Stem Cells International

Transcriptional and Genomic Control of Stem Cells in Development and Cancer

Lead Guest Editor: Jinsong Zhang
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Editorial Board

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Editorial

Transcriptional and Genomic Control of Stem Cells in Development and Cancer

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Stem cells play important roles in normal physiology, and their deregulated functions are involved in diseases such as cancer. The normal stem cells have the potential to differentiate into different cell types during development [1]. In adults, somatic stem cells are a valuable source for tissue-specific cell types to replace aged or damaged cells [2]. Cancer stem cells are cells within a tumor which possess some stem cell properties [3]. A cancer stem cell removed from its original tumor and seeded in a new organ or tissue type can form a brand new tumor in the new location, a process known as metastasis [4]. Stem cells can be regulated at both transcriptional and genomic levels. Transcription factors and their coactivators or corepressors are very important both in normal stem cell function and in cancer stem cell maintenance [5–7]. Genetic mutations resulted from replication errors or defective repair mechanisms affect genome stability and stem cell functions [8, 9]. Therapeutic targeting of stem cell regulation has the potential to alleviate disease condition and to find a cure in the fight against cancer.

This special issue contains review articles that offer new insight into the current status of the research areas. In one of the reviews, M. Wang et al. presented the common types of molecular mutations that can occur as a single mutation or as combinations of two or three mutations in cytogenetically normal acute myeloid leukemia (CN-AML). They analyzed available AML databases using bioinformatics tools at their disposal and found that mutations in stem cell regulatory factors such as FLT3, TET2, DNMT3A, and IDH1 often have an unfavorable clinical outcome and may predict relapse of leukemia in CN-AML patients. In another review, S. Zhang et al. described the cancer stem cell properties of polyploidy giant cancer cells (PGCC) and presented evidence from the literature showing that tumor budding and micropapillary pattern are recognized indicators of tumor aggressiveness in colorectal cancer. Literature review by K. M. Beach et al. discussed the role of Müller glia and Müller glial-derived stem cells in retina regeneration after injury in mammals. They summarized recent progress made in the field and emphasized that discovery of intrinsic and extrinsic factors that regulate JAK/STAT and MAPK signaling pathways take priority in future research to promote switch in the direction of regenerative responses of Müller glia in the retina after injury in mammals. In a review on neural stem cells, L. Zhang et al. summarized recent progress on the regulation of neural stem cells at both the genomic and transcriptional levels. They used bioinformatics methods to predict factors that may serve as novel therapeutic targets for functional recovery after hemorrhagic stroke. In one other review, S.-L. Cheng et al. examined 15 clinical trials using stem cells from different tissue/organ sources for the treatment of chronic

obstructive pulmonary disease. Available data from three trials indicate that administration of mesenchymal stem cells (MSCs) in patients with degenerative lung diseases is well tolerated and may improve overall health and quality of life for patients.

This special issue also includes original research articles of various aspects related to the topic. L. Guo et al. observed the effect of resveratrol treatment on human umbilical cord mesenchymal stem cells (hUC-MSCs). They found that resveratrol at relatively high concentrations (15.0 and 30.0 mg/L) stimulated the hUC-MSC differentiation into neuron-like cells as evidenced by the increased expression of nestin and neuron-specific enolase (NSE) at protein and mRNA levels four hours after treatment. On the other hand, expression of glial fibrillary acidic protein (GFAP) showed no change after resveratrol treatment, indicating that differentiation of hUC-MSCs into glial cells did not occur after resveratrol stimulation. The discovery may be of therapeutic importance. One other research article by C. Yang et al. investigated the maintenance of stemness in triple-negative breast cancer (TNBC) which is enriched in cancer stem cells. They analyzed available TCGA datasets and several smaller-size datasets of breast cancer and demonstrated that cadherin family members CDH2, 4, 6, and 17 were upregulated when E-cadherin (CDH1) was lost or downregulated. Changes in the expression of CDH2/4/6/17 were associated with increased expression of several important stem cell-related transcription factors including FOXM1, MCM2, WWTR1, and Sox9. Further analysis by connectivity map search indicated that small compounds may be of therapeutic use in targeting TNBC when expression of CDHs is altered. In another research article, A. Po et al. studied the maintenance of stemness in normal cerebellar stem cells (CSC). They showed that silencing of intragenic miR-326 and its host gene β-arrestin1 by epigenetic modification, specifically by CpG islands methylation, of β-arrestin1 promoter contributes to the maintenance of stem cell properties of CSCs. They further showed that the use of demethylating agents triggered differentiation program and promoted cell growth, but inhibited cell proliferation.

In summary, enormous progress has been made in genomic and transcriptional control of stem cells in development and cancer. Reviews and original research articles presented in this special issue bring to light recent development and future challenges ahead.

Jinsong Zhang
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Research Article

Resveratrol Induces Differentiation of Human Umbilical Cord Mesenchymal Stem Cells into Neuron-Like Cells

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Objective. Human umbilical cord mesenchymal stem cells (hUC-MSCs) potentially differentiate to various types of cells including neuron-like cells. The natural polyphenol resveratrol benefits patients with many diseases including ischemic brain injury. We hypothesize that resveratrol induces differentiation of hUC-MSCs into neuron-like cells. *Methods*. Flow cytometry was used to determine the surface antigens in different stage of hUC-MSCs (P2, P5, and P10). Nestin, neuron-specific enolase (NSE), and glial fibrillary acidic protein (GFAP) were detected by immunocytochemistry, Western blotting, and real time RT-PCT. The cultured hUC-MSCs were treated with resveratrol at different concentrations (0, 7.5, 15.0, and 30.0 mg/L). Nestin, GFAP, and NSE protein and mRNA were measured at posttreatment time points of 2 h, 4 h, 6 h, 12 h, and 24 h. *Results*. Neuron-like cells were found in hUC-MSCs treated by resveratrol at concentrations of 15.0 and 30.0 mg/L, but not in hUC-MSCs treated with vehicle and 7.5 mg/L resveratrol. Furthermore, immunocytochemical staining revealed that nestin and NSE immunoreactivities were positive in resveratrol-treated hUC-MSCs at concentrations of 15.0 and 30.0 mg/L. Resveratrol treatment significantly increased nestin and NSE protein and mRNA levels 4 h after the treatment. However, resveratrol treatment did not change GFAP immunoreactivities and protein and mRNA expression levels in cultured hUC-MSCs. *Conclusions*. Taken together, resveratrol treatment induces a differentiation of hUC-MSCs into neuron-like cells at relatively high concentrations.

