microRNAs. A complex interaction map of interplay among 29 genes and top 10 miRNAs which showed maximum number of interaction with Parkinson disease associated genes was constructed.

2.2. Physico-Chemical Properties of Top 10 miRNAs. Important physico-chemical properties of 10 miRNAs (based on multiplicity) such as molecular weight (Kilo Dalton), free energy (δG in Kcal/mol), and composition were calculated by

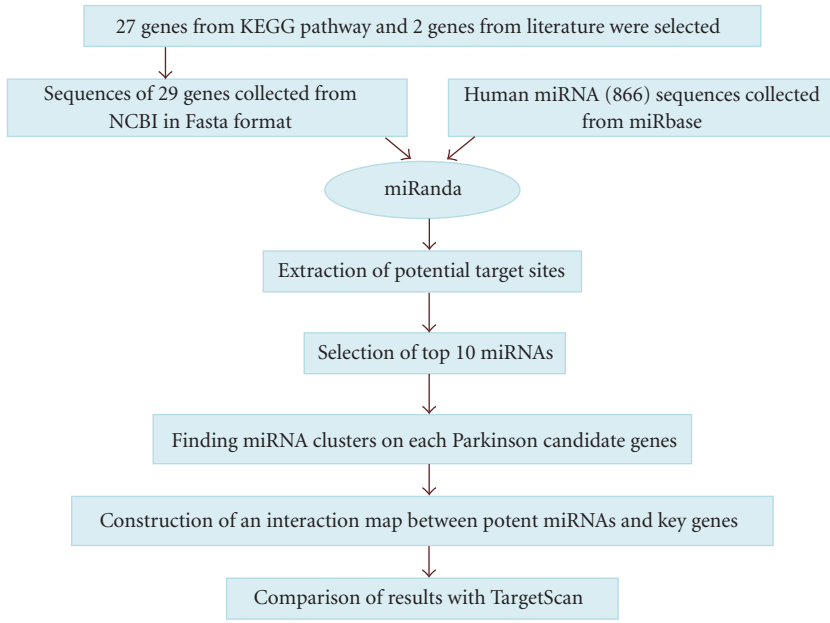


Figure 1: Schematic representation of the workflow.

employing Oligo Calc: Oligonucleotide Properties Calculator [30].

Molecular weight and free energy were calculated by the following formulae:

$$\text{Molecular weight } (Mw) = (A_n \text{x } 329.21) + (U_n \text{ x } 306.17) + (C_n \text{ x } 305.18) + (G_n \text{ x } 329.21) + 159.0, \quad (1)$$

where A_n, U_n, C_n and G_n are the number of each respective nucleotide of the RNA molecule under consideration. Additionally, weight 159.0 gm/mole was added that accounts to 5′triphosphat.

$$\text{Free energy } (\delta G) : \delta G = \text{RT } l_n \left[\frac{(\text{RNA} \cdot \text{template})}{(\text{RNA})(\text{template})}\right]. \quad (2)$$

Both (1) and (2) assume that the annealing occurs under the standard conditions of 50 nM primer, 50 mM Na^+, and pH 7.0.

2.3. Validation of miRNA Target Prediction Using Multiple Programmes. In order to verify target sites of microRNA predicted solely by miRanda, TargetScan (Release 5.1) (31) was employed for prediction of target sites. miRNA targets in 3′UTR that are common in two miRNA prediction algorithms and their comparative analysis was provided.

Information regarding specific microRNAs that are expressed in midbrain was collected from WALK database (http://web.bioinformatics.ic.ac.uk/MSc07/WALK/mirna.html) and whole data was screened for target sites of midbrain-specific miRNAs.

3. Results and Discussion

Complex interplay of genes and miRNA appears to be a key factor in determining the delicate balance of disease controlling genes expression. Prediction of miRNAs using computer-based methods serves many advantages and aid in recognition of molecular hallmarks of the disease that can lead to development of effective screens for miRNA targets.

However, prediction of microRNA targets might suffer from numerous problems including tissue specific expression and lack of validation. Such weakness of *in silico* studies can be partially compensated by predicting targets using multiple programmes. The outputs can be effectively utilized for development of a molecular marker in diagnosis and prognosis.

3.1. miRNA Target Sites in Parkinson's Disease. Total 5501 miRNA binding target sites were predicted for 29 genes selected and the dataset of 866 human miRNA used for the analysis.

Initially, top 20 miRNA targets were selected on basis of top scores and stringent parameters for each gene. These sites were distributed diversely in 5′untranslated regions; coding region and 3′untranslated regions (UTR) (Figure 3, Figure SF1-29, and Table S2 in Supplementary Material). Surprisingly, more target sites were predicted in CDS region as compared to 3′UTR and 5′UTR. It was found that target sites predicted for CASP3, COX6B2, CYCS, SEPT5, SLC6A3, UBE2J2, UBE2L3 genes were more in 3′UTR. In contrast, no target was predicted in 3′UTR for GPR37, PINK1, SLC25A4, UQCRFSL1, LOC100133737 genes. Till recently it was believed that microRNA can target all the regions including 5′UTR, CDS, and 3′ UTR in plants and are restricted to 3′UTR in animals. But there is growing

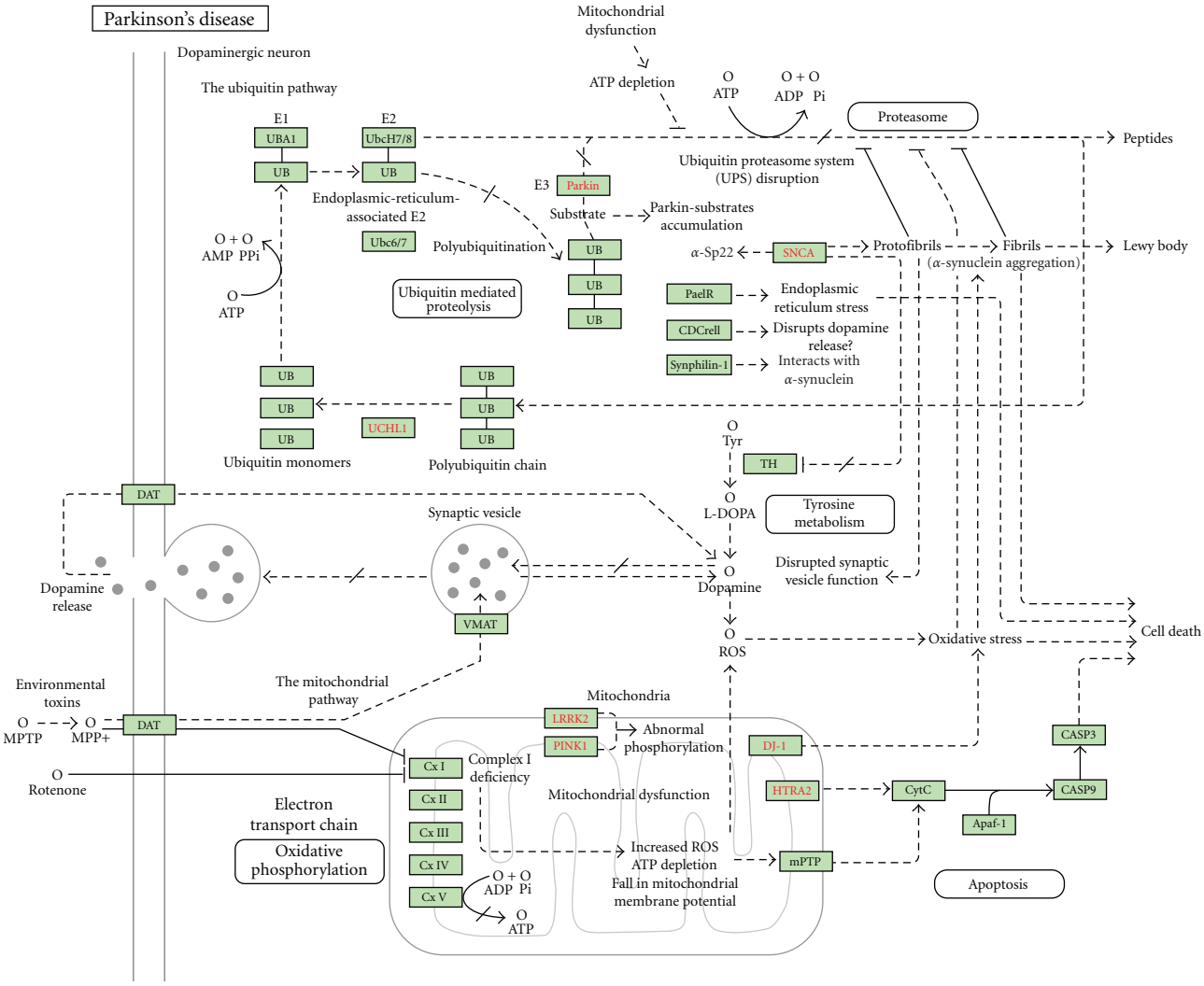


Figure 2: Pathway showing genes involved in Parkinson's disease (Courtesy: KEGG pathway).

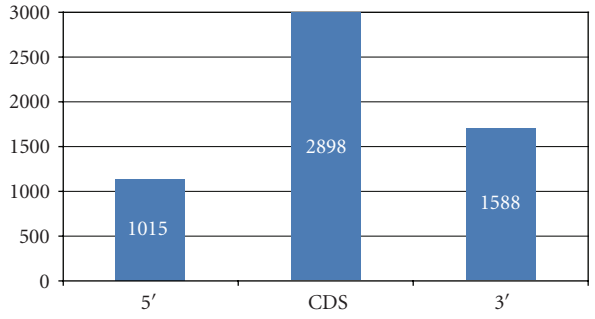


Figure 3: Distribution of predicted target sites in 5′UTR, CDS and 3′UTR as predicted using miRanda.

evidence that micro RNA can target the CDS [31–35] as well as 5′region [35, 36]. Location of target sites in a specified region also provides clues about mode of action of microRNAs, whether they are involved in transcriptional, posttranscriptional, and translational inhibition. Figure 4 shows the distribution pattern of miRNA target sites on the NDUFS7 genes.

3.2. Identification of Hotspots. Many microRNAs share either same target sites or sites located in vicinity of other microRNAs. Gathering of many miRNAs in the same site or vicinity area is commonly known as "hotspot." Usually, miRNAs that occupy the same spot are coregulated and coexpressed [37, 38] and are involved in important biological functions [39–41]. In contrast, it is reasonable to anticipate that sequences in hotspot region might be occupied only by a predominant microRNA among the cluster members and a microRNA having affinity towards such sites may outcast other microRNAs by competitive selection. But how such selection of single microRNA occurs is still unknown. This may provide a reasonable clue regarding the variable effectiveness of microRNA pools. Researchers employ different criteria for defining cluster, mostly ranging from the presence of more than two miRNAs in a same chromosomal sites [36] occurrence in same orientation,

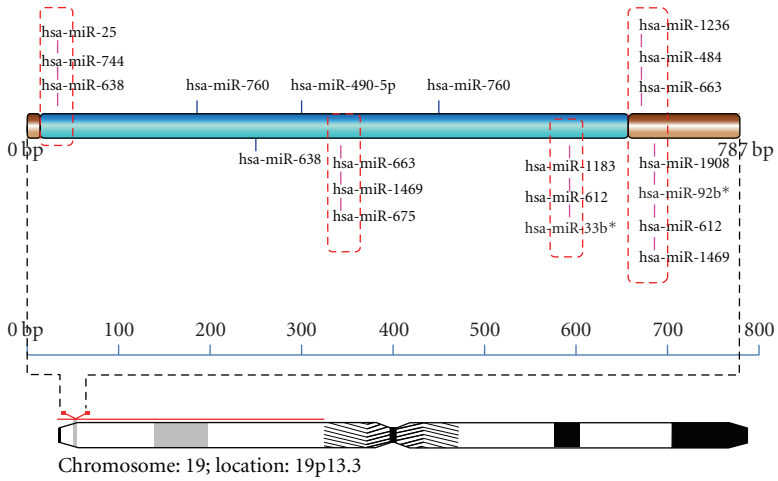
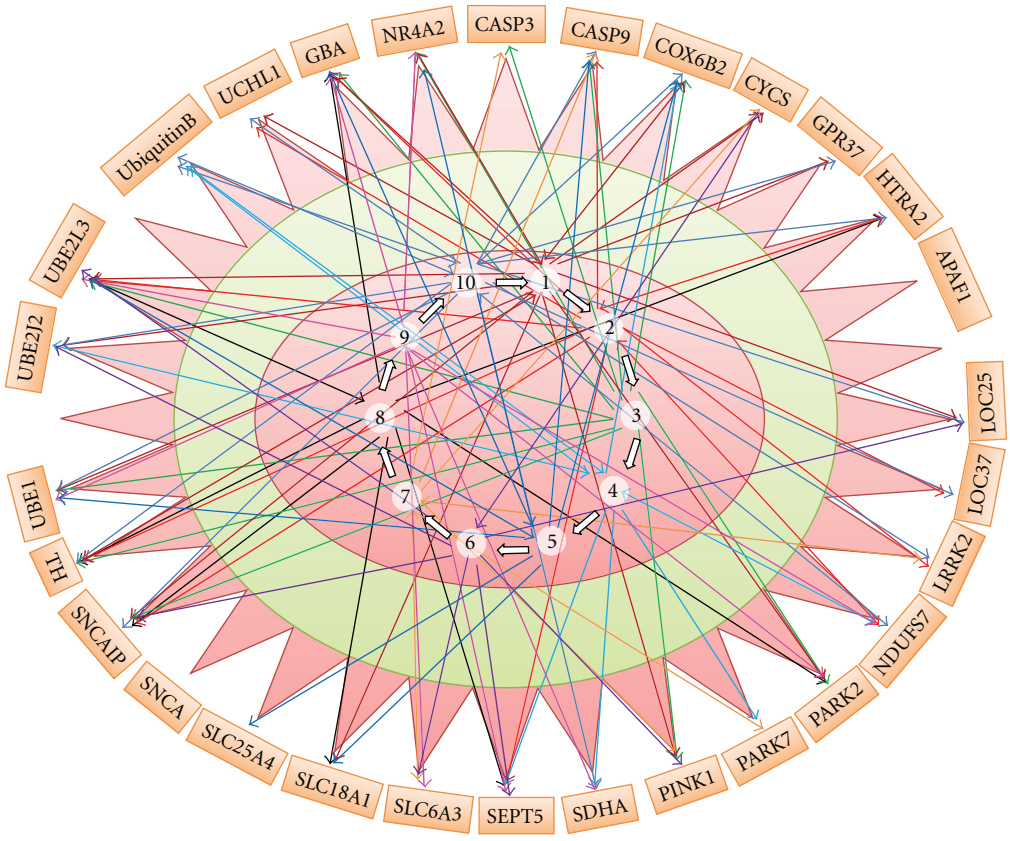


Figure 4: Schematic representation of miRNA targets on NDUFS7.