1. Introduction

The mesenchymal stem cells (MSCs), derived from the mesoderm in early development, are pluripotent stem cells that have the properties of multiline age differentiation [1, 2]. These MSCs are able to differentiate into bone cells, adipose cells, skeletal muscle cells, cardiac muscle cells, nerve cells, and epithelial cells [1, 2]. MSCs can be found in many tissues including skin, fat, muscle, placenta, amniotic fluid, umbilical vein endothelial under layer, liver, and blood [3]. Although MSCs are enriched in bone marrow, the practical source of obtaining MSCs is fetus umbilical cord blood [4]. Recent studies have shown that the human umbilical cord mesenchymal stem cells (hUC-MSCs) have many advantages including excellent potential of differentiation, low immunogenicity, convenient transfection, rich source, and less trauma, no ethical constraints, and relatively easier accessibility, when they are compared with the MSCs from other sources. Thus, hUC-MSCs have great potentials for clinical application.

The most commonly used approaches to induce differentiation of MSCs into neuron-like cell include (1) antioxidant agents such as mercaptoethanol, dimethylsulfoxide (DMSO), and beta hydroxy acid (BHA); (2) neurotropic factors such as retinoic acid (RA), nerve growth factor (NGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), brain derived neurotropic factor (BDNF); (3) Chinese herbs and their effective components such as astragalus glycosides, berberine, tanshinone, lycium barbarum polysaccharide, acanthopanax; (4) cultural method; and (5) gene transfection: transfection of specific genes such as

Noggin [5] and Notch into hUC-MSCs to get neuron-like cell. However, these differentiation approaches are of low efficiency.

Resveratrol (3,5,4′-trihydroxy stilbene) is one of the polyphenols extracted from grape, polygonum cuspidatum, semen cassia, or peanut. It has been used clinically to treat cardiovascular disease, slow down cancer process, reduce ischemic brain injury, enhance antibiotic action, and exert estrogenic effects [6–9]. Furthermore, resveratrol modulates immune responses, reduces allergic reaction, delays aging process, and dispels chloasma [10, 11]. In addition, resveratrol has relatively strong antioxidative effect through removing free radicals [12]. Resveratrol can increase the nerve protective effect [13]. On the basis of previous studies, we tested our hypothesis that resveratrol induces differentiation of hUC-MSCs into neuron-like cells. In this study, we also determine the optimized concentration for resveratrol induced differentiation of hUC-MSCs into neuron-like cells.

2. Materials and Methods

2.1. Materials. Resveratrol (purity: 99.2%) was purchased from Nanjing Zelang Pharmaceutical Co., Ltd. (Nanjing, China). Dulbecco's Modified Eagle's Medium (L-DMEM)/F-12 and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA) and trypsin was obtained from Amresco LLC (Solon, OH, USA). Fluorescein isothiocyanate (FITC)-CD19, FITC-CD34, phycoerythrin- (PE-) CD11b, PE-CD73, PE-CD90, PE-CD45, PE-CD105, and glial fibrillary acidic protein (GFAP) were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Nestin and neuron-specific enolase (NSE) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-rabbit immunoglobulin G (IgG) (1 : 2,000) was purchased from Affinity Biosciences (Cincinnati, OH, USA). PS immunohistochemistry kit was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China). Taq polymerase chain reaction (PCR) star mix was purchased from Beijing GenStar Biosolutions Co. Ltd. (Beijing, China); EasyScript First-Strand cDNA synthesis supermix was obtained from Trans-Gen Biotech Co., Ltd. (Beijing, China).

2.2. Isolation and Culture of hUC-MSCs. Umbilical cord was rinsed with D-Hank's medium thoroughly and then stored in H-DMEM/F-12 culture medium under aseptic conditions at 4°C. The blood vessel including artery and vein were removed. The umbilical cord mesenchymal tissue was cut into small pieces in the size of 1 mm and was digested by using 0.2% collagenase II. To obtain the primary cells, the digested tissue in the solution was placed in a culture flask containing 2.0 ng/ml EGF, 20% FBS, 25.0 mM L-Glu, and 100.0 U/ml penicillin-streptomycin mixture at 37°C with 5% CO_2 and saturation humidity. The culture medium was then half-changed every 24 hour and replenished every 3 days. When 80–90% confluence was achieved, cells were then rinsed twice with phosphate-buffered saline (PBS) containing trypsin (0.25%) and EDTA (0.2 g/l) to further digest into single cells for passaging at the ratio of 1 : 3. The culture medium of H-DMEM/F-12 contained 100 U/ml of a penicillin-streptomycin mixture and 10% FBS [14].

2.3. Analysis of Cellular Phenotype of hUC-MSCs. In the logarithmic phase of hUC-MSCs growth, trypsin was used to digest the hUC-MCCs to individual cells, and the suspension was aliquot into tubes containing 1×10^6 cells/tube. The mouse anti-human monoclonal antibodies CD11-PE, CD105-PE, CD73-PE, CD45-PE, CD90-PE, CD19-FITC, human leukocyte antigen- (HLA-) DR-PE, and CD34-FITC (each 5 μl) were, respectively, added to 8 tubes. Anti-mouse IgG1-FITC and anti-mouse IgG1-PE (each 7 μl) were applied in other 2 tubes as isotype controls. These tubes were then well mixed and incubated for 30 min at 4°C. The isolated cells were then analyzed by flow cytometry.

2.4. Differentiation of hUC-MSCs into Neuron-Like Cells. At the P5 stage, the hUC-MSCs were passed into six-well plates and 25 cm^2 cell-culture bottle with the complete culture medium. One six-well plate and three 25 cm^2 culture bottles were selected randomly into one group. Four groups of hUC-MSCs were treated with resveratrol at concentrations of 0.0, 7.5, 15.0, and 30.0 mg/L in L-DMEM culture medium. The expressions of GFAP, nestin, and NSE were detected by immunohistochemical staining at the 4 h treatment. The mRNA and protein expressions of GFAP, nestin, and NSE were determined by RT-PCR and Western blot at 2 h, 4 h, 6 h, 12 h, and 24 h after induction.

2.5. Identification of Differentiated Cells. The nestin, NSE, and GFAP were detected and analysis by immunocytochemistry after 4-hour incubation. Briefly, the cultured cells were gently rinsed once with PBS and then fixed with 4% paraformaldehyde at room temperature for 20 min. After rinsing three times with PBS, the cells were immersed into 0.5% Triton X-100 in PBS for 15 min followed by incubation with 3% H_2O_2 at room temperature for 5 min. The cells were blocked with normal goat serum blocking agent for 15 min and then incubated with primary antibodies against NSE, GFAP, and nestin at 4°C overnight. The appropriate biotinylated secondary antibodies were then applied for 15 min and then incubated with horseradish peroxidase-conjugated streptavidin for 1 min. Finally, the cells were stained with freshly prepared DAB for 1 min and counterstained with hematoxylin.