1: miR-1226*
2: miR-638
3: miR-608
4: miR-1469
5: miR-658
6: miR-939
7: miR-1301
8: miR-1207-5p
9: miR-1183
10: miR-612

Figure 5: Interaction map of miRNA and selected 29 genes. Interactions among genes and miRNAs are depicted with arrows.

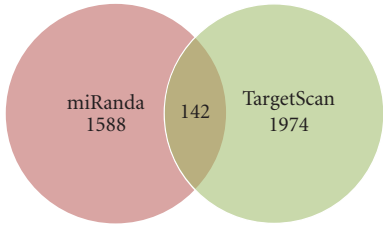


Figure 6: Intersection of miRanda and TargetScan.

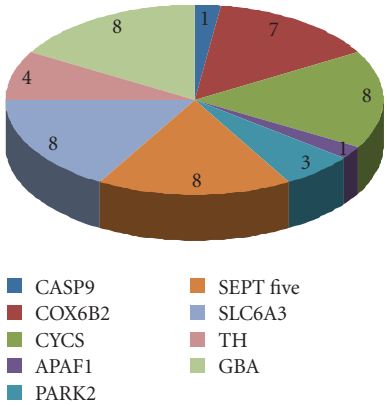


Figure 7: Common target sites predicted using miRanda and Targetscan, where datalabels show the number of target sites predicted for each gene.

absence of interfering transcription unit [42] or in some cases on the basis of phylogenetic relationships [37]. Keeping in view, the coannotation of these spots in important biological processes, 5501 miRNA targets were then further analyzed for identifying target prone regions in all Parkinson associated genes.

In the present study, we employed some preset criteria, which define a region as a hotspot if the region showed minimum 10 nucleotide overlap at starting position and possesses minimum overlapping five miRNA targets (see Supplementary Material). Total 288 regions were identified on all 29 genes spanned across 5′UTR, 3′UTR, and coding regions of the genes (Figure SF30-57 in supplement). Among all miRNA hotspots, 81, 77, and 130 regions were found in 5′UTR, 3′UTR, and coding region, respectively. Any region prone to multiple microRNA targeting could not be identified on CYCS and LOC100133737 genes. For all other genes, the number of hotspots varies between 1 and 26. It is anticipated that the genes that show the highest number of hotspots possibly undergo highest sensitivity in miRNA mediated regulation at transcriptional level because most of the sensitive sites reside in the 5′ regulatory domains of the genes. Genes such as PINK1, UBE2J2, SEPT5, and TH carried 26, 24, 23, 20 hotspots for miRNA action, respectively. The details of miRNA target prone sites against each gene are shown in Table S3. For example, in PINK1, 23 of the 26 sensitive target sites were found in the 5′UTR region, 3 regions are found in CDS only. Surprisingly, no hotspots could be identified in 3′UTR for PINK1. This revealed that PINK1 is not prone to translational inhibition. In reality, microRNA mediated transcriptional regulation is lacking broadly in animals, but frequent is plants. Abundance of predicted miRNA targets in the PINK1 regulatory regions depicts complexity of microRNA mediated target selection.

Conversely in UBE2J2, majority of such hotspots (19) were identified in 3′UTR while CDS region and 5′UTR showed only 1 and 4 hotspots, respectively. Depending on location of target sites, most likely UBE2J2 was regulated by the translational initiation. These results show that miRNAs can functionally target endogenous mRNAs in any region including coding region and 5′UTR and not restricted to the 3′UTR as described earlier. However, such dispersed distribution of miRNA target can generate more complexity in the nature of interaction, which counteracts the possibility of selection of most efficient miRNA required for functional knockdown of the PD genes.

3.3. Multiplicity and Cooperativity. In general term, one miRNA can target more than one gene (multiplicity), and one gene can be controlled by more than one miRNA (cooperativity) [29]. As reported earlier single miRNA can control hundreds of genes [43, 44]. Here, top 20 miRNAs displaying high scores were initially selected. After assembling all data, multiplicity and cooperativity were calculated. From the dataset, miRNAs that displayed maximum number of interactions with PD related genes were selected and used for constructing an interaction map. miRNAs showing maximum number of targets in PD related genes were selected as top 10 miRNA. Maximum interactions were found in 6 miRNAs, that is, hsa-miR-638, hsa-miR-1226*, hsa-miR-612, hsa-miR-612, hsa-miR1469, hsa-miR-608 and hsa-miR-939 that show high value of multiplicity. For instance, hsa-miR-638 exhibited high multiplicity showing total 119 interactions with 26 genes while no hsa-miR-638 target sites were found on three genes, namely, CASP3, PARK7, and SNCA. Defects in SNCA have been implicated in the pathogenesis of Parkinson's disease while autosomal recessive mutations in PARK7 cause early onset of Parkinson disease. CASP3 gene encodes a predominant caspase involved in cleavage of amyloid-beta 4A precursor protein, belonging to cysteine-aspartic acid protease (caspase) family which is associated with neuronal death in other neurological disorders. hsa-miR-1226* displayed 107 interactions with 25 genes but no complementary target sites could be identified for LOC100133737, PARK7, SNCA, ubiquitinB. Therefore, it can be inferred that these miRNAs may not be involved in regulation of activity of these genes.

Similarly, we analyzed the same dataset for estimating cooperativity. It was found that PINK1, SEPT5, TH exhibited high cooperativity towards top 10 miRNAs. All top 10 miRNA displayed 974 targets on the selected genes. It provides a complex picture which is difficult to comprehend the interactions. For example, PINK1 is regulated by 10 miRNAs at 81 positions, so these top 10 miRNAs demonstrate high cooperativity towards PINK1. Similarly, top 10 miRNAs exhibited 79, 78 targets in SEPT5 and TH genes. In contrast,

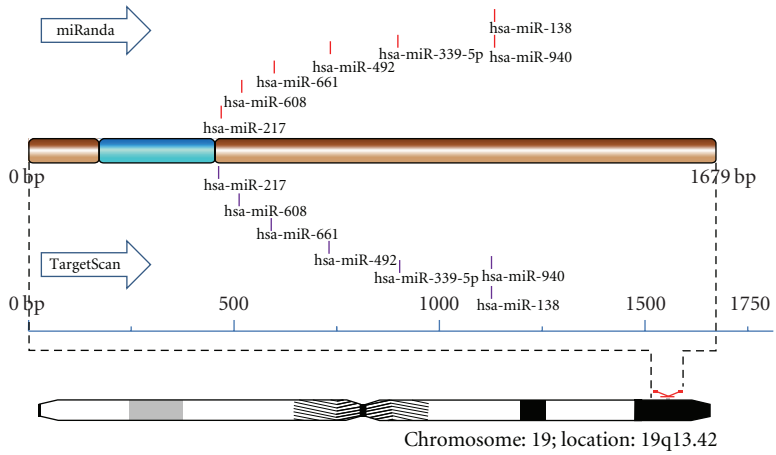


Figure 8: Schematic representation of miRNA targets on COX6B2.

Table 1: Multiplicity and cooperativity for all miRNA data.

miRNA/Genes	1-CASP3	2-CASP9	3-COX6B2	4-CYCS	5-GPR37	6-HTRA2	7-APAF1	8-LOC100128525	9-LOC100133737	10-LRRK2	11-NDUFS7	12-PARK2	13-PARK7	14-PINK1	15-SDHA	16-SEPT5	17-SLC6A3	18-SLC18A1	19-SLC25A4	20-SNCA	21-SNCAIP	22-TH	23-UBE1	24-UBE2J2	25-UBE2L3	26-ubiquitinB	27-UCHL1	28-GBA8	29-NR4A2	Total
hsa-miR-638	—	3	6	5	8	10	3	2	1	2	9	3	—	4	1	12	5	1	4	—	3	9	4	6	2	2	2	6	6	119
hsa-miR-1226*	4	3	7	3	1	4	1	2	—	3	5	1	—	7	3	4	7	5	3	—	2	10	2	5	4	—	2	9	10	107
hsa-miR-612	1	1	8	—	3	7	—	2	1	1	5	4	1	12	4	7	5	3	2	1	5	8	2	8	3	4	2	4	2	106
hsa-miR-1469	1	2	3	1	6	12	—	2	—	1	6	3	1	9	4	14	5	1	2	1	1	8	2	7	2	3	2	3	4	106
hsa-miR-939	—	4	7	6	2	4	—	3	—	2	1	2	—	5	2	1	7	5	3	1	8	10	6	3	5	—	1	5	6	99
hsa-miR-661	4	2	6	1	6	9	5	—	1	1	3	2	—	7	4	11	3	—	2	—	2	3	4	4	2	5	1	3	2	93
hsa-miR-1538	2	6	4	1	6	7	—	2	—	1	3	2	—	11	6	3	3	3	3	1	1	7	4	5	5	—	2	3	1	92
hsa-miR-663	3	2	3	5	5	6	1	2	—	1	7	3	—	7	2	10	3	1	2	—	2	8	4	3	2	—	2	4	2	90
hsa-miR-663b	1	1	3	—	4	—	—	1	—	—	5	2	1	11	4	12	7	—	4	—	2	9	2	6	2	—	2	3	4	86
hsa-miR-608	2	1	3	1	4	7	—	—	—	2	3	3	1	8	1	5	4	6	1	—	1	6	1	3	5	—	—	3	5	76
Total	18	25	50	23	45	66	10	16	3	14	47	25	4	81	31	79	49	25	26	4	27	78	31	50	32	14	16	43	42	974

a less degree of cooperativity was found in LOC100133737, SNCA genes suggesting a low sensitivity to, microRNA mediated regulation. Only three of 10 microRNA that is, hsa-miR-638, hsa-miR-612, and hsa-miR-661 exhibited miRNA targets on LOC10013373 (for details, see Table 1). Therefore, multiplicity and cooperativity add more complexity of microRNA selection and their sensitivity to large number of targets on PD genes.

Next, top 10 miRNAs were predicted based on the highest scores. hsa-miR-612 showed the maximum score 212. All the miRNAs having a score >=196 were selected and it was found that 6 miRNAs were common among both datasets, namely, miRNAs with the highest multiplicity and highest scores. Based on *in silico* prediction, we anticipated that these microRNAs may be master controller for regulating of PD pathway genes.

3.4. Unique miRNA Targets. microRNA targeting a single gene at single site can be termed as unique miRNA for a particular dataset and holds much importance, being specific towards a specific gene. Therefore, for targeting a specific gene these microRNAs might be useful, but single site targeting may not be sufficient to repress the activity of that gene to a desired level. It was also found that only 100 of 866 miRNAs displayed single interaction with Parkinson associated genes. Surprisingly, 3 out of 29 genes, SLC25A4, SNCAIP, and ubiquitin, did not exhibit targets for these unique miRNAs. They are not involved in single miRNA mediated regulation. One more gene UBE1 that encodes protein in the first step of ubiquitin conjugation to mark cellular proteins for degradation showed the highest no of single miRNA targets implying the need of the highest degree of specificity (Table S4 in Supplementary Material). These

Table 2: Common microRNA target sites for various genes predicted using miRanda and TargetScan.

Gene	microRNA	Start position of target sites predicted by miRanda	End position of target sites predicted by miRanda	Start position of target sites predicted by TargetScan	End position of target sites predicted by TargetScan
CASP9	hsa-miR-224	240	264	255	261
COX6B2	hsa-miR-138	676	701	693	699
	hsa-miR-217	6	36	26	32
	hsa-miR-339-5p	454	479	471	477
	hsa-miR-492	274	293	285	291
	hsa-miR-608	55	86	77	83
	hsa-miR-661	163	187	180	186
	hsa-miR-940	672	688	681	687
CYCS	hsa-miR-1301	1812	1840	1830	1836
	hsa-miR-25	2191	2214	2204	2210
	hsa-miR-510	2244	2273	2262	2268
	hsa-miR-591	1332	1352	1341	1347
	hsa-miR-658	2198	2232	2221	2227
	hsa-miR-663	2283	2304	2286	2292
	hsa-miR-769-5p	1943	1974	1964	1970
	hsa-miR-939	1990	2018	2007	2013
APAF1	hsa-miR-650	639	662	654	660
PARK2	hsa-miR-361-3p	21	40	31	37
	hsa-miR-574-5p	96	118	110	116
	hsa-miR-614	263	291	282	288
SEPT 5	hsa-miR-1207-5p	643	664	657	663
	hsa-miR-1250	671	696	687	693
	hsa-miR-1825	600	622	614	620
	hsa-miR-484	871	894	887	893
	hsa-miR-572	877	899	880	886
	hsa-miR-637	81	104	95	101
	hsa-miR-663	160	183	176	182
	hsa-miR-663b	74	93	86	92
SLC6A3	hsa-miR-1301	1613	1635	1626	1632
	hsa-miR-193b	1518	1541	1533	1539
	hsa-miR-331-3p	331	351	342	348
	hsa-miR-34a	684	706	698	704
	hsa-miR-449a	686	706	698	704
	hsa-miR-486-3p	324	347	337	343
	hsa-miR-601	406	432	423	429
	hsa-miR-637	883	912	883	889
TH	hsa-miR-34c-5p	211	240	230	236
	hsa-miR-658	217	245	236	242
	hsa-miR-885-3p	96	115	108	114
	hsa-miR-920	107	125	116	122
GBA	hsa-miR-1233	12	33	25	31
	hsa-miR-212	363	386	376	382
	hsa-miR-331-3p	109	133	124	130
	hsa-miR-331-3p	159	178	169	175
	hsa-miR-519e	4	26	18	24
	hsa-miR-637	197	223	215	221
	hsa-miR-661	452	476	467	473
	hsa-miR-766	160	183	175	181

results predict a coherent interplay between variation of microRNA target and functional efficacy of PD genes. It is possible that simple computational prediction of microRNA targets may shed some light on the behavior of target genes involved in Parkinson disease.

3.5. Deciphering Complexity through miRNA and Gene Interactions Map. To envisage the interrelationship of multiplicity of top 10 miRNA and their association with selected 29 genes, a gene-miRNA interaction map for Parkinson disease was constructed (Figure 5). Hsa-miR-612 showed highest number of interactions against 19 genes while four miRNAs hsa-miR-939, hsa-miR-1301, hsa-miR-1207-5p, hsa-miR-1183 showed interactions with only 9 genes. In contrast, counting the miRNA hits for individual gene, it was found that UBE2L3 exhibited 9 interactions with top scoring miRNAs whereas APAF1 and SNCA did not show any interaction with these miRNAs. These genes might not be under miRNA-based control. These findings suggest that computer-based prediction of microRNA target selection undoubtedly reduces the noise but is not sufficient to estimate efficiency of each microRNA on the multiple targets.

Next, several important physico-chemical properties such as molecular weight (MW), free energy (δG in Kcal/mole), and the sequence composition features for the top ten miRNAs (based on multiplicity) were calculated employing Oligo Calc: Oligonucleotide tool to identify any potential specificity related to Parkinson's disease. The size of the miRNAs varied between 22 and 26 nucleotides. A narrow range of difference in properties was found among top 10 miRNAs. All microRNAs displayed a high GC content with 2 miRNAs, namely, hsa-miR-1469 and hsa-miR-663 showing exceptionally high GC composition of 91% (Table S5 in supplement). GC and AU content % of the human miRNome as taken from sequence feature statistics of 782 microRNAs from Argonaute database [45] ranges from 19.05% to 95% and 5 to 80.95%, respectively. The difference in properties is not enough for preferential selection of one miRNA from the others.

Finally, to investigate the sequence conservation of microRNA, Multiple Sequence Alignment (MSA) available at miRBase (http://microrna.sanger.ac.uk/) was used. A clear predominance of *G*-nucleotide site at 20th position was observed in all the top 10 miRNAs and a strong bias towards *G* was also found at 8th, 12th, and 15th positions.

Intersection of miRanda and TargetScan prediction. In order to verify our above-mentioned findings depicted exclusively by miRanda, we have predicted only 3′UTR target sites using TargetScan [46] since prediction of TargetScan is limited to target sites only in 3′UTR. TargetScan relies on perfect seed complementarity and thus reduces false positive rate. Target sites predicted using miRanda and TargetScan were matched and compared (Figure 6 and Table S6).

Two genes LOC100128525 and LOC100133737 in Parkinson pathways were devoid of any predicted target sites in the 3′UTR regions as predicted using both the algorithms. Moreover, miRanda failed to predict target sites in PINK1 and SLC25A4 and resulted in prediction of target sites for only 25 genes while TargetScan predicted target sites for all remaining 27 genes. The number of targets sites predicted by two separate programmes is quite large as compared to common microRNA targets envisaged by both programmes. Target sites predicted based on only seed sequence similarity in TargetScan are greater than the 1588 sites predicted by miRanda. However, intersection includes a small number (142) of common target sites which is limited to only 12 genes (Table S7).

Using both algorithms, 7 microRNAs were predicted to have target sites in vicinity. Target sites for 7 microRNAs, namely, hsa-mir-138 (miRanda: 676–701, TargetScan: 693–699), hsa-miR-217 (miRanda: 6–36, TargetScan: 26–32), hsa-miR-339-5p (miRanda: 454–479,TargetScan: 471–477), hsa-miR-492 (miRanda: 274–293, TargetScan: 285–291), hsa-miR-608 (miRanda: 55–86, TargetScan: 77–83), hsa-miR-661 (miRanda: 163–187, TargetScan: 180–186), hsa-miR-940 (miRanda: 672–688, TargetScan: 681–687) were found in specified region. Similar results were found for other 8 genes and are summarized in Table 2.

Midbrain Specific microRNAs. The predicted microRNA targets were also screened for a subset of microRNA expressed in the midbrain. It comprises of 112 microRNAs. In all, 378 target sites were predicted for 60 midbrain-specific miRNAs for miRanda across the entire length of gene (Table S8). Among them, 343 target sites were predicted for 99 midbrain-specific microRNAs using TargetScan while only 115 target sites were identified for 43 midbrain-specific microRNAs using miRanda for 3′UTR (Tables S9 and S10 in Supplementary Material). Only 39 miRNAs were found to be common in both algorithms for which 105 and 136 target sites were predicted by miRanda and TargetScan, respectively. Only target sites for hsa-miR-138 in COX6B2 (miRanda: 676–701, TargetScan: 693–699) and hsa-miR-25 in CYCS (miRanda: 2191–2214, TargetScan: 2204–2210) were predicted to occur in close proximity. The variation in results can be attributed to difference in set parameters in two independent algorithms.

4. Conclusion

Neurological disorders are complex diseases in which a wealth of information remains hidden owing to a variety of regulatory complexity. Despite of numerous studies on Parkinson diseases, existence of multiple parallel pathways or their convergence at a point is still a puzzle. The previous studies have pointed out the role of miRNAs in the etiology of PD. Absence of efficient treatment methods tends us to broaden our understanding of the candidate genes involved in disease pathway. Knowledge of putative miRNA targets on these genes achieved using fast computer-based assays will be a reasonable and powerful advancement for understanding PD. Repression of candidate genes involved in pathogenesis by a single miRNA or a group of miRNAs may aid in combating this disease. In order to offer a simplistic view of perplexing relationship of gene and miRNAs, we propose an intricate and comprehensive microRNA-gene interaction map in the

PD pathway. For gaining an insight about the mechanism of these miRNA actions, distribution of these target sites at different regions was explored. Importantly, it was found that miRNA target sites are not restricted only to 3′UTR but are distributed across the entire length of gene. These hotspots may be representing favored sites for miRNA-based regulation. The miRNA and gene networks of a particular trait are poorly understood. Interaction map also provides a way for selecting important miRNAs markers required for diagnosis and therapeutics. Finally, though physiochemical properties of microRNA narrate some indicative parameters yet it is not enough to understand the complex relationship of Parkinson's disease genes and microRNAs. The weakness of the work is that as most of the computational miRNA prediction algorithms focus on 3′UTR and a number of miRNA in these search algorithms represent only a fraction of total miRNA available in microRNA repositories like miRBase, it is difficult to validate the target sites for those miRNAs and also in 5′UTR and CDS. Therefore, computational prediction may not guide optimize selection of any single microRNA for efficient knock down and level of action and further validation of these findings using experimental approaches is required. Further analysis and experimental validation of these results is mandatory for resolving complexity for the selection of microRNA targets in future.

Acknowledgments

This work was funded by grants from DBT and Wellcome Trust Senior International Fellowship to M. Pal-Bhadra and U. Bhadra, Human Frontier Science Program and DST CSIR Network (NWP 34, NWP35) projects to U. Bhadra. The authors thank anonymous reviewers for their critical and useful comments for improvement of manuscript.

References

[1] J. C. Fitzgerald and H. Plun-Favreau, "Emerging pathways in genetic Parkinson's disease: autosomal-recessive genes in Parkinson's disease—a common pathway?" *FEBS Journal*, vol. 275, no. 23, pp. 5758–5766, 2008.

[2] A. Wood-Kaczmar, S. Gandhi, and N. W. Wood, "Understanding the molecular causes of Parkinson's disease," *Trends in Molecular Medicine*, vol. 12, no. 11, pp. 521–528, 2006.

[3] C. A. Davie, "A review of Parkinson's disease," *British Medical Bulletin*, vol. 86, no. 1, pp. 109–127, 2008.

[4] B. Thomas and M. F. Beal, "Parkinson's disease," *Human Molecular Genetics*, vol. 16, no. 2, pp. 183–194, 2007.

[5] T. Gasser, "Genetics of Parkinson's disease," *Journal of Neurology*, vol. 248, no. 10, pp. 833–840, 2001.

[6] A. H. V. Schapira, E. Bezard, J. Brotchie, et al., "Novel pharmacological targets for the treatment of Parkinson's disease," *Nature Reviews Drug Discovery*, vol. 5, no. 10, pp. 845–854, 2006.

[7] M. R. Cookson, "The biochemistry of Parkinson's disease," *Annual Review of Biochemistry*, vol. 74, pp. 29–52, 2005.

[8] J. Jankovic, "Parkinson's disease: clinical features and diagnosis," *Journal of Neurology, Neurosurgery and Psychiatry*, vol. 79, no. 4, pp. 368–376, 2008.

[9] D. J. Moore, A. B. West, V. L. Dawson, and T. M. Dawson, "Molecular pathophysiology of Parkinson's disease," *Annual Review of Neuroscience*, vol. 28, pp. 57–87, 2005.

[10] N. Rajewsky, "microRNA target predictions in animals," *Nature Genetics*, vol. 38, supplement 1, pp. S8–S13, 2006.

[11] M. Garofalo, C. Quintavalle, G. Di Leva, et al., "MicroRNA signatures of TRAIL resistance in human non-small cell lung cancer," *Oncogene*, vol. 27, no. 27, pp. 3845–3855, 2008.

[12] S. N. Bhattacharyya, R. Habermacher, U. Martine, E. I. Closs, and W. Filipowicz, "Relief of microRNA-mediated translational repression in human cells subjected to stress," *Cell*, vol. 125, no. 6, pp. 1111–1124, 2006.

[13] G. A. Calin, C. D. Dumitru, M. Shimizu, et al., "Frequent deletions and downregulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia," *Proceedings of the National Academy of Sciences*, vol. 99, pp. 15524–15529, 2002.

[14] W. J. Lukiw, "Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus," *NeuroReport*, vol. 18, no. 3, pp. 297–300, 2007.

[15] R. Saba, C. D. Goodman, R. L. C. H. Huzarewich, C. Robertson, and S. A. Booth, "A miRNA signature of prion induced neurodegeneration," *PLoS ONE*, vol. 3, no. 11, article e3652, 2008.

[16] N. Bushati and S. M. Cohen, "microRNAs in neurodegeneration," *Current Opinion in Neurobiology*, vol. 18, no. 3, pp. 292–296, 2008.

[17] J. Kim, K. Inoue, J. Ishii, et al., "A microRNA feedback circuit in midbrain dopamine neurons," *Science*, vol. 317, no. 5842, pp. 1220–1224, 2007.

[18] F. Gillardon, M. Mack, W. Rist, et al., "MicroRNA and proteome expression profiling in early-symptomatic α-synuclein(A30P)-transgenic mice," *Proteomics-Clinical Applications*, vol. 2, no. 5, pp. 697–705, 2008.

[19] P. T. Nelson, W.-X. Wang, and B. W. Rajeev, "MicroRNAs (miRNAs) in neurodegenerative diseases," *Brain Pathology*, vol. 18, no. 1, pp. 130–138, 2008.

[20] I. Bentwich, "Prediction and validation of microRNAs and their targets," *FEBS Letters*, vol. 579, no. 26, pp. 5904–5910, 2005.

[21] J. R. Brown and P. Sanseau, "A computational view of microRNAs and their targets," *Drug Discovery Today*, vol. 10, no. 8, pp. 595–601, 2005.

[22] E. Berezikov, V. Guryev, J. van de Belt, E. Wienholds, R. H. A. Plasterk, and E. Cuppen, "Phylogenetic shadowing and computational identification of human microRNA genes," *Cell*, vol. 120, no. 1, pp. 21–24, 2005.

[23] J. Aharon-Peretz, H. Rosenbaum, and R. Gershoni-Baruch, "Mutations in the glucocerebrosidase gene and Parkinson's disease in Ashkenazi Jews," *The New England Journal of Medicine*, vol. 351, no. 19, pp. 1972–1977, 2004.

[24] Z. Gan-Or, N. Giladi, U. Rozovski, et al., "Genotype-phenotype correlations between GBA mutations and Parkinson's disease risk and onset," *Neurology*, vol. 70, no. 24, pp. 2277–2283, 2008.

[25] E.-K. Tan, J. Tong, S. Fook-Chong, et al., "Glucocerebrosidase mutations and risk of Parkinson's disease in Chinese patients," *Archives of Neurology*, vol. 64, no. 7, pp. 1056–1058, 2007.

[26] D. G. Healy, P. M. Abou-Sleiman, K. R. Ahmadi, et al., "NR4A2 genetic variation in sporadic Parkinson's disease: a gene wide approach," *Movement Disorders*, vol. 21, pp. 1960–1963, 2006.

[27] S. Wuchty, W. Fontana, I. L. Hofacker, and P. Schuster, "Complete suboptimal folding of RNA and the stability of

secondary structures," *Biopolymers*, vol. 49, no. 2, pp. 145–165, 1999.

[28] A. J. Enright, B. John, U. Gaul, T. Tuschl, C. Sander, and D. S. Marks, "MicroRNA targets in Drosophila," *Genome Biology*, vol. 5, no. 1, pp. R1–R12, 2003.

[29] B. John, A. J. Enright, A. Aravin, T. Tuschl, C. Sander, and D. S. Marks, "Human microRNA targets," *PLoS Biology*, vol. 2, no. 11, article e363, 2004.

[30] W. A. Kibbe, "OligoCalc: an online oligonucleotide properties calculator," *Nucleic Acids Research*, vol. 35, no. 2, pp. W43–W46, 2007.

[31] X. Zhou, X. Duan, J. Qian, and F. Li, "Abundant conserved microRNA target sites in the 5′-untranslated region and coding sequence," *Genetica*, vol. 137, no. 2, pp. 159–164, 2009.

[32] A. M. Duursma, M. Kedde, M. Schrier, C. le Sage, and R. Agami, "miR-148 targets human DNMT3b protein coding region," *RNA*, vol. 14, no. 5, pp. 872–877, 2008.

[33] J. J. Forman, A. Legesse-Miller, and H. A. Coller, "A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 39, pp. 14879–14884, 2008.