2.6. Western Blot Analysis. Cell lysates were obtained by using RIPA buffer and phenylmethylsulfonyl fluoride (Beijing Solarbio Science & Technology Co., Ltd.). The protein concentrations were quantified by an ultramicro ultraviolet visible light meter (Gene Company, Ltd.). Each sample of 16 μg protein was loaded and electrophoresed in 10% SDS-PAGE gels, which were transferred to polyvinylidene fluoride membrane (PVDF) (Beijing Solarbio Science & Technology Co., Ltd.). After being blocked with 10% nonfat milk for 2 hours, the PVDF membranes were incubated with primary antibodies against GFAP, nestin, and NSE at 4°C overnight.

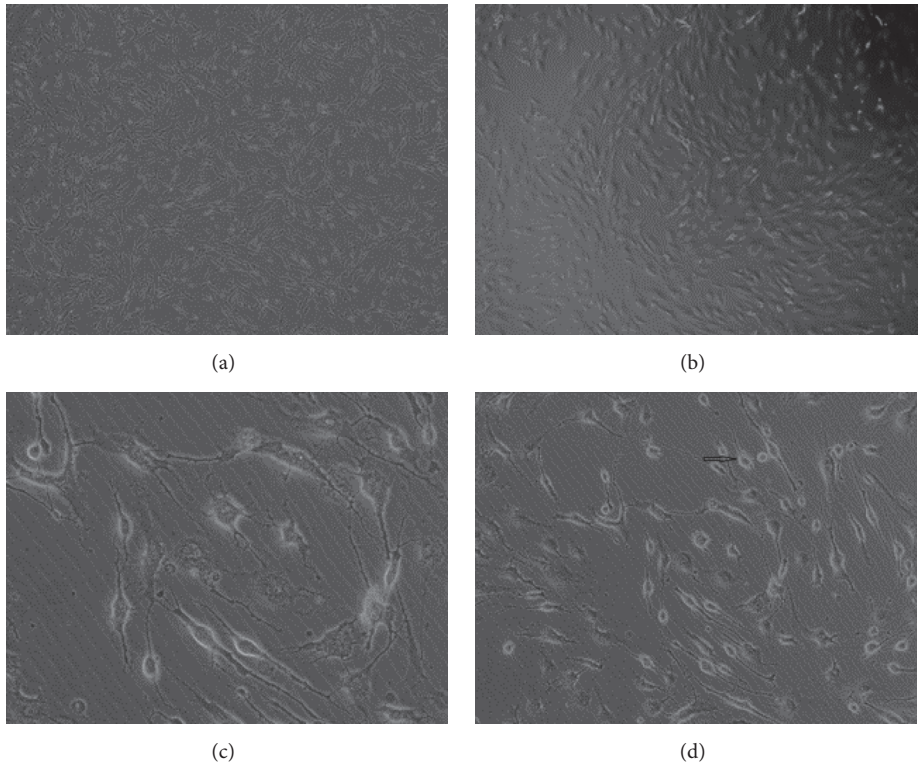


Figure 1: Images showing that different concentrations of resveratrol (0.0 mg/L (a), 7.5 ml/L (b), 15.0 mg/L (c), and 30.0 mg/L (d)) induce differentiation of hUC-MSCs into neuron-like cells.

On the second day, the membranes were incubated with anti-rabbit IgG (1 : 2,000; Affinity Biosciences). Images were visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and captured by using the Image AlphaEaseFC system (Alpha Innotech Co., San Leandro, CA, USA).

2.7. RT-PCR. Total mRNA was extracted by TriPure Reagent (Aidlab Biotechnologies Co., Ltd., Beijing, China) and quantified by ultramicro ultraviolet visible light meter (Gene Company, Ltd., Hong Kong, China). Total mRNA were first reversely transcripted to cDNA by using EasyScript First-Strand cDNA synthesis supermix (TransGen Biotech Co., Ltd.). Then the following protocol was used for semiquantitative PCR: 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 30 sec, and 72°C for 30 sec; and a final extension at 72°C for 10 min. The PCR product was separated by electrophoresis, and the interested genes were normalized by GAPDH transcription. The primers were designed by Primer 5.0 as the below: nestin Forward: TCCAGAAACTCAAGCACCACT Reverse: TCCACCGTATCTTCCCACCT 342 bp; NSE Forward: GGCACTCTACCAGGACTTTG Reverse: GCGATGACTCACCATAACCC 286 bp; GFAP Forward: GTCCATGTGGAGCTTGAC Reverse: CATTGAGCAGGTCCTGGTAC 406 bp; GAPDH Forward: AGAAGGCTGGGGCTCATTTG Reverse: AGGGGCCATCCACAGTCTTC 258 bp.

2.8. Statistical Analysis. All the data were expressed as Mean ± SEM. Statistical analysis was performed by SPSS 21.0. One-way ANOVA was performed in three or more groups comparison followed by Student-Newman-Keuls method (q-test) between groups. If the data distribution did not fit the normality or homogeneity of variance, the nonparametric rank sum test (K-W H test) was the performed. $P < 0.05$ was considered to be significant difference.

3. Result

3.1. Resveratrol Induced Morphological Changes of hUC-MSCs into Neuron-Like Cells. Primary cultured cells were passaged at a ratio of 1 : 1 and the hUC-MSCs were passaged at the ratio of 1 : 3 or 1 : 2. After being passaged continuously, the hUC-MSCs still had strong ability of proliferation. There was no significant change in hUC-MSCs treated with resveratrol at 7.5 mg/L compared with the control condition. Treatment with resveratrol (15.0 mg/L) for 6 h changed the shape of hUC-MSCs from circular to bipolar cells in oval or quasi-circular. The ratio of neuron-like cells was about 5% (Figure 1). However, with resveratrol (30.0 mg/L) treatment of hUC-MSCs for 1 h, the ratio of neuron-like cells reached 50%. The number of multistage cells is significantly higher than the numbers in hUC-MSCs treated with resveratrol at 15.0 mg/L. Furthermore, the cells appeared connected into a net-like pattern (Figure 1). After 6 h treatment with

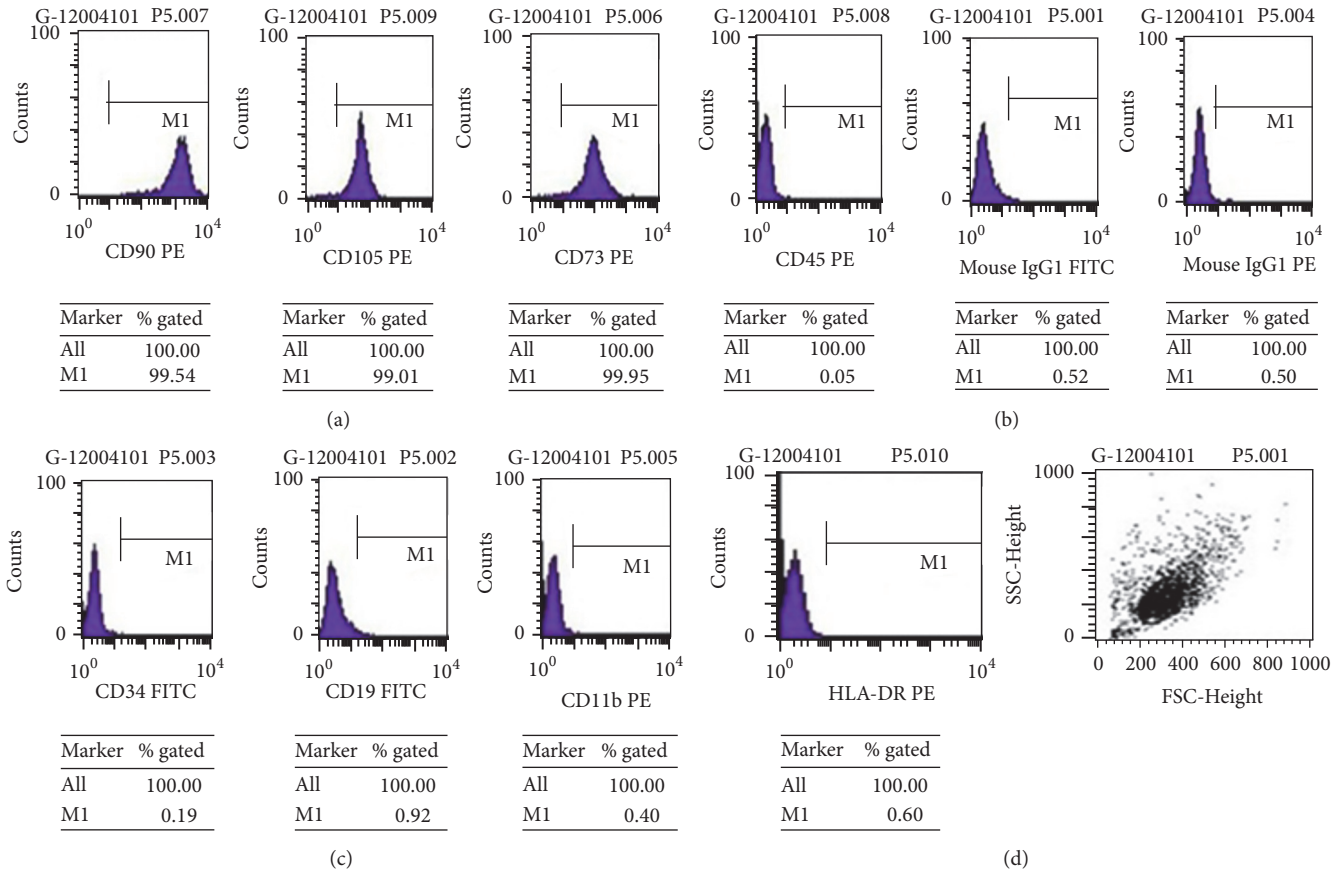


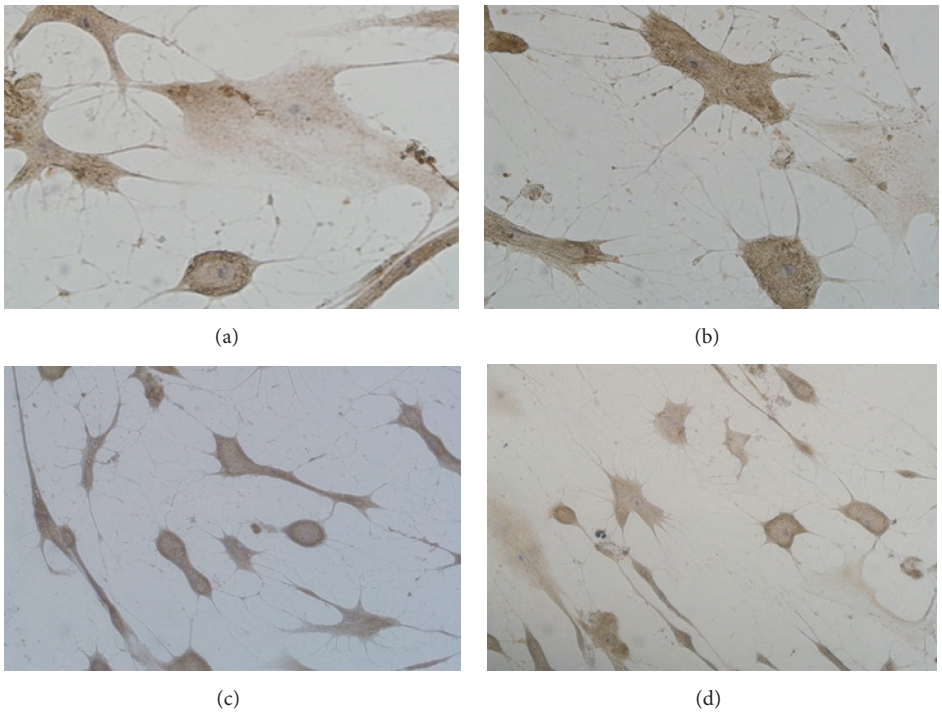
Figure 2: Fluorescence-labeled cell sorting analysis from hUC-MSCs of (a) CD90, CD105, and CD73; (b) CD34, CD19, and CD11b; (c) CD45; and HLA-DR and FSC-Height.

resveratrol (30 mg/L), 85%~90% of the hUC-MSCs displayed neuron-like shape (Figure 1). However, the ratio of neuron-like cells reduced to 70% and 80% after 24 h treatment with resveratrol.