[34] A. Lal, H. H. Kim, K. Abdelmohsen, et al., "$p16^{INK4a}$ translation suppressed by miR-24," *PLoS ONE*, vol. 3, no. 3, article e1864, 2008.

[35] Y. Tay, J. Zhang, A. M. Thomson, B. Lim, and I. Rigoutsos, "MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation," *Nature*, vol. 455, no. 7216, pp. 1124–1128, 2008.

[36] C. L. Jopling, M. Yi, A. M. Lancaster, S. M. Lemon, and P. Sarnow, "Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA," *Science*, vol. 309, no. 5740, pp. 1577–1581, 2005.

[37] A. Tanzer and P. F. Stadler, "Molecular evolution of a microRNA cluster," *Journal of Molecular Biology*, vol. 339, no. 2, pp. 327–335, 2004.

[38] A. Tanzer and P. F. Stadler, "Evolution of microRNAs," *Methods in Molecular Biology*, vol. 342, pp. 35–350, 2006.

[39] Y. Hayashita, H. Osada, Y. Tatematsu, et al., "A polycistronic MicroRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation," *Cancer Research*, vol. 65, no. 21, pp. 9628–9632, 2005.

[40] M. A. Samols, J. Hu, R. L. Skalsky, and R. Renne, "Cloning and identification of a MicroRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus," *Journal of Virology*, vol. 79, no. 14, pp. 9301–9305, 2005.

[41] R. Zhang, Y.-Q. Wang, and B. Su, "Molecular evolution of a primate-specific microRNA family," *Molecular Biology and Evolution*, vol. 25, no. 7, pp. 1493–1502, 2008.

[42] J. R. Lytle, T. A. Yario, and J. A. Steitz, "Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 23, pp. 9667–9672, 2007.

[43] J. Brennecke, A. Stark, R. B. Russell, and S. M. Cohen, "Principles of microRNA-target recognition," *PLoS Biology*, vol. 3, no. 3, article e85, 2005.

[44] A. Krek, D. Grün, M. N. Poy, et al., "Combinatorial microRNA target predictions," *Nature Genetics*, vol. 37, no. 5, pp. 495–500, 2005.

[45] P. Shahi, S. Loukianiouk, A. Bohne-Lang, et al., "Argonaute—a database for gene regulation by mammalian microRNAs," *Nucleic Acids Research*, vol. 34, pp. D115–D118, 2006.

[46] B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell*, vol. 120, no. 1, pp. 15–20, 2005.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 634520, 11 pages
doi:10.1155/2009/634520

Review Article

TIS11 Family Proteins and Their Roles in Posttranscriptional Gene Regulation

Maria Baou,[1,2] Andrew Jewell,[3] and John J. Murphy[1]

[1] *Division of Immunology Infection and Inflammatory Disease, King's College London, London WC2R 2LS, UK*
[2] *MRC Toxicology Unit, University of Leicester, Leicester LE1 7RH, UK*
[3] *Faculty of Health and Social Care Sciences, Kingston University and St George's Hospital Medical School, London SW17 ORE, UK*

Correspondence should be addressed to John J. Murphy, john.murphy@kcl.ac.uk

Received 25 February 2009; Accepted 18 May 2009

Recommended by Bibekanand Mallick

Posttranscriptional regulation of gene expression of mRNAs containing adenine-uridine rich elements (AREs) in their 3′ untranslated regions is mediated by a number of different proteins that interact with these elements to either stabilise or destabilise them. The present review concerns the TPA-inducible sequence 11 (TIS11) protein family, a small family of proteins, that appears to interact with ARE-containing mRNAs and promote their degradation. This family of proteins has been extensively studied in the past decade. Studies have focussed on determining their biochemical functions, identifying their target mRNAs, and determining their roles in cell functions and diseases.

1. Introduction

Mechanisms of posttranscriptional gene regulation by micro-RNA (miRNA) and short interfering RNA (siRNA) have been highlighted in recent years as having important roles in control of a variety of developmental and functional processes. The present review concerns a small family of proteins, the 12-O-tetradecanoylphorbol-13-acetate (TPA) inducible sequence 11 (TIS11) family, which also function in posttranscriptional gene regulation and whose functions may overlap and interact with miRNA and siRNA control mechanisms. The TIS11 family consists of four mammalian members and include TIS11 (ZFP36, TTP, Nup475, GOS24), TIS11b (Berg36, ERF-1, ZFP36L1, BRF-1), and TIS11d (ZFP36L2, ERF-2, BRF-2). The fourth family member described in rodents, Zfp36l3, was expressed in mouse placenta, but was not detected in human placenta or other human tissues [1]. TIS11-like proteins have also been identified in *drosophila* and yeast [2–4]. These proteins contain two tandemly repeated zinc finger motifs through which they bind to adenine uridine (AU) rich elements (AREs) in mRNA and mediate ARE-mediated mRNA decay [5]. Table 1 lists the three human TIS11 family members, their chromosomal locations as well as reported mRNA targets. This family of proteins have been reported to promote deadenylation, decapping, and finally degradation of mRNAs by either exosome (3′-5′ degradation) or XRN1 exonuclease (5′-3′ degradation) [6].

AREs are found mostly at the 3′ end of many mRNAs encoding cytokines, transcription factors, cell cycle regulators, and apoptosis regulators. It is estimated that up to 8% of mRNAs may contain AREs [7]. AREs are subdivided into three classes: Class I, Class II, and Class III. Class II is further subdivided into Class IIA, IIB, IIC, IID, IIE [6]. Class I is characterised by the AUUUA motif and a U-rich region. Class III is characterised by a U-rich sequence found in, for example, the mRNA of c-jun. Class IIA contains five consecutive AUUUA pentamer sequences; Class IIB contains four consecutive AUUUA pentamers; Class IIC contains three consecutive AUUUA pentamers; Class IID contains two consecutive AUUUA pentamers, and finally Class IIE contains one AUUUA motif [6].

2. The TIS11 Family in Posttranscriptional Gene Regulation

One of the first indications that the prototype TIS11 protein functions as an AU binding protein came from work which showed that TIS11 inhibited TNFα production from

macrophages by destabilising its messenger RNA (mRNA) and this appeared to be due to direct binding of TIS11 to the TNFα ARE [8]. It was later shown that the optimal and minimally required RNA sequence for TIS11 binding is UUAUUUAUU [9–12]. The adenine residues and the spacing between them are critical in ensuring a stable association between the TIS11 peptide and RNA, even though TIS11 was still able to strongly bind to an AUUUUA peptide, and intermediately to AUUA and AUUUUUA peptides [9, 10]. TIS11 interaction with the RNA sequence is of relatively high affinity [10]. It should be noted that AREs are found at the 3′ end of each of the TIS11 family mRNAs suggesting that they may regulate themselves by a negative feedback loop [13, 14].

Overexpression of human TIS11 in HEK293 cells caused significant reduction in the levels of an artificial reporter mRNA containing part of the TNF-α 3′ untranslated region (3′ UTR), and this was dependent on the amount of TIS11 plasmid transfected [32]. Similar effects were seen for rat TIS11b or Xenopus TIS11d, although TIS11d was less efficient in inducing TNF-α reporter mRNA decay than TIS11 and TIS11b [32]. Two zinc finger motifs in TIS11 are necessary and sufficient for binding to the AREs, and also mediate TNF-α mRNA decay [32]. Similarly, other studies reported that TIS11 and other members of the TIS11 family can also mediate decay of mRNA for GM-CSF and IL-3 [15, 33]. Critical residues in the human TIS11 family zinc finger domains for binding to the TNF-α ARE probe or GM-CSF ARE probes were Cys^{124}, Cys^{147}, His^{128}, Cys^{162}, and His^{166}, and mutations of these residues to other amino acids completely abolished TIS11 binding [39]. In the same study it was shown that coexpression of wild type TIS11 and a Cys^{124R} nonbinding mutant resulted in stabilisation of artificial reporter TNF-α mRNA, even though wild type TIS11 was able to bind reporter TNF-α mRNA [39]. This finding suggested that the presence of a nonbinding mutant acts as a dominant negative over TIS11 destabilising activity, possibly by interacting with proteins that regulate TIS11 destabilising function [39].

TIS11-dependent degradation of mRNA requires deadenylation [40]. Deadenylation is strongly induced by TIS11 when two nonamers (UUAUUUAUU) are present in a sequence, whereas deadenylation and mRNA degradation by TIS11 in probes containing only one nonamer is much weaker [12]. The ability of TIS11 to promote deadenylation was dependent on the presence of Mg^{+2} and it was suggested that there is involvement of PolyA specific ribonuclease (PARN) in the process. It was shown that TIS11 requires PARN to promote deadenylation of an ARE containing mRNA probe [40]. Association between TIS11 and PARN is indirect rather than direct and other proteins may form the bridge between them. Alternatively, the presence of TIS11 may displace an ARE stabilising factor that inhibits deadenylation caused by PARN [40]. Another study using immunoprecipitation demonstrated that TIS11 does not associate directly with PARN [41]. It was also shown that TIS11 co-immunoprecipitates with hDcp1 and hDcp2 (decapping enzymes), hXrn1 (5′-3′ exonuclease), hCcr4 (deadenylase), hRrp4 (a component of the exosome), or hEdc3 (enhances the activity of the decapping enzymes) through the N-terminal domain, whereas the zinc fingers and the C-terminal domain are not involved in these interactions [41, 42]. Additionally, deletion of the N-terminal domain of TIS11 partially abrogated induction of mRNA decay by TIS11. It was therefore proposed that TIS11 is involved in deadenylation and degradation of mRNA by recruiting enzymes such as hDcp1, hDcp2 and hCcr4 through the N-terminal domain [41]. Other transacting elements may also be involved in TIS11 function recruited through the C-terminal domain, since deletion of this domain in both TIS11 and TIS11b resulted in stabilisation of a β-globin mRNA containing AREs [41]. One such element may be the exosome, a multicomponent complex with 3′-5′ exonuclease activity, and it was reported that TIS11 requires and recruits the exosome specifically in ARE containing mRNA probes, to mediate degradation of a deadenylated mRNA [43]. It was also shown that the exosome consists of 14 different components, among which are hRrp4, hRrp40, hRrp41, hRrp42, hRrp43, hRrp46, hCsl4, and hMtr3 [43]. It seems likely that TIS11 mediated mRNA degradation involves multiple enzymes including PARN, decapping enzymes such as hDcp1, hDcp2, hEdc3, exonucleases including the exosome, and Xrn1 (5′-3′ exonuclease) as well, to preferentially degrade ARE containing mRNAs [42, 43]. Figure 1 shows the pathways and major components of ARE mediated mRNA decay.

Reported mRNA targets of the TIS11 family include TNF-α in many cell types [14, 24, 44–47], GM-CSF [15], IL-3 [16], IL-2 [21], IL-6 [17], IL-10 [30], Ccl2 and Ccl3 [24], 1,4-galactosyltransferase [22], vascular endothelial growth factor (VEGF) [29], cyclooxygenase-2 [18], plasminogen activator inhibitor type 2 [19], paired-like homeodomain 2 (Pitx2) [20], E47 [28], polo-like kinase 3 [31], and TIS11 itself [14]. VEGF mRNA was also reported to be a target of TIS11b [35] although in TIS11b deficient fibroblasts VEGF levels were shown to be elevated as a consequence of increased translation rather than an effect on VEGF mRNA [37]. More recently, TIS11b has been reported to target and degrade steroid acute regulatory (STAR) protein mRNA [38]. Utilisation of siRNA inhibition for TIS11 revealed that it targets FOS and p21 in the THP-1 cell line and primary human monocytes [26] and IL-12 and MIP-2 in macrophages [23]. Similarly, TIS11 is involved in the regulation of c-myc and cyclin-D following treatment of glioblastoma and prostate cancer cell lines with rapamycin [25]. This suggests that TIS11 also regulates certain transcription factors and cell cycle regulators. A global search for novel mRNA targets of TIS11 identified 250 potential targets and characterised immediate early response gene 3 (Ier3) mRNA as a novel target [27]. Notably, in this study Ier3 was clearly shown not to be a target for TIS11b [27] and in a later study polo-like kinase 3 was shown also to be a target for TIS11 but not TIS11b or TIS11d [31]. These examples illustrate that mRNA targets of TIS11 family members may be different for different family members. In fact, there is growing awareness in the field that many in vitro assays designed to measure mRNA interaction and decay may identify "nonphysiological targets" and that "physiological targets" of TTP family members can best be determined by analysis of mRNA

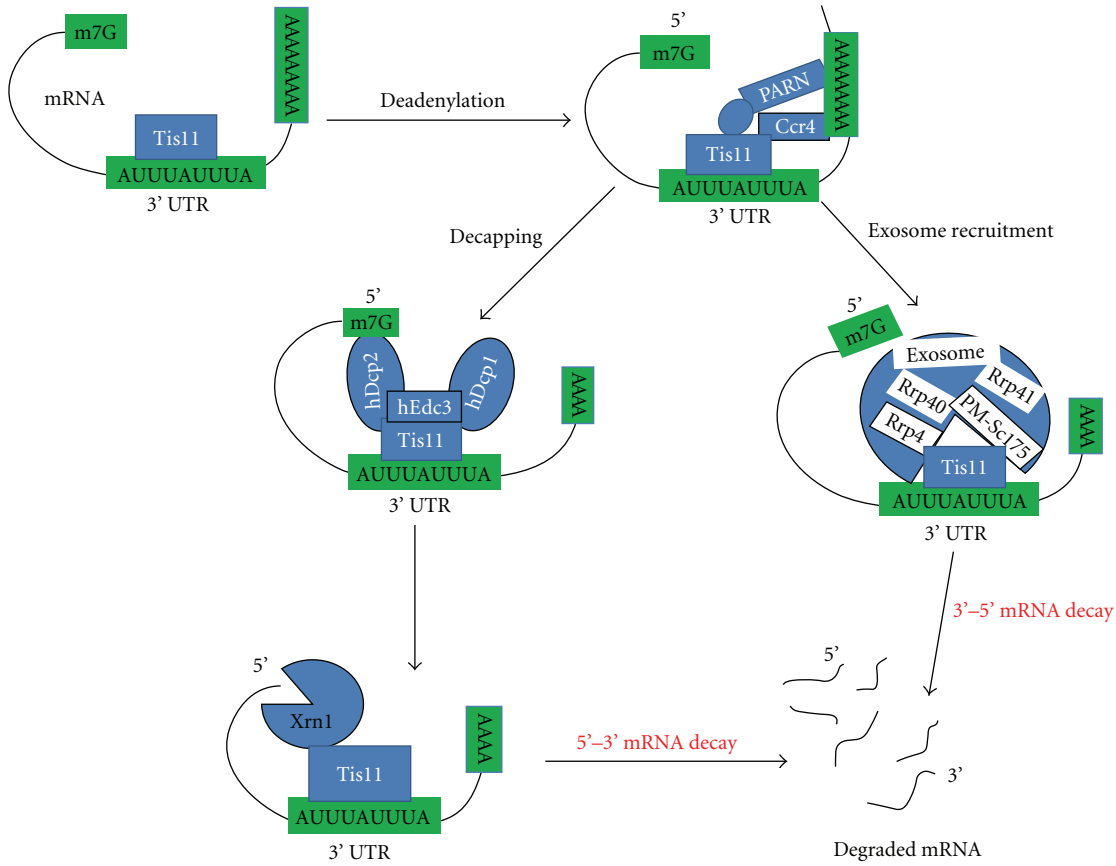


Figure 1: Pathways and major components of ARE-mediated mRNA decay by TIS11 proteins. TIS11 protein binds to the ARE sequence in the 3′ UTR and recruits deadenylases either directly (hCcr4) or indirectly (PARN). Deadenylated mRNA can be recruited by the exosome, a multiprotein structure containing proteins such as Rrp4, Rrp40, Rrp41, and PM-Scl75 that form the 3′ to 5′ exoribonuclease complex. Decapping of mRNA can follow deadenylation mediated by decapping enzymes such as Dcp1 and Dcp2 and degradation of mRNA can then be mediated by the 5′ to 3′ Xrn1 exonuclease.