3.2. Cell Surface Markers. We analyzed the surface antigens at the stages of P2, P5, and P10 of hUC-MSCs with flow cytometry. CD105, CD73, and CD90 were expressed on hUC-MSCs at the stages of P2, P5, and P10. Stages P7 and P8 showed signs of reduced ability of proliferation compared with P2 to P5. Thus, we choose to use P5 to represent the early passages to test the effect of resveratrol on the cell surface markers. In addition, no apparent morphological changes of hUC-MSCs were observed from stages of P5 to P8. CD11b, CD45, CD34, CD19, and HLA-DR were negative in hUC-MSCs at stages of P2, P5, and P10 (Figure 2).

3.3. Resveratrol Induced Immunoreactivities of Nestin, NSE, and GFAP. Nestin and NSE are specifically expressed on neurons and have been used as putative neuronal markers; we determine the effect of resveratrol on immunoreactivities of nestin and NSE in cultured hUC-MSCs. The hUC-MSCs were immunostained with antibodies against nestin and NSE. The brown color cell bodies and purple nucleus were nestin- or NSE-positive cells (Figure 3). Nestin or NSE-positive immunoreactivities were found in hUC-MSCs treated with resveratrol at a concentration of 15.0 and 30 mg/L. To determine if resveratrol differentiates hUC-MSCs into glia, GFAP immunoreactivity was also determined by using antibodies against GFAP. GFAP immunoreactivities were negative in hUC-MSCs treated with vehicle and resveratrol at all concentrations (Figure 3). Resveratrol treatment increased the nestin and NSE-positive cell number at concentrations of 15.0 and 30.0 mg/L compared with the vehicle-treated cells ($P < 0.01$). However, 7.5 mg/L resveratrol treatment did not change nestin and NSE-positive cell numbers ($P > 0.05$).

3.4. Resveratrol Increased mRNA and Protein Levels of Nestin and NSE. We then measured mRNA level of GFAP, nestin, and NSE in vehicle-treated and resveratrol-treated cultured hUC-MSCs. The GFAP, nestin, and NSE mRNA levels were measured by RT-PCR before and after treatment with resveratrol (30 mg/L) for 2 h, 4 h, 6 h, 12 h, and 24 h. The GFAP mRNA was not detectable in hUC-MSCs treated with vehicle and resveratrol at all concentrations. Nestin mRNA levels were gradually increased and reached a peak value at 4 h after resveratrol treatment. Furthermore, NSE mRNA levels were also increased and reached the peak value when hUC-MSCs were treated for 6 h (Figure 4).



(a) (b) (c) (d)

Figure 3: Immunostaining images showing the effect of resveratrol (15.0 and 30.0 mg/L) on neuron markers nestin and NSE on hUC-MSCs. (a) Nestin and (b) NSE immunochemical staining of hUC-MSCs treated with resveratrol at a concentration of 15.0 mg/L. (c) Nestin and (d) NSE immunochemical staining of hUC-MSCs treated with 30.0 mg/L resveratrol.

Consistently with these changes of nestin or NSE mRNA levels in response to treatment with resveratrol, the protein levels of nestin or NSE have similar changes in response to resveratrol treatment. Resveratrol treatment increased the protein levels of nestin and reached a peak value 4 h after resveratrol treatment. Also, resveratrol treatment increased NSE protein levels and reached a peak value 6 h after resveratrol treatment (Figure 4).

4. Discussion

Many agents induce differentiation of MSCs to neuron-like cells through distinct mechanisms. For example, β-mercaptoethanol induces MSCs to differentiate into neuron-like cells through modulating cAMP concentration to increase protein kinase A (PKA) activity [15]. It has been shown that EGF directly induces differentiation of MSCs and promotes mitosis to maintain growth and survival of synapses [16]. The acanthopanax suppresses p65 trafficking from the cytoplasm towards the cell nucleus to inhibit the downregulation of IκBα. Also, acanthopanax inhibits NF-κB to relieve the obstruction of differentiation of stem cells to promote differentiation [17, 18]. Chen et al. [19] have demonstrated that the activation of gene GAP-43 importantly regulates differentiation through influencing the growth and extension of synapses, from which the injured nerve starts to repair. Wu et al. [20] have confirmed that miRNA-128 induces differentiation of BMSCs into neuron-like cells when Wnt3a gene expression is downregulated.

Resveratrol exerts many pharmacological functions such as anticardiovascular diseases, antitumor, anti-inflammation, neuron-protection, antiaging, and antioxidation. Previous studies have shown that resveratrol affects the expression levels of proangiogenic related factor in the recovery phase of ischemia-reperfusion brain tissues and exerts positive effect on hemodynamics and revascularization in ischemic brain tissue [21]. Furthermore, resveratrol inhibits oxidative stress via activation of SIRT1 [22–24]. It has been shown that resveratrol may decrease the expression levels of MMP-2 through inhibiting the activity of NF-κB and subsequently reducing the invasion of glioma [25, 26]. In addition, resveratrol reduces the number of macrophages and neutrophils, inhibits H-thymidine, and induces tumor cells to differentiate into nonpropagative phenotype cells. These studies suggest that resveratrol inhibits development of tumor [27]. Resveratrol at different doses can alleviate the injury in rat cortical neural stem cells, which is caused by oxygen glucose deprivation/reoxygenation (OGD/R) in vitro in different extent. In addition, resveratrol can promote the proliferation of cortical neural stem cells [28].

This is the first study showing that resveratrol treatment induced differentiation of hUC-MSCs into neuron-like cells. In this study, we observed the cellular markers CD 105, CD73, and CD90 rather than other cellular and molecular traits for cell senescence. These markers did not show any changes in response to resveratrol treatment compared with vehicle treatment. However, we cannot rule out the possibility that other cellular and molecular traits are altered