degradation kinetics in knockout mice or cells derived from knockout mice [27]. Another more recent global analysis of TIS11 targets identified 137 potential TIS11 mRNA targets [30]. Interestingly, only a subset of the putative TIS11 targets contained the UUAUUUAUU sequence. This implies that either the RNA binding specificity of TIS11 proteins is not fully understood or that TIS11 proteins can be indirectly recruited to mRNA targets via other RNA-binding proteins, or that many false positives have been indentified in target screens. Table 1 summarises the reported mRNA targets for TIS11 family proteins and reported mechanisms.

A number of recent studies have reported links between TIS11 and miRNA functions and processes. Dicer, an enzyme required in the processing of miRNAs, was needed for TIS11 mediated degradation of TNFα ARE [48]. Furthermore, these studies revealed that miRNA16 can pair with eight bases in the TNFα ARE and this pairing is required for TIS11 mediated degradation of TNFα ARE [48]. TIS11 and miR16 do not bind directly to each other but interact through association with Argonaute/eiF2C family members [48]. Thus, TIS11 mediated mRNA degradation may require components of the miRNA processing pathway and indeed miRNA and TIS11 proteins may act codependently in mediating degradation. TIS11 was also identified as a component of the RNAi pathway by a genome wide screen in *drosophila melanogaster* [49]. *TIS11* genes contain many predicted targets for miRNA suggesting another level of complexity in the interrelationship between TIS11 family members and miRNAs [50]. A picture is therefore emerging of potentially complex interplay of different mechanisms of posttranscriptional gene regulation which include the TIS11 family.

3. Identification of Human TIS11 Family Members

Human *TIS11* is located on chromosome 19q13.1, and was isolated and cloned by several groups after stimulation of cells with serum, TPA, or insulin [51–53]. Human *TIS11b* is located on chromosome 14q22-24 and was isolated from chronic lymphocytic leukemia cells (CLL) after stimulation with TPA [54, 55], from human cDNA libraries probed with the rat cMG1 [13] or as a gene that reversed the phenotype of mutant H1080 cells which were unable to degrade IL-3 mRNA [34]. Finally human *TIS11d* is located on 2p22.3-p21 and was isolated from human cDNA libraries probed with the mouse TIS11d mRNA [56]. The sequence of each

Table 1: Human TIS11 family members and reported mRNA targets of the TIS11 family.

Gene	Alternative names	Chromosomal location	Reported mRNA targets	reference	mechanism
TIS11	*ZFP36, TTP, Nup475, GOS24*	19q13.1	**TNF***	[8]	mRNA stability
			GM-CSF	[15]	mRNA stability
			IL-3	[16]	mRNA stability
			IL-6	[17]	mRNA stability
			cyclooxygenase	[18]	mRNA stability
			PAI type 2	[19]	mRNA stability
			Pitx2	[20]	mRNA stability
			TIS11	[14]	mRNA stability
			IL-2	[21]	mRNA stability
			1,4galactosyltransferase	[22]	mRNA stability
			IL-12	[23]	?
			Ccl2	[24]	mRNA stability
			Ccl3	[24]	mRNA stability
			c-myc	[25]	mRNA stability
			cyclin D1	[25]	mRNA stability
			Fos	[26]	mRNA stability
			Ier3	[27]	mRNA stability
			Genome analysis 250 mRNAs	[27]	mRNA stability
			MIP-2	[23]	?
			p21	[26]	mRNA stability
			E47	[28]	mRNA stability
			VEGF	[29]	mRNA stability
			IL-10	[30]	mRNA stability
			Genome analysis 137 mRNAs	[30]	mRNA stability
			polo-like kinase 3	[31]	mRNA stability
TIS11b	*ZFP36L1, Berg36, ERF-1, BRF-1*	14q22-24	TNF	[32]	mRNA stability
			GMCSF	[33]	mRNA stability
			IL-3	[34]	mRNA stability
			VEGF	[35]	mRNA stability
			c-IAP2	[36]	mRNA stability
			VEGF	[37]	translation
			STAR	[38]	mRNA stability
TIS11d	*ZFP36L2, ERF-2, BRF-2*	2p22.3-p21	TNF	[32]	mRNA stability
			GM-CSF	[33]	mRNA stability
			IL-3	[33]	mRNA stability

* Targets in bold confirmed in cells derived from knockout animals are so-called "physiological" targets.

of the three human genes shows little variation between individuals, and most polymorphisms are detected in the introns, promoter, and 3′ UTR regions [57]. Thirteen polymorphisms were detected in the protein-coding regions of the three *TIS11* family genes and six of these would result in amino acid changes. In *TIS11b* a polymorphism generating a dinucleotide substitution was detected that would prevent splicing of the single intron of *TIS11b* but to date the significance of this is unknown [57]. Further polymorphisms of *TIS11* were identified in a more recent study and one, an C to T transition in the protein coding domain of *TIS11*, was significantly associated with rheumatoid arthritis in African-Americans [58].

TIS11, TIS11b, and TIS11d have all been associated with induction of mRNA decay (see Table 1), and even though redundancy in their function has been suggested, studies with knock-out mice and analysis of tissue distribution suggest that they may have cell specific regulation and functions. TIS11 knock-out mice appear normal at birth but later develop severe cachexia, autoimmunity, patchy alopecia, arthritis, and conjunctivitis, which can be reversed by administration of anti-TNF-α antibody [44]. TIS11b knock-out mice do not survive to birth but die in utero between days E8 and E13, due either to failure in chorioallontoic fusion [59], or to defective extraembryonic vasculogenesis [37]. Mice in which a truncated form of TIS11d was generated, which left the RNA binding domain and the nuclear export signal intact [60], appeared normal at birth, but females were infertile. Expression of the TIS11 family in mouse tissues has shown that the TIS11 protein is highly expressed in spleen,

thymus, lung, large intestine, and liver, weakly expressed in brain and pancreas but is not expressed at all in the testis and uterus [61]. TIS11b is highly expressed in kidneys, liver, lung, pancreas, and heart, and weakly in skeletal muscle, colon, thymus, spleen, small intestine, brain, and peripheral blood leukocytes [62]. TIS11d is highly expressed in lung, liver, skeletal muscle, kidneys, pancreas, placenta, and less strongly in heart and brain [63].

4. Structure of TIS11 Family Members

The structures of the first zinc finger motif of TIS11 [64] and human TIS11d before and after binding to an ARE probe have been reported [65]. The spacing between the cysteines and histidine in the zinc fingers ($CX_8CX_5CX_3H$) and the length of the linker between the zinc finger motifs are conserved between TIS11, TIS11b, and TIS11d. Because of the similarity of the zinc finger motifs between the three family members, it is expected that the model of mRNA binding to TIS11d will apply to all family members. The first zinc finger motif of TIS11 was found to be very different in comparison with other metal and zinc binding core domains, in terms of diameter and thickness [64]. For human TIS11d each of the two zinc fingers is folded independently into a compact domain connected by a linker sequence [65]. The structure is stabilised by binding of zinc to side chains of Cys^{159}, Cys^{168}, Cys^{174}, His^{178} for the first zinc finger (ZF1) motif and Cys^{197}, Cys^{206}, Cys^{212}, His^{216} for the second zinc finger (ZF2). Hydrogen bonds and van der Waals forces are involved in further stabilisation of this structure [65]. The orientation of the fingers is fixed, because the linker region has a relatively rigid structure stabilised by hydrogen bonds at each end and has little flexibility apart from the central region (residues 186-189) [65]. TNF-α ARE was used as a probe in this study, with the sequence 5′-UUAUUUAUU-3′. Zinc finger 1 (Arg^{153}-Phe^{180}) interacted with a single UAUU subsite on the 3′ end while zinc finger 2 (Lys^{191}-Ala^{218}) interacted with the same subsite on the 5′ end.

5. Regulation of the TIS11 Family at the mRNA Level

TIS11 family mRNAs are induced by TPA in a wide variety of cells including CLL cells, mouse fibroblasts, human epithelial cells, and PC phaeochromocytoma cells [53, 54]. Insulin can induce TIS11 in NIH3T3 mouse fibroblasts [66] but in RIE-1 cells only TIS11b is up regulated by insulin while the other two family members remain unaffected [67]. Lipopolysaccharide (LPS) induces TIS11 as early as 30 minutes poststimulation in murine macrophage RAW264.7 cells or human macrophages (THP-1) [14, 68, 69]. TIS11 was also induced by LPS in primary human white blood cells including lymphocytes, monocytes, and neutrophils [70]. TIS11 can be induced by GM-CSF in myeloid cells [71] and by TGF-β in a T-cell line [72] or a combination of TPA and ionomycin in T lymphocytes [73]. TIS11 can be induced by anisomycin (a p38 activator) and further induced by a combination of anisomycin plus IFN-γ or IFN-β as early as 1 hour poststimulation in mouse embryonic fibroblasts [24]. In the same study it was shown that addition of IFN-γ to LPS treated primary mouse bone marrow macrophages further increased induction of TIS11 mRNA in comparison with LPS treated cells [24]. Glucocorticoids have also been reported to induce TIS11 mRNA and protein in lung epithelial cells and this induction may well be important for glucocorticoid mediated control of inflammatory gene expression [74, 75]. The second family member, TIS11b, has been shown to be induced by TPA, bryostatin, and rituximab in CLL cells [54, 76, 77], calcium ionophore, anti-IgM and anti-CD20 in Burkitt's lymphoma cell lines [55, 78], insulin in RIE-1 cells [67] parathyroid hormone in human primary osteoblasts [79] or adrenocorticotropic hormone (ACTH) hormone in adrenocortical cells [80].

An important consideration is that the kinetics of induction of the mRNA for TIS11 genes appears to be dependent on the type of stimulus and cell type. For example, TIS11 mRNA is evident at 15 minutes after insulin or serum stimulation in mouse fibroblasts returning to almost baseline levels at two hours [66, 81]. In RAW264.7 cells, TIS11 mRNA induction by LPS is biphasic with the first peak at one hour and the second peak at five hours poststimulation [69]. It has been shown that both phases of the biphasic TIS11 mRNA induction after LPS treatment in RAW264.7 are regulated through the p38 pathway at the mRNA level [69] and p38 and ERK pathway at the protein level [82]. Similarly TIS11b peaks at two hours poststimulation and is clearly above basal levels at four hours after stimulation in insulin treated RIE-1 cells [67] but in ACTH treated human adrenocortical cells, peak induction of the gene occurs at three hours poststimulation and is above the basal levels for up to 24 hours poststimulation [80]. TPA stimulation of CLL cells induced TIS11b more strongly and earlier than bryostatin [76] and in the BL60-2 Burkitts lymphoma cell line, anti-IgM induced TIS11b more strongly than anti-CD20 stimulation [78]. H7 (a broad specificity inhibitor for the PKC and PKA pathways) did not have any effect on induction of TIS11b mRNA by TPA or bryostatin in CLL cells [76]. Similarly, induction of TIS11b in RIE-1 cell line by insulin was unaffected by PKC inhibition, but inhibition of PI3K pathway by wortmannin partially abrogated induction, whereas the same PI3K inhibitor had no effect on induction of TIS11b by TPA [67].

6. Regulation of the TIS11 Family at the Protein Level

The TIS11 family is also regulated at the protein level through either phosphorylation and/or localisation in different cellular compartments.

6.1. Phosphorylation. The TIS11 protein was suggested to be phosphorylated since it was noticed that stimulation of the NIH3T3 cell line with various stimuli such as serum, TPA, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) for 2 hours, induced TIS11 protein detected as 45 kDa protein and not as the predicted 33 kDa protein [83]. In this particular study, phosphorylation of TIS11 was shown to be partially regulated by the p42 mitogen activated protein

kinase (MAPK) pathway, which phosphorylates Ser^{220} in mouse TIS11 [83]. In another study in LPS stimulated murine macrophages, TIS11 protein was evident at 75 minutes poststimulation as a 36 kDa protein, and increased in size over time up to 45 kDa at 240 minutes poststimulation [84]. TIS11 protein is a substrate for MAPK p38α and p38β isoforms which probably control multiple phosphorylation events [84]. TIS11 phosphorylation was also reported to be mediated by the p38-regulated MAPK activated protein kinase 2 (MK2) [68]. Ser^{52}, Ser^{178}, Thr^{249}, Thr^{250}, Ser^{264}, $Ser^{80/82}$ were suggested to be the sites of phosphorylation by MK2 in murine TIS11 and Ser^{264} and Thr^{250} were suggested to be regulated by the p38a pathway [85]. Addition of a p38 inhibitor to RAW 264.7 cells previously stimulated by LPS for 2 hours resulted in rapid TIS11 dephosphorylation and degradation [82]. TIS11 phosphorylation is therefore in equilibrium with dephosphorylation probably mediated by protein phosphatase 2A [86]. Major phosphorylation sites responsible for murine TIS11 stabilisation were found to be Ser^{52} and Ser^{178} which were phosphorylated by MK2 [82, 87].