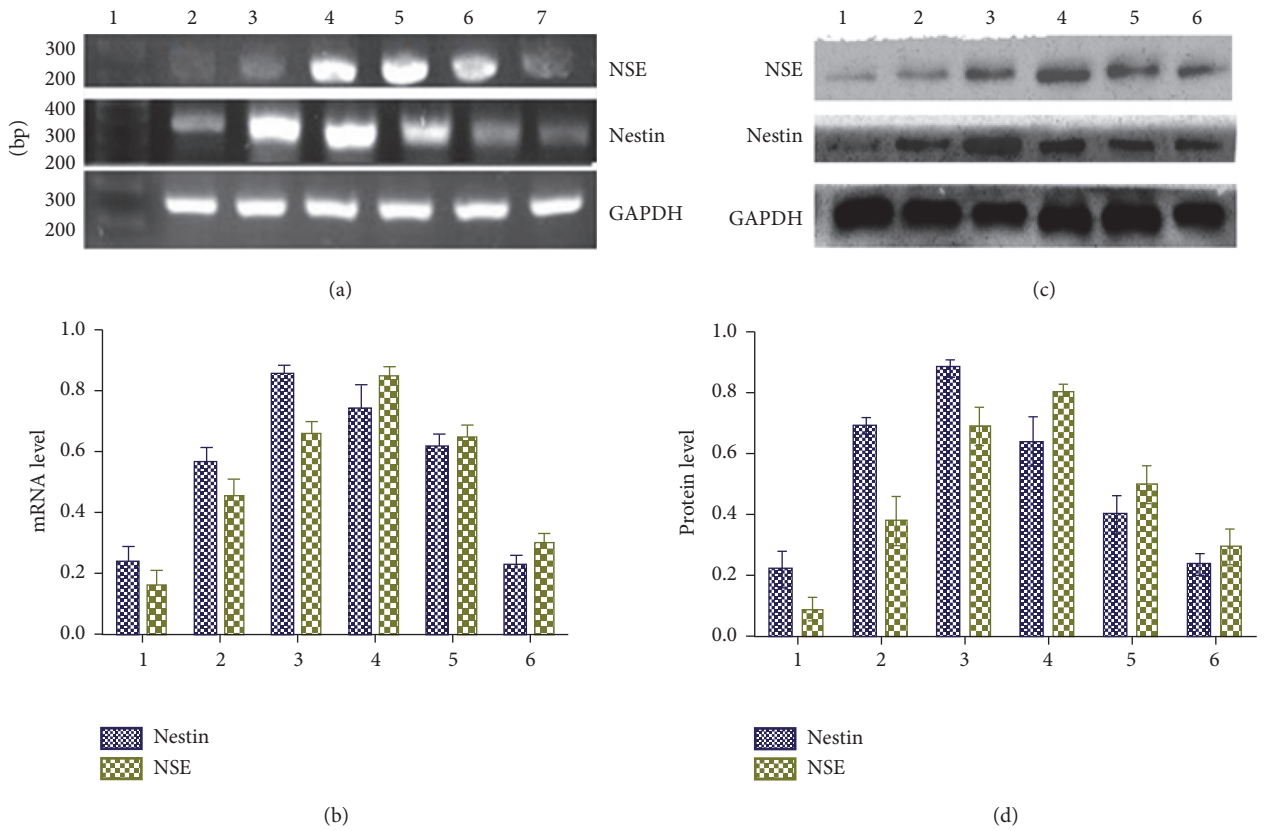


Figure 4: mRNA and protein levels of nestin and NSE in resveratrol-treated hUC-MSCs. (a) Original gel images and (b) summary data showing mRNA levels of nestin and NSE in resveratrol-treated hUC-MSCs at different time points. (c) Gel images and (d) summary data showing protein levels of nestin and NSE in resveratrol-treated hUC-MSCs at different time points. 1–6: period of resveratrol treatment for 0 (control), 2 h (3), 4 h (4), 6 h (5), 12 h (6), and 24 h (7).

in response to resveratrol treatment. It is very important to consider if hUC-MSCs at stage of P5 exhibit several signs of senescence in the future studies. In this study, we detected the corresponding neural markers nestin and NSE by observation of related-positive immunohistochemistry staining and the expression levels of their mRNA and protein. The negative expression of GFAP in hUC-MSCs indicates that resveratrol did not induce differentiation of hUC-MSCs into glia cells. We also determined the optimal treatment time and concentrations for resveratrol to induce hUC-MSCs differentiating into neuron-like cells. We found that resveratrol treatment significantly increased the mRNA and protein expression levels of neuronal markers nestin and NSE. Furthermore, the nestin mRNA and protein expression levels reached peak values after treatment with resveratrol for 4 h, while the NSE mRNA and protein expression levels reached peak values after treatment with resveratrol for 6 h. In addition, we found that the expression of GFAP was negative in both resveratrol-treated and vehicle-treated hUC-MSCs, indicating that resveratrol treatment did not induce hUC-MSCs differentiation into glia cells. Although previous studies have shown that hUC-MSCs induce themselves to express their associated neural markers partially [29], we found very low expression levels of neuronal markers nestin and NSE in vehicle-treated and low resveratrol-treated hUC-MSCs in our preparation.

In this study, we found that a suitable concentration for resveratrol (30 mg/L) induced hUC-MSCs differentiation into neuron-like cells. Although resveratrol activates signaling pathways involving cAMP, Epac1, and AMPK to activate SIRT1 indirectly, it is well accepted that the beneficial effect of resveratrol is through directly activating SIRT1 [30–32]. It is not clear which signaling pathways are involved in resveratrol induced differentiation of hUC-MSCs into neuron-like cells. Thus, further studies are warranted to determine these mechanisms.

In summary, findings from our study suggest that the resveratrol can induce hUC-MSCs to differentiate into neuron-like cells effectively at certain concentrations. This novel finding provides detailed information on resveratrol induced hUC-MSCs differentiation into neuron-like cells. This information may help to develop stem cell implantation-based therapy for ischemic neuronal damage.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Li Guo, Liang Wang, and Li Wang are co-first authors.

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Research Article

Cadherins Associate with Distinct Stem Cell-Related Transcription Factors to Coordinate the Maintenance of Stemness in Triple-Negative Breast Cancer

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Triple-negative breast cancer (TNBC) is an aggressive type of breast cancer with poor prognosis and is enriched in cancer stem cells (CSCs). However, it is not completely understood how the CSCs were maintained in TNBC. In this study, by analyzing The Cancer Genome Atlas (TCGA) provisional datasets and several small-size breast datasets, we found that cadherins (CDHs) 2, 4, 6, and 17 were frequently amplified/overexpressed in 47% of TNBC while E-cadherin (CDH1) was downregulated/mutated at 10%. The alterations of CDH2/4/6/17 were strongly associated with the elevated levels of several stem cell-related transcription factors (SC-TFs) including FOXM1, MCM2, WWTR1, SNAI1, and SOX9. CDH2/4/6/17-enriched genes including FOXM1 and MCM2 were also clustered and regulated by NFY (nuclear transcription factor Y) and/or EVI1/MECOM. Meanwhile, these SC-TFs including NFYA were upregulated in TNBC cells, but they were downregulated in luminal type of cells. Furthermore, small compounds might be predicted via the Connectivity Map analysis to target TNBC with the alterations of CDH2/4/6/17 and SC-TFs. Together with the important role of these SC-TFs in the stem cell regulation, our data provide novel insights into the maintenance of CSCs in TNBC and the discovery of these SC-TFs associated with the alterations of CDH2/4/6/17 has an implication in targeted therapy of TNBC.