The importance of Ser^{52} phosphorylation for the cytoplasmic localisation of murine TIS11 was demonstrated in a study which also suggested that Ser^{178} is important for this localisation [82]. Interestingly, Ser^{178} is also present in murine TIS11b and phosphorylation of this site creates a binding site for the 14-3-3 β isoform [85]. This is not the only isoform of 14-3-3 that the TIS11 family can bind to and it has been shown by a yeast two hybrid system that all TIS11 family members bind equally well to 14-3-3 η and β isoforms [88]. A study using MudPIT or MALDI/MS methods showed that the major phosphorylation sites in human TIS11 when purified from HEK293 cells were Ser^{66}, Ser^{88}, Thr^{92}, Ser^{169}, Ser^{186}, Ser^{197}, Ser^{218}, Ser^{228}, Ser^{276}, Ser^{296} [89]. Ser^{197}, Ser^{218}, and Ser^{228} were predicted to be potential sites for protein kinase A, glycogen synthase kinase-3, and extracellular-signal-regulated kinase 1 (both Ser^{218} and Ser^{228}), respectively [89].

Phosphorylation of TIS11 may alter its function as a destabilizing AU binding protein. One study showed that transfection of cells with TIS11 and MKK6, which activates p38, resulted in phosphorylation of TIS11 protein but this did not affect TIS11 ability to induce mRNA decay [69]. Another study using a series of serine or threonine mutants, alone or in combination, showed that these phosphorylation events did not alter the function of TIS11 in terms of TNF-α induced destabilisation or mRNA binding and deadenylation [47]. A further study showed that cotransfection of HEK293 cells with TIS11 and MKK6(E) dominant active MKK6 resulted in a slight increase in the half life of a TNF-α construct in comparison with cells transfected with TIS11 alone, suggesting some deactivation or inhibition of TIS11 function following phosphorylation [84]. Furthermore, it was reported that phosphorylation of murine TIS11 especially at Ser^{178} inhibited its binding ability to TNF-α ARE and thus that the unphosphorylated TIS11 is active in mRNA degradation [87]. It was proposed that reduced binding activity of phosphorylated murine TIS11 could be due to competitive binding with 14-3-3 proteins because phosphorylation of Ser^{52} and Ser^{178} has been reported to induce binding to 14-3-3 proteins [87]. More recently, it was reported that phosphorylation of human TIS11b did not affect its ability to bind to AREs and recruit RNA degradation enzymes but did nonetheless inhibit its ability to degrade ARE-containing mRNA [90]. Finally, another study involving human TIS11b, showed that phosphorylation of TIS11b by protein kinase B at Ser^{92} abrogated mRNA decay of an IL-3 ARE containing probe induced by overexpression of TIS11b [91].

6.2. Subcellular Localisation of TIS11 Family Members. In many cell types, TIS11 is expressed at very low/undetectable levels in the nucleus and cytoplasm of unstimulated cells and is almost entirely expressed in the cytoplasm of stimulated cells [82]. However, there was one early report showing that TIS11 is nuclear and remains nuclear after stimulation with serum [51]. Another exception appears to be human umbilical vein endothelial cells (HUVEC) in which TIS11 is cytoplasmic in resting HUVEC and becomes nuclear after stimulation with TNF-α [22]. Nuclear to cytoplasmic shuttling of murine Tis11 has been shown to be regulated through induction of phosphorylation at Ser^{52} and Ser^{178}, primarily by the p38 pathway [82]. Hypophosphorylated forms of TIS11 were found to be nuclear and highly susceptible to proteasomal degradation [82].

Murine TIS11 has a nuclear export signal (NES) between amino acids 1–15 [92]. For murine TIS11b B the NES has been mapped between amino acids 305–313 and for murine TIS11d between amino acids 471–479 [92]. In a separate study, the rat TIS11 nuclear localisation signal (NLS) was mapped to the region containing the tandem repeats of the zinc finger motif (amino acids 88–161), and Arg^{127} and Arg^{131}, which are located in the linker region between the two zinc finger motifs, were shown to be critical residues whereas Leu^{3}, Iso^{116} and Leu^{10} were critical for nuclear export [93]. It has been shown by a yeast two hybrid system that TIS11 specifically associates with nucleoporin/Nup214 which is part of the nuclear pore on the cytoplasmic face of the pore complex [94] and this protein probably associates with the linker region between the zinc finger motifs to mediate nuclear localisation of TIS11. On the other hand nuclear export seems to be regulated through CRM1 since inhibition of CRM1 resulted in nuclear accumulation of TIS11 [93].

When cells are stimulated with a strong stress signal such as heat shock, TIS11 co-localises with TIA-1 in stress granules [95]. Localisation of TIS11 in stress granules also occurs when cells are treated with FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydrazone, a mitochondrial inhibitor), and oxidative stress but MK2 phosphorylation of TIS11 promotes its complex formation with 14-3-3 protein and excludes it from stress granules [96, 97]. Both zinc fingers of rat TIS11 are absolutely necessary for localisation in stress granules, especially amino acids Tyr^{105}, Tyr^{113}, Phe^{119}, Gly^{109}, and Gly^{114} [95]. On the contrary it was shown that arsenite treatment excludes TIS11 from stress granules through MK2 mediated or p38 and c-jun N-terminal kinase (JNK) mediated phosphorylation [47, 96]. TIS11 and TIS11b also localise to processing bodies (P-bodies), which are cytoplasmic foci of mRNAs and enzymes that have been proposed as

sites of mRNA decay and translational silencing [42, 97, 98]. TIS11 and TIS11b appear to deliver ARE containing mRNAs to processing bodies for translational silencing and mRNA decay [98]. Overexpression of TIS11 and TIS11b resulted in stable association between stress granules and processing bodies suggesting that the TIS11 family plays an active role in tethering stress granules to processing bodies [97]. Stress granules are considered sites where mRNA is sorted for storage, reinitiation of translation or degradation [97, 99]. Interestingly, formation of processing bodies is absolutely necessary for RNA interference activity and translation arrest or mRNA degradation through siRNA and miRNA [100]. Thus, TIS11 family and miRNA posttranscriptional functions take place in the same subcellular location as well as possibly being interdependent and sharing mRNA degradation machinery [48].

7. TIS11 Family in Cell Functions and Disease

Overexpression of TIS11 family members induces apoptosis in a variety of cell lines including HeLa, U20S, SAOS2, and 3T3 [101, 102] and B-cell lymphoma cells [77] cells. Induction of apoptosis by all three TIS11 family members was completely abrogated in the presence of Bcl-2 or CrmA [101]. TIS11 synergistically induced apoptosis with TNF-α in 3T3 cells and the zinc fingers and the N-terminal domain of TIS11 are absolutely critical for this effect [102]. Mutant TIS11 lacking the zinc finger motifs failed to induce apoptosis and localised in the nucleus, whereas the wild type TIS11 localised in the cytoplasm [102]. The TIS11 family may regulate degradation of cell survival proteins such as, Bcl-2, directly. Bcl-2 mRNA contains a Class II ARE motif in the 3′UTR with at least one UUAUUUAUU nonamer which is conserved between different species and this sequence controls the stability of Bcl-2 [103]. RNA electrophoretic shift assays have confirmed an interaction between TIS11b and the Bcl-2 ARE (Murphy et al., unpublished observations). Because TIS11 also destabilises several mRNAs whose overexpression is related to malignant change, it has been suggested that they may form a class of tumour suppressor genes. In support of this hypothesis, it has been reported that injection of mast cells transformed with v-H-Ras resulted in tumour formation in mice as early as 6 weeks after injection due to production of autocrine IL-3 [104]. Transfection with v-H-Ras clones overexpressing TIS11 delayed tumour formation by at least 4 weeks [104]. Another study correlated response to cisplatin in head and neck squamous cell carcinoma cell lines with levels of TIS11b expression. Tumour cells expressing TIS11b underwent apoptosis following drug exposure, while tumour cells with low/absent levels of TIS11b were resistant to apoptosis, but became sensitive after transfection of cells with a TIS11b expressing plasmid [36]. Interestingly, significant downregulation of inhibitor of apoptosis protein 2 (cIAP2) but not Bcl-2 mRNA was seen in these cells even though both these mRNAs contain class II AREs [36].

TIS11 (but not TIS11b) specifically interacts with the Tax protein from either bovine leukaemia virus or human T lymphotropic virus 1 (HTLV-1) through the C-terminal domain [46]. In the presence of TIS11, Tax was less oncogenic probably due to inhibition of Tax transactivation activity [46]. Interestingly TIS11 was able to abrogate TNF-α induction by Tax in unstimulated RAW264.7 cells [46]. TIS11b expression is upregulated in human HTLV-1-infected cells by Tax transactivator protein which binds to two TIS11b upstream control elements [105].

TIS11b was found to be one of the genes that are over expressed in cell lines and primary cells expressing the AML1-ETO fusion protein, that is, found in 40% of Acute Myeloid Leukaemia subtype M2 [62]. When ectopically expressed TIS11b promoted myeloid progenitor L-G cell proliferation and inhibited differentiation [62]. Intriguingly, siRNA mediated TIS11b knockdown in murine embryonic stem cells promoted their differentiation to cardiomyocytes [106]. Increased expression of TIS11b was reported in primary breast tumours [107] and hepatocellular carcinomas [108]. In addition, TIS11, TIS11b, and TIS11d have been reported to be over-expressed in a variety of the NCI 60 panel of human cancer cell lines [109].

The involvement of the TIS11 family in inflammatory disease was one of the earliest reported disease associations of this family. TIS11 knockout mice developed a generalised autoimmune-like disease that was largely controlled by the addition of neutralising anti-TNF antibodies [44]. TIS11, in particular, has been reported to mediate degradation of inflammatory cytokine mRNAs (see Table 1 and references therein). Recently, targeting TIS11 has been shown to have therapeutic potential in an animal model of inflammatory bone loss [110].

8. Conclusion

The TIS11 family of proteins mediate posttranscriptional gene regulation, which appears to be important in a range of physiological and pathological processes. A number of important gaps in our knowledge still exist and require further study. We have limited knowledge of mRNA targets of TIS11 family members and whether individual TIS11 family members target distinct, overlapping, or identical targets to other family members. A few studies have suggested inhibition of translation may also be mediated by the TIS11 family as well as mRNA degradation and this requires further elucidation. A most interesting line of enquiry concerns the relationship of TIS11 family mediated posttranscriptional regulation to miRNA and siRNA mediated posttranscriptional regulation. Finally, their role in cell functions and disease have already provided evidence that they may be useful therapeutic targets in inflammatory diseases and cancer. Further studies are likely to identify other important regulatory mechanisms for this family in health and disease.

Abbreviations

ARE: Adenine uridine rich elements
AU: Adenine uridine
CLL: Chronic lymphocytic leukemia
LPS: Lipopolysaccharide
MAPK: Mitogen activated protein kinase

miRNA: Micro RNA
MK2: p38-regulated MAPK activated protein kinase 2
PARN: PolyA specific ribonuclease
siRNA: Short interfering RNA
TPA: 12-O-tetradecanoylphorbol-13-acetate.

References

[1] P. J. Blackshear, R. S. Phillips, S. Ghosh, S. V. B. Ramos, E. K. Richfield, and W. S. Lai, "Zfp36l3, a rodent X chromosome gene encoding a placenta-specific member of the tristetraprolin family of CCCH tandem zinc finger proteins," *Biology of Reproduction*, vol. 73, no. 2, pp. 297–307, 2005.

[2] Q. Ma, D. Wadleigh, T. Chi, and H. Herschman, "The Drosophila TIS11 homologue encodes a developmentally controlled gene," *Oncogene*, vol. 9, no. 11, pp. 3329–3334, 1994.

[3] Q. Ma and H. R. Herschman, "The yeast homologue YTIS11, of the mammalian TIS11 gene family is a non-essential, glucose repressible gene," *Oncogene*, vol. 10, no. 3, pp. 487–494, 1995.

[4] M. J. Thompson, W. S. Lai, G. A. Taylor, and P. J. Blackshear, "Cloning and characterization of two yeast genes encoding members of the CCCH class of zinc finger proteins: zinc finger-mediated impairment of cell growth," *Gene*, vol. 174, no. 2, pp. 225–233, 1996.

[5] P. J. Blackshear, "Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover," *Biochemical Society Transactions*, vol. 30, no. 6, pp. 945–952, 2002.

[6] C. J. Wilusz, M. Wormington, and S. W. Peltz, "The cap-to-tail guide to mRNA turnover," *Nature Reviews Molecular Cell Biology*, vol. 2, no. 4, pp. 237–246, 2001.

[7] T. Bakheet, B. R. G. Williams, and K. S. A. Khabar, "ARED 2.0: an update of AU-rich element mRNA database," *Nucleic Acids Research*, vol. 31, no. 1, pp. 421–423, 2003.

[8] E. Carballo, W. S. Lai, and P. J. Blackshear, "Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin," *Science*, vol. 281, no. 5379, pp. 1001–1005, 1998.

[9] M. T. Worthington, J. W. Pelo, M. A. Sachedina, J. L. Applegate, K. O. Arseneau, and T. T. Pizarro, "RNA binding properties of the AU-rich element-binding recombinant Nup475/TIS11/tristetraprolin protein," *The Journal of Biological Chemistry*, vol. 277, no. 50, pp. 48558–48564, 2002.

[10] P. J. Blackshear, W. S. Lai, E. A. Kennington, et al., "Characteristics of the interaction of a synthetic human tristetraprolin tandem zinc finger peptide with AU-rich element-containing RNA substrates," *The Journal of Biological Chemistry*, vol. 278, no. 22, pp. 19947–19955, 2003.

[11] B. Y. Brewer, J. Malicka, P. J. Blackshear, and G. M. Wilson, "RNA sequence elements required for high affinity binding by the zinc finger domain of tristetraprolin: conformational changes coupled to the bipartite nature of AU-rich mRNA-destabilizing motifs," *The Journal of Biological Chemistry*, vol. 279, no. 27, pp. 27870–27877, 2004.

[12] W. S. Lai, D. M. Carrick, and P. J. Blackshear, "Influence of nonameric AU-rich tristetraprolin-binding sites on mRNA deadenylation and turnover," *The Journal of Biological Chemistry*, vol. 280, no. 40, pp. 34365–34377, 2005.

[13] S. A. Bustin, X.-F. Nie, R. C. Barnard, et al., "Cloning and characterization of ERF-1, a human member of the Tis11 family of early-response genes," *DNA and Cell Biology*, vol. 13, no. 5, pp. 449–459, 1994.

[14] S. A. Brooks, J. E. Connoly, and W. F. C. Rigby, "The role of mRNA turnover in the regulation of tritetraprolin expression: evidence for an extracellular signal-regulated kinase-specific, AU-rich element-dependent, autoregulatory pathway," *The Journal of Immunology*, vol. 172, no. 12, pp. 7263–7271, 2004.

[15] E. Carballo, W. S. Lai, and P. J. Blackshear, "Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability," *Blood*, vol. 95, no. 6, pp. 1891–1899, 2000.

[16] G. Stoecklin, X.-F. Ming, R. Looser, and C. Moroni, "Somatic mRNA turnover mutants implicate tristetraprolin in the interleukin-3 mRNA degradation pathway," *Molecular and Cellular Biology*, vol. 20, no. 11, pp. 3753–3763, 2000.

[17] G. Stoecklin, P. Stoeckle, M. Lu, O. Muehlemann, and C. Moroni, "Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements," *RNA*, vol. 7, no. 11, pp. 1578–1588, 2001.

[18] H. Sawaoka, D. A. Dixon, J. A. Oates, and O. Boutaud, "Tristetraprolin binds to the 3′-untranslated region of cyclooxygenase-2 mRNA: a polyadenylation variant in a cancer cell line lacks the binding site," *The Journal of Biological Chemistry*, vol. 278, no. 16, pp. 13928–13935, 2003.

[19] H. Yu, S. Stasinopoulos, P. Leedman, and R. L. Medcalf, "Inherent instability of plasminogen activator inhibitor type 2 mRNA is regulated by tristetraprolin," *The Journal of Biological Chemistry*, vol. 278, no. 16, pp. 13912–13918, 2003.

[20] P. Briata, C. Ilengo, G. Corte, et al., "The Wnt/β-catenin → Pitx2 pathway controls the turnover of Pitx2 and other unstable mRNAs," *Molecular Cell*, vol. 12, no. 5, pp. 1201–1211, 2003.

[21] R. L. Ogilvie, M. Abelson, H. H. Hau, I. Vlasova, P. J. Blackshear, and P. R. Bohjanen, "Tristetraprolin down-regulates IL-2 gene expression through AU-rich element-mediated mRNA decay," *The Journal of Immunology*, vol. 174, no. 2, pp. 953–961, 2005.

[22] S. I. Gringhuis, J. J. García-Vallejo, B. van Het Hof, and W. van Dijk, "Convergent actions of IκB kinase β and protein kinase C δ modulate mRNA stability through phosphorylation of 14-3-3 β complexed with tristetraprolin," *Molecular and Cellular Biology*, vol. 25, no. 15, pp. 6454–6463, 2005.

[23] U. Jalonen, R. Nieminen, K. Vuolteenaho, H. Kankaanranta, and E. Moilanen, "Down-regulation of tristetraprolin expression results in enhanced IL-12 and MIP-2 production and reduced MIP-3α synthesis in activated macrophages," *Mediators of Inflammation*, vol. 2006, Article ID 40691, 8 pages, 2006.

[24] I. Sauer, B. Schaljo, C. Vogl, et al., "Interferons limit inflammatory responses by induction of tristetraprolin," *Blood*, vol. 107, no. 12, pp. 4790–4797, 2006.

[25] M. Marderosian, A. Sharma, A. P. Funk, et al., "Tristetraprolin regulates cyclin D1 and c-Myc mRNA stability in response to rapamycin in an Akt-dependent manner via p38 MAPK signaling," *Oncogene*, vol. 25, no. 47, pp. 6277–6290, 2006.

[26] W. D. Patino, J.-G. Kang, S. Matoba, O. Y. Mian, B. R. Gochuico, and P. M. Hwang, "Atherosclerotic plaque macrophage transcriptional regulators are expressed in blood and modulated by tristetraprolin," *Circulation Research*, vol. 98, no. 10, pp. 1282–1289, 2006.

[27] W. S. Lai, J. S. Parker, S. F. Grissom, D. J. Stumpo, and P. J. Blackshear, "Novel mRNA targets for tristetraprolin (TTP)

identified by global analysis of stabilized transcripts in TTP-deficient fibroblasts," *Molecular and Cellular Biology*, vol. 26, no. 24, pp. 9196–9208, 2006.

[28] D. Frasca, A. M. Landin, J. P. Alvarez, P. J. Blackshear, R. L. Riley, and B. B. Blomberg, "Tristetraprolin, a negative regulator of mRNA stability, is increased in old B cells and is involved in the degradation of E47 mRNA," *The Journal of Immunology*, vol. 179, no. 2, pp. 918–927, 2007.

[29] K. Essafi-Benkhadir, C. Onesto, E. Stebe, C. Moroni, and G. Pagès, "Tristetraprolin inhibits ras-dependent tumor vascularization by inducing vascular endothelial growth factor mRNA degradation," *Molecular Biology of the Cell*, vol. 18, no. 11, pp. 4648–4658, 2007.

[30] G. Stoecklin, S. A. Tenenbaum, T. Mayo, et al., "Genome-wide analysis identifies interleukin-10 mRNA as target of tristetraprolin," *The Journal of Biological Chemistry*, vol. 283, no. 17, pp. 11689–11699, 2008.

[31] T. J. Horner, W. S. Lai, D. J. Stumpo, and P. J. Blackshear, "Stimulation of polo-like kinase 3 mRNA decay by tristetraprolin," *Molecular and Cellular Biology*, vol. 29, no. 8, pp. 1999–2010, 2009.

[32] W. S. Lai, E. Carballo, J. M. Thorn, E. A. Kennington, and P. J. Blackshear, "Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to AU-rich elements and destabilization of mRNA," *The Journal of Biological Chemistry*, vol. 275, no. 23, pp. 17827–17837, 2000.

[33] W. S. Lai and P. J. Blackshear, "Interactions of CCCH zinc finger proteins with mRNA. Tristetraprolin-mediated AU-rich element-dependent mRNA degradation can occur in the absence of a poly(A) tail," *The Journal of Biological Chemistry*, vol. 276, no. 25, pp. 23144–23154, 2001.

[34] G. Stoecklin, M. Colombi, I. Raineri, et al., "Functional cloning of BRF1, a regulator of ARE-dependent mRNA turnover," *The EMBO Journal*, vol. 21, no. 17, pp. 4709–4718, 2002.

[35] D. Ciais, N. Cherradi, S. Bailly, et al., "Destabilization of vascular endothelial growth factor mRNA by the zinc-finger protein TIS11b," *Oncogene*, vol. 23, no. 53, pp. 8673–8680, 2004.

[36] S. K. Lee, S. B. Kim, J. S. Kim, et al., "Butyrate response factor 1 enhances cisplatin sensitivity in human head and neck squamous cell carcinoma cell lines," *International Journal of Cancer*, vol. 117, no. 1, pp. 32–40, 2005.

[37] S. E. Bell, M. J. Sanchez, O. Spasic-Boskovic, et al., "The RNA binding protein Zfp36l1 is required for normal vascularisation and post-transcriptionally regulates VEGF expression," *Developmental Dynamics*, vol. 235, no. 11, pp. 3144–3155, 2006.

[38] H. Duan, N. Cherradi, J.-J. Feige, and C. Jefcoate, "cAMP-dependent posttranscriptional regulation of steroidogenic acute regulatory (STAR) protein by the zinc finger protein ZFP36L1/TIS11b," *Molecular Endocrinology*, vol. 23, no. 4, pp. 497–509, 2009.

[39] W. S. Lai, E. A. Kennington, and P. J. Blackshear, "Interactions of CCCH zinc finger proteins with mRNA: non-binding tristetraprolin mutants exert an inhibitory effect on degradation of AU-rich element-containing mRNAs," *The Journal of Biological Chemistry*, vol. 277, no. 11, pp. 9606–9613, 2002.

[40] W. S. Lai, E. A. Kennington, and P. J. Blackshear, "Tristetraprolin and its family members can promote the cell-free deadenylation of AU-rich element-containing mRNAs by poly(A) ribonuclease," *Molecular and Cellular Biology*, vol. 23, no. 11, pp. 3798–3812, 2003.

[41] J. Lykke-Andersen and E. Wagner, "Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1," *Genes and Development*, vol. 19, no. 3, pp. 351–361, 2005.

[42] M. Fenger-Grøn, C. Fillman, B. Norrild, and J. Lykke-Andersen, "Multiple processing body factors and the ARE binding protein TTP activate mRNA decapping," *Molecular Cell*, vol. 20, no. 6, pp. 905–915, 2005.

[43] C.-Y. Chen, R. Gherzi, S.-E. Ong, et al., "AU binding proteins recruit the exosome to degrade ARE-containing mRNAs," *Cell*, vol. 107, no. 4, pp. 451–464, 2001.

[44] G. A. Taylor, E. Carballo, D. M. Lee, et al., "A pathogenetic role for TNFα in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency," *Immunity*, vol. 4, no. 5, pp. 445–454, 1996.

[45] K. Suzuki, H. Nakajima, K. Ikeda, et al., "IL-4-Stat6 signaling induces tristetraprolin expression and inhibits TNF-α production in mast cells," *Journal of Experimental Medicine*, vol. 198, no. 11, pp. 1717–1727, 2003.

[46] J.-C. Twizere, V. Kruys, L. Lefèbvre, et al., "Interaction of retroviral tax oncoproteins with tristetraprolin and regulation of tumor necrosis factor-α expression," *Journal of the National Cancer Institute*, vol. 95, no. 24, pp. 1846–1859, 2003.

[47] W. F. C. Rigby, K. Roy, J. Collins, et al., "Structure/function analysis of tristetraprolin (TTP): p38 stress-activated protein kinase and lipopolysaccharide stimulation do not alter TTP function," *The Journal of Immunology*, vol. 174, no. 12, pp. 7883–7893, 2005.

[48] Q. Jing, S. Huang, S. Guth, et al., "Involvement of microRNA in AU-Rich element-mediated mRNA instability," *Cell*, vol. 120, no. 5, pp. 623–634, 2005.

[49] S. Dorner, L. Lum, M. Kim, R. Paro, P. A. Beachy, and R. Green, "A genomewide screen for components of the RNAi pathway in *Drosophila* cultured cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 32, pp. 11880–11885, 2006.

[50] A. J. Asirvatham, C. J. Gregorie, Z. Hu, W. J. Magner, and T. B. Tomasi, "MicroRNA targets in immune genes and the Dicer/Argonaute and ARE machinery components," *Molecular Immunology*, vol. 45, no. 7, pp. 1995–2006, 2008.

[51] R. N. DuBois, M. W. McLane, K. Ryder, L. F. Lau, and D. Nathans, "A growth factor-inducible nuclear protein with a novel cysteine/histidine repetitive sequence," *The Journal of Biological Chemistry*, vol. 265, no. 31, pp. 19185–19191, 1990.

[52] G. A. Taylor, W. S. Lai, R. J. Oakey, et al., "The human TTP protein: sequence, alignment with related proteins, and chromosomal localization of the mouse and human genes," *Nucleic Acids Research*, vol. 19, no. 12, p. 3454, 1991.

[53] B. C. Varnum, R. W. Lim, V. P. Sukhatme, and H. R. Herschman, "Nucleotide sequence of a cDNA encoding TIS11, a message induced in Swiss 3T3 cells by the tumor promoter tetradecanoyl phorbol acetate," *Oncogene*, vol. 4, no. 1, pp. 119–120, 1989.

[54] J. J. Murphy and J. D. Norton, "Cell-type-specific early response gene expression during plasmacytoid differentiation of human B lymphocytic leukemia cells," *Biochimica et Biophysica Acta*, vol. 1049, no. 3, pp. 261–271, 1990.

[55] Z.-Q. Ning, J. D. Norton, J. Li, and J. J. Murphy, "Distinct mechanisms for rescue from apoptosis in Ramos human B cells by signaling through CD40 and interleukin-4 receptor: role for inhibition of an early response gene, Berg36,"

European Journal of Immunology, vol. 26, no. 10, pp. 2356–2363, 1996.

[56] X.-F. Nie, K. N. Maclean, V. Kumar, I. A. McKay, and S. A. Bustin, "ERF-2, the human homologue of the murine TIS11d early response gene," *Gene*, vol. 152, no. 2, pp. 285–286, 1995.

[57] P. J. Blackshear, R. S. Phillips, J. Vazquez-Matias, and H. Mohrenweiser, "Polymorphisms in the genes encoding members of the tristetraprolin family of human tandem CCCH zinc finger proteins," *Progress in Nucleic Acid Research and Molecular Biology*, vol. 75, pp. 43–68, 2003.

[58] D. M. Carrick, P. Chulada, R. Donn, et al., "Genetic variations in ZFP36 and their possible relationship to autoimmune diseases," *Journal of Autoimmunity*, vol. 26, no. 3, pp. 182–196, 2006.

[59] D. J. Stumpo, N. A. Byrd, R. S. Phillips, et al., "Chorioallantoic fusion defects and embryonic lethality resulting from disruption of Zfp36L1, a gene encoding a CCCH tandem zinc finger protein of the tristetraprolin family," *Molecular and Cellular Biology*, vol. 24, no. 14, pp. 6445–6455, 2004.

[60] S. B. V. Ramos, D. J. Stumpo, E. A. Kennington, et al., "The CCCH tandem zinc-finger protein Zfp36l2 is crucial for female fertility and early embryonic development," *Development*, vol. 131, no. 19, pp. 4883–4893, 2004.