1. Introduction

Breast cancer can be classified depending on the status of estrogen receptor (ER) and/or progesterone receptor (PGR) and epidermal growth factor receptor 2 (ERBB2/HER2) in clinic [1]. When all three markers ER, PGR and HER2 are negative in a tumor, it is called triple-negative breast cancer (TNBC). TNBC represents an aggressive type of breast cancer with poor prognosis [1]. The TNBC group is heterogeneous in nature, consisting of at least two major molecular subtypes including basal-like and claudin-low [2–4]. Both basal-like and claudin-low subtypes are enriched in cancer stem cells (CSCs) [4]. Although a lot of efforts have been made in this field, it is not fully understood how the stem cell population was enriched in TNBC.

Cadherins (CDHs) are a family of adhesion proteins consisting of more than 20 subtypes. These CDHs play important roles in cell-cell or cell-matrix junctions and regulate multiple aspects of fundamental cellular events such as cell polarity, motility, embryonic stem cell self-renewal and differentiation [5]. Among them, E-cadherin (CDH1) is a prototype and has been well characterized in stem cell maintenance and differentiation [6–8]. However, different subtypes of cadherins are expressed in distinct tissue types during development, and may involve in different aspect of cellular behavior. For instance, CDH1 is highly expressed in epithelial cells and modulate epithelial structure remodeling and the maintenance of epithelial stemness [6–8]. CDH2 (N-cadherin) is mostly expressed in the neuronal cells, and also involves in the process of epithelial-to-mesenchymal

transition (EMT), which is correlated to the development of cancer stem cells (CSCs) [9–12]. Since CDH1 is critical for stem cell maintenance and regulation of epithelial cells, it raises a question whether other CDHs are involved in the maintenance of CSCs in breast cancer in addition to CDH1, especially under circumstances when CDH1 is deleted or lost, which remains relatively unexplored. In this study, we analyzed The Cancer Genome Atlas (TCGA) datasets containing over 1000 invasive breast cancer cases. We found that CDH2, 4, 6, and 17 were frequently amplified/overexpressed in breast cancer while CDH1 was downregulated/mutated. These changes affected the expression of several stem cell-related transcription factors (SC-TFs) such as NFYA and WWTR1, etc. Moreover, based on CDH2/4/6/17-enriched gene profiling, several small compounds might be predicted via the Connectivity Map (CMap) analysis to target TNBC with the alterations of CDH2/4/6/17 and SC-TFs. Altogether, our findings of these SC-TFs associated with the alterations of CDH2/4/6/17 may have an implication in targeted therapy of TNBC.

2. Materials and Methods

2.1. Breast Cancer Samples and Cell Lines Datasets. Breast cancer sample data used in this study were in whole or part based upon data generated by the TCGA Research Network (http://cancergenome.nih.gov/), which include the complete tumors group (n = 960) and triple-negative group (n = 116) from the Breast Invasive Carcinoma (TCGA provisional, n = 1105). TCGA datasets, as of December 15, 2016, contained the experimental data including gene mutations, copy number alterations (CNA), mRNA and protein expression and clinical data, and were retrieved from cBioPortal for Cancer Genomics (http://www.cbioportal.org/) [13]. Other breast cancer datasets used here including Gene Expression Omnibus (GEO) GSE3971 [14] and European Genome-phenome Archive (EGA) EGAS00000000083 [15] were accessed and retrieved from Oncomine (https://www.oncomine.org/). Breast cancer cell lines datasets (GEO GSE36139 and ArrayExpress E-MTAB-181) [16, 17] were retrieved from Cancer Cell Line Encyclopedia (CCLE) portal (http://www.broadinstitute.org/ccle) or UCSC Cancer Genomics Browser (http://xena.ucsc.edu/).

2.2. Data Analysis. Alterations of interested genes including amplifications, deletions, mutations, and up- or downregulation were retrieved from TCGA datasets with cBioPortal [13] or from other indicated datasets with Oncomine (https://www.oncomine.org/). Gene set enrichment analysis data (including mRNA level or RPPA [reverse phase protein assay]-based protein level) and survival data were also retrieved from cBioPortal with default parameters unless otherwise indicated. Gene Ontology (GO)/KEGG (Kyoto Encyclopedia of Genes and Genomes) or Reactome pathway analysis and transcription factor discovery were performed through the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.8 (https://david-d.ncifcrf.gov/). Heatmap was created from GenePattern according to the instructions (https://genepattern.broadinstitute.org/). Expression levels of stem cell-related transcription factors (SC-TFs) and several CDHs were retrieved from UCSC Cancer Browser with default parameters.

All CDH genes (n = 27) screened in this study include CDH1 (E-cadherin), CDH2 (N-cadherin), CDH3 (P-cadherin), CDH4 (R-cadherin), CDH5 (VE-cadherin), CDH6 (K-cadherin), CDH7 (cadherin 7), CDH8 (cadherin 8), CDH9 (T1-cadherin), CDH10 (T2-cadherin), CDH11 (OB-cadherin), CDH12 (N-cadherin 2), CDH13 (H-cadherin), CDH15 (M-cadherin), CDH16 (KSP-cadherin), CDH17 (LI-cadherin), CDH18 (cadherin 18), CDH20 (cadherin 20), PCDHGA12 (CDH21), CDH22 CDH23 CDH24 DCHS1 (CDH19, or CDH25), CDH26 DCHS2 (CDH27), CDHR3 (CDH28), and CDHR4 (CDH29).

SC-TFs used here include CTNNB1 (β-catenin), FOXM1, FOXO3, GLI2, HIF1A, HMGA1B, KLF4, MAF (c-MAF), MCM2, NANOG, POU5F1 (Oct-3/4), PRDM14, SNAI1 (Snail), SOX2, SOX9, STAT3, WWTR1, TBX3, TWIST1, ZEB1, LIN28A, LIN28B, and MYC (c-Myc) [18–30].

2.3. Flow Cytometry. Stem cell population of triple-negative (or claudin-low) breast cancer cells MDA-MB231 and luminal cells SKBR3 was determined by flow cytometry with stem cell marker $CD24^{-/low}CD44^{+/high}$. Fluorochrome-conjugated monoclonal antibodies against CD24 (FITC, Cat# 555478) and CD44 (PE, Cat# 555428) were purchased from BD Biosciences (San Diego, CA, USA). Fluorescent staining of CD24 and CD44 was performed as described elsewhere [31]. The labeled cells were finally analyzed on a FACS LSRII (BD Biosciences). The experiments were independently repeated.