[61] H. Cao, J. S. Tuttle, and P. J. Blackshear, "Immunological characterization of tristetraprolin as a low abundance, inducible, stable cytosolic protein," *The Journal of Biological Chemistry*, vol. 279, no. 20, pp. 21489–21499, 2004.

[62] H. Shimada, H. Ichikawa, S. Nakamura, et al., "Analysis of genes under the downstream control of the t(8;21) fusion protein AML1-MTG8: overexpression of the TIS11b (ERF-1, cMG1) gene induces myeloid cell proliferation in response to G-CSF," *Blood*, vol. 96, no. 2, pp. 655–663, 2000.

[63] T. Ino, H. Yasui, M. Hirano, and Y. Kurosawa, "Identification of a member of the TIS11 early response gene family at the insertion point of a DNA fragment containing a gene for the T-cell receptor β chain in an acute T-cell leukemia," *Oncogene*, vol. 11, no. 12, pp. 2705–2710, 1995.

[64] B. T. Amann, M. T. Worthington, and J. M. Berg, "A Cys3His zinc-binding domain from Nup475/tristetraprolin: a novel fold with a disklike structure," *Biochemistry*, vol. 42, no. 1, pp. 217–221, 2003.

[65] B. P. Hudson, M. A. Martinez-Yamout, H. J. Dyson, and P. E. Wright, "Recognition of the mRNA AU-rich element by the zinc finger domain of TIS11d," *Nature Structural and Molecular Biology*, vol. 11, no. 3, pp. 257–264, 2004.

[66] W. S. Lai, D. J. Stumpo, and P. J. Blackshear, "Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein," *The Journal of Biological Chemistry*, vol. 265, no. 27, pp. 16556–16563, 1990.

[67] A. N. Corps and K. D. Brown, "Insulin and insulin-like growth factor I stimulate expression of the primary response gene cMG1/TIS11b by a wortmannin-sensitive pathway in RIE-1 cells," *FEBS Letters*, vol. 368, no. 1, pp. 160–164, 1995.

[68] K. R. Mahtani, M. Brook, J. L. E. Dean, G. Sully, J. Saklatvala, and A. R. Clark, "Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability," *Molecular and Cellular Biology*, vol. 21, no. 19, pp. 6461–6469, 2001.

[69] C. R. Tchen, M. Brook, J. Saklatvala, and A. R. Clark, "The stability of tristetraprolin mRNA is regulated by mitogen-activated protein kinase p38 and by tristetraprolin itself," *The Journal of Biological Chemistry*, vol. 279, no. 31, pp. 32393–32400, 2004.

[70] A. M. Fairhurst, J. E. Connolly, K. A. Hintz, et al., "Regulation and localization of endogenous human tristetraprolin," *Arthritis Research & Therapy*, vol. 5, no. 4, pp. R214–R225, 2003.

[71] B. C. Varnum, R. W. Lim, D. A. Kujubu, et al., "Granulocyte-macrophage colony-stimulating factor and tetradecanoyl phorbol acetate induce a distinct, restricted subset of primary-response TIS genes in both proliferating and terminally differentiated myeloid cells," *Molecular and Cellular Biology*, vol. 9, no. 8, pp. 3580–3583, 1989.

[72] K. Ogawa, F. Chen, Y.-J. Kim, and Y. Chen, "Transcriptional regulation of tristetraprolin by transforming growth factor-β in human T cells," *The Journal of Biological Chemistry*, vol. 278, no. 32, pp. 30373–30381, 2003.

[73] A. Raghavan, R. L. Robison, J. McNabb, C. R. Miller, D. A. Williams, and P. R. Bohjanen, "HuA and tristetraprolin are induced following T cell activation and display distinct but overlapping RNA binding specificities," *The Journal of Biological Chemistry*, vol. 276, no. 51, pp. 47958–47965, 2001.

[74] K. Smoak and J. A. Cidlowski, "Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling," *Molecular and Cellular Biology*, vol. 26, no. 23, pp. 9126–9135, 2006.

[75] F. T. Ishmael, X. Fang, M. R. Galdiero, et al., "Role of the RNA-binding protein tristetraprolin in glucocorticoid-mediated gene regulation," *The Journal of Immunology*, vol. 180, no. 12, pp. 8342–8353, 2008.

[76] Z.-Q. Ning, T. Hirose, R. Deed, J. Newton, J. J. Murphy, and J. D. Norton, "Early response gene signalling in bryostatin-stimulated primary B chronic lymphocytic leukaemia cells in vitro," *Biochemical Journal*, vol. 319, part 1, pp. 59–65, 1996.

[77] M. Baou, A. Jewell, A. Muthurania, et al., "Involvement of TIS11b, an AU-rich binding protein, in induction of apoptosis by rituximab in B cell chronic lymphocytic leukemia cells," *Leukemia*, vol. 23, pp. 986–989, 2009.

[78] S. Mathas, A. Rickers, K. Bommert, B. Dörken, and M. Y. Mapara, "Anti-CD20-and B-cell receptor-mediated apoptosis: evidence for shared intracellular signaling pathways," *Cancer Research*, vol. 60, no. 24, pp. 7170–7176, 2000.

[79] S. Reppe, O. K. Olstad, E. Rian, V. T. Gautvik, K. M. Gautvik, and R. Jemtland, "Butyrate response factor 1 is regulated by parathyroid hormone and bone morphogenetic protein-2 in osteoblastic cells," *Biochemical and Biophysical Research Communications*, vol. 324, no. 1, pp. 218–223, 2004.

[80] A. M. Chinn, D. Ciais, S. Bailly, E. Chambaz, J. LaMarre, and J.-J. Feige, "Identification of two novel ACTH-responsive genes encoding manganese-dependent superoxide dismutase (SOD2) and the zinc finger protein TIS11b [tetradecanoyl phorbol acetate (TPA)-inducible sequence 11b]," *Molecular Endocrinology*, vol. 16, no. 6, pp. 1417–1427, 2002.

[81] G. A. Taylor, M. J. Thompson, W. S. Lai, and P. J. Blackshear, "Mitogens stimulate the rapid nuclear to cytosolic translocation of tristetraprolin, a potential zinc-finger transcription factor," *Molecular Endocrinology*, vol. 10, no. 2, pp. 140–146, 1996.

[82] M. Brook, C. R. Tchen, T. Santalucia, et al., "Posttranslational regulation of tristetraprolin subcellular localization and protein stability by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways," *Molecular and Cellular Biology*, vol. 26, no. 6, pp. 2408–2418, 2006.

[83] G. A. Taylor, M. J. Thompson, W. S. Lai, and P. J. Blackshear, "Phosphorylation of tristetraprolin, a potential zinc finger transcription factor, by mitogen stimulation in intact cells

and by mitogen-activated protein kinase in vitro," *The Journal of Biological Chemistry*, vol. 270, no. 22, pp. 13341–13347, 1995.

[84] W. Zhu, M. A. Brauchle, F. Di Padova, et al., "Gene suppression by tristetraprolin and release by the p38 pathway," *American Journal of Physiology*, vol. 281, no. 2, pp. L499–L508, 2001.

[85] C. A. Chrestensen, M. J. Schroeder, J. Shabanowitz, et al., "MAPKAP kinase 2 phosphorylates tristetraprolin on in vivo sites including Ser178, a site required for 14-3-3 binding," *The Journal of Biological Chemistry*, vol. 279, no. 11, pp. 10176–10184, 2004.

[86] L. Sun, G. Stoecklin, S. Van Way, et al., "Tristetraprolin (TTP)-14-3-3 complex formation protects TTP from dephosphorylation by protein phosphatase 2a and stabilizes tumor necrosis factor-α mRNA," *The Journal of Biological Chemistry*, vol. 282, no. 6, pp. 3766–3777, 2007.

[87] E. Hitti, T. Iakovleva, M. Brook, et al., "Mitogen-activated protein kinase-activated protein kinase 2 regulates tumor necrosis factor mRNA stability and translation mainly by altering tristetraprolin expression, stability, and binding to adenine/uridine-rich element," *Molecular and Cellular Biology*, vol. 26, no. 6, pp. 2399–2407, 2006.

[88] B. A. Johnson, J. R. Stehn, M. B. Yaffe, and T. K. Blackwell, "Cytoplasmic localization of tristetraprolin involves 14-3-3-dependent and -independent mechanisms," *The Journal of Biological Chemistry*, vol. 277, no. 20, pp. 18029–18036, 2002.

[89] H. Cao, L. J. Deterding, J. D. Venable, et al., "Identification of the anti-inflammatory protein tristetraprolin as a hyperphosphorylated protein by mass spectrometry and site-directed mutagenesis," *Biochemical Journal*, vol. 394, no. 1, pp. 285–297, 2006.

[90] S. Maitra, C.-F. Chou, C. A. Luber, K.-Y. Lee, M. Mann, and C.-Y. Chen, "The AU-rich element mRNA decay-promoting activity of BRF1 is regulated by mitogen-activated protein kinase-activated protein kinase 2," *RNA*, vol. 14, no. 5, pp. 950–959, 2008.

[91] M. Schmidlin, M. Lu, S. A. Leuenberger, et al., "The ARE-dependent mRNA-destabilizing activity of BRF1 is regulated by protein kinase B," *The EMBO Journal*, vol. 23, no. 24, pp. 4760–4769, 2004.

[92] R. S. Phillips, S. B. V. Ramos, and P. J. Blackshear, "Members of the tristetraprolin family of tandem CCCH zinc finger proteins exhibit CRM1-dependent nucleocytoplasmic shuttling," *The Journal of Biological Chemistry*, vol. 277, no. 13, pp. 11606–11613, 2002.

[93] T. Murata, Y. Yoshino, N. Morita, and N. Kaneda, "Identification of nuclear import and export signals within the structure of the zinc finger protein TIS11," *Biochemical and Biophysical Research Communications*, vol. 293, no. 4, pp. 1242–1247, 2002.

[94] J. A. Carman and S. G. Nadler, "Direct association of tristetraprolin with the nucleoporin CAN/Nup214," *Biochemical and Biophysical Research Communications*, vol. 315, no. 2, pp. 445–449, 2004.

[95] T. Murata, N. Morita, K. Hikita, K. Kiuchi, K. Kiuchi, and N. Kaneda, "Recruitment of mRNA-destabilizing protein TIS11 to stress granules is mediated by its zinc finger domain," *Experimental Cell Research*, vol. 303, no. 2, pp. 287–299, 2005.

[96] G. Stoecklin, T. Stubbs, N. Kedersha, et al., "MK2-induced tristetraprolin:14-3-3 complexes prevent stress granule association and ARE-mRNA decay," *The EMBO Journal*, vol. 23, no. 6, pp. 1313–1324, 2004.

[97] N. Kedersha, G. Stoecklin, M. Ayodele, et al., "Stress granules and processing bodies are dynamically linked sites of mRNP remodeling," *Journal of Cell Biology*, vol. 169, no. 6, pp. 871–884, 2005.

[98] T. M. Franks and J. Lykke-Andersen, "TTP and BRF proteins nucleate processing body formation to silence mRNAs with AU-rich elements," *Genes and Development*, vol. 21, no. 6, pp. 719–735, 2007.

[99] N. Cougot, S. Babajko, and B. Séraphin, "Cytoplasmic foci are sites of mRNA decay in human cells," *Journal of Cell Biology*, vol. 165, no. 1, pp. 31–40, 2004.

[100] A. Jakymiw, S. Lian, T. Eystathioy, et al., "Disruption of GW bodies impairs mammalian RNA interference," *Nature Cell Biology*, vol. 7, no. 12, pp. 1267–1274, 2005.

[101] B. A. Johnson, M. Geha, and K. T. Blackwell, "Similar but distinct effects of the tristetraprolin/TIS11 immediate-early proteins on cell survival," *Oncogene*, vol. 19, no. 13, pp. 1657–1664, 2000.

[102] B. A. Johnson and T. K. Blackwell, "Multiple tristetraprolin sequence domains required to induce apoptosis and modulate responses to TNFα through distinct pathways," *Oncogene*, vol. 21, no. 27, pp. 4237–4246, 2002.

[103] N. Schiavone, P. Rosini, A. Quattrone, et al., "A conserved AU-rich element in the 3′ untranslated region of bcl-2 mRNA is endowed with a destabilizing function that is involved in bcl-2 down-regulation during apoptosis," *The FASEB Journal*, vol. 14, no. 1, pp. 174–184, 2000.

[104] G. Stoecklin, B. Gross, X.-F. Ming, and C. Moroni, "A novel mechanism of tumor suppression by destabilizing AU-rich growth factor mRNA," *Oncogene*, vol. 22, no. 23, pp. 3554–3561, 2003.

[105] B. Li, T. Fink, P. Ebbesen, X. D. Liu, and V. Zachar, "Expression of butyrate response factor 1 in HTLV-1-transformed cells and its transactivation by tax protein," *Archives of Virology*, vol. 148, no. 9, pp. 1787–1804, 2003.

[106] D. Wegmüller, I. Raineri, B. Gross, E. J. Oakeley, and C. Moroni, "A cassette system to study embryonic stem cell differentiation by inducible RNA interference," *Stem Cells*, vol. 25, no. 5, pp. 1178–1185, 2007.

[107] M. C. Abba, H. Sun, K. A. Hawkins, et al., "Breast cancer molecular signatures as determined by SAGE: correlation with lymph node status," *Molecular Cancer Research*, vol. 5, no. 9, pp. 881–890, 2007.

[108] P. J. Zindy, A. L'Helgoualc'h, D. Bonnier, et al., "Upregulation of the tumor suppressor gene menin in hepatocellular carcinomas and its significance in fibrogenesis," *Hepatology*, vol. 44, no. 5, pp. 1296–1307, 2006.

[109] D. M. Carrick and P. J. Blackshear, "Comparative expression of tristetraprolin (TTP) family member transcripts in normal human tissues and cancer cell lines," *Archives of Biochemistry and Biophysics*, vol. 462, no. 2, pp. 278–285, 2007.

[110] C. S. Patil, M. Liu, W. Zhao, et al., "Targeting mRNA stability arrests inflammatory bone loss," *Molecular Therapy*, vol. 16, no. 10, pp. 1657–1664, 2008.