2.4. Connectivity Map (CMap). CDH2/4/6/17-enriched gene signature identified in this study, which included 101 genes with 12 downregulated and 89 upregulated, was used as a query for the CMap analysis according to the instructions (http://www.broadinstitute.org/cmap/) [32, 33]. CMap analyzes the association (i.e., the positive or negative correlation) between the given test signature and gene expression profiles of cell lines treated with specific concentrations of various drugs (perturbagens).

3. Results

3.1. CDH2/4/6/17 High/Amplified in Cooccurrence with CDH1 Low/Mutated Is Associated with TNBC. As previously known, CDH1 is highly expressed in ductal invasive breast cancer, but low or absent in lobular invasive breast cancer [34, 35]. In terms of clinical classification based on ESR1/PGR or HER2 status, CDH1 expression is reduced in TNBC [36, 37]. Then the question arises what are the status of other CDHs in TNBC. Are they upregulated or downregulated or unchanged? Here, we wanted to determine whether the expression of other CDHs is altered in breast cancer, especially in TNBC since CDH1 is mostly downregulated or mutated in TNBC [36, 37]. To investigate the status of CDHs in breast cancer, we screened 27 members

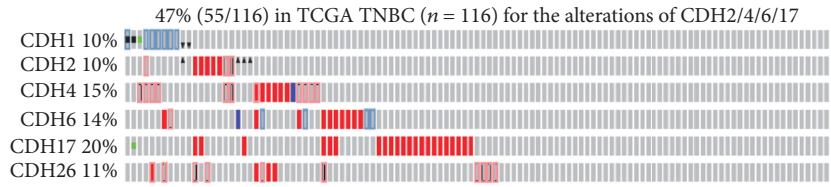
of CDHs in TCGA datasets and found that CDH1 was indeed low expressed or mutated in 16% of TCGA samples, and other CDHs (especially CDH17, CDH4, CDH26, CDH3/8, and CDH2/6/12) were almost highly expressed or amplified (Supplementary Figure S1 in Supplementary Material at https://doi.org/10.1155/2017/5091541). In TNBC, CDH1, CDH2, CDH4, CDH6, CDH17, and CDH26 also exhibited marked alterations (Figure 1(a), Supplementary Figure S2). Since CDH26 (11%) had a tendency to cooccur with CDH4 (15%) (Figure 1(a)), we herein selected CDH1/2/4/6/17 for further study. As for CDH1, our data showed that it had positive correlations with ESR1 or HER2 (Supplementary Table S1), implying that CDH1 might be downregulated in TNBC (ESR1-/PGR- and HER2-). Actually, we found CDH1 was low expressed in TNBC than other breast cancers in a TCGA cohort (Figure 1(b)). However, CDH2/4/17 were remarkably altered in TNBC with high expression in most cases (Figure 1(b)). Also, we observed that CDH1 mRNA level was downregulated in most triple-negative cell lines from CCLE datasets (Figure 1(c)). Conversely, CDH2/4/6/17 (especially CDH2, 4, and 6) mRNA levels were aberrantly upregulated in these triple-negative cell lines (Figure 1(c)). Furthermore, CDH1 low was associated with poor overall survival of TNBC patients (Figure 1(d)). Therefore, these findings indicated that CDH2/4/6/17 high combined with CDH1 low might be of importance in TNBC.

3.2. Altered Expression of Stem Cell-Related Transcription Factors Is Concurrent with the Alterations of CDH2/4/6/17. Since CDH1 is vital for breast epithelial stem cell remodeling [5–7, 38–40], we wanted to answer the question if CDH2/4/6/17 are involved in breast CSCs under the condition of loss of CDH1. One way that the alterations of CDH2/4/6/17 influence the behavior of CSCs is through the changes in stem cell-related transcription factors (SC-TFs). Here, we selected 23 SC-TFs (listed in the Methods section) and screened their associations with the alterations of CDH2/4/6/17 in TCGA datasets via cBioPortal. As shown in Table 1, there were 5 SC-TFs including MCM2, WWTR1, FOXM1, SNAI1, and SOX9, having a tendency to be cooccurrent with the alterations of 3-4 CDHs of CDH2, 4, 6, and 17. Meanwhile, RPPA data confirmed that 4 SC-TFs (FOXM1, WWTR1, SNAI1, and MYC) were highly expressed with the alterations of CDH2/4/6/17 (Table 2); 3 out of these 4 SC-TFs belonged to the aforementioned 5 SC-TFs. In contrast, the alteration events (mutations or deletions) of CDH1 were mutually exclusive with high-level expressions of 9 out of 14 SC-TFs (Supplementary Tables S2), suggesting that CDH1 might be not a key protein for the alterations of SC-TFs. Taken together, these findings indicated that the alterations of SC-TFs were cooccurring with the alteration events of CDH2/4/6/17.

3.3. CDH2/4/6/17-Associated Gene Enrichment Is Regulated by Stem Cell-Related Transcription Factors. We next established CDH2/4/6/17-enriched gene signature to analyze whether the enriched genes can be regulated by SC-TFs. This is a different way to show the relationship between the alterations of CDH2/4/6/17 and SC-TFs. As shown in Figure 2(a), we first found that 199 genes were overlapped among CDH1- and CDH2-enriched genes; after CDH2-, CDH4-, CDH6-, and CDH17-enriched genes were considered together, we generated a refined list of CDH2/4/6/17-associated genes (101 genes) (Figure 2(a), Supplementary Table S3), which were all contained in CDH1/2-enriched genes except 2 genes (KCNE4 and ZDHHC1). CDH2/4/6/17-associated genes were mainly involved in mitotic cell cycle and DNA replication/repair (Figure 2(b)). Further analysis showed that these genes possibly participated into several signaling pathways including the MAPK-ERK pathway and the PI3K-mTOR pathway (data not shown). Among the CDH2/4/6/17-associated 101 genes, top 24 genes were strongly correlated with the alterations of CDH2/4/6/17 (Figure 2(c)). Also, 20 out of these 24 genes were highly altered in TNBC (Figure 2(d), compared to Supplementary Figure S3). Meanwhile, the aforementioned two transcription factors (MCM2 and FXOM1) were identified in this gene signature and highly expressed in the samples with the alterations of CDH2/4/6/17 (Supplementary Table S3).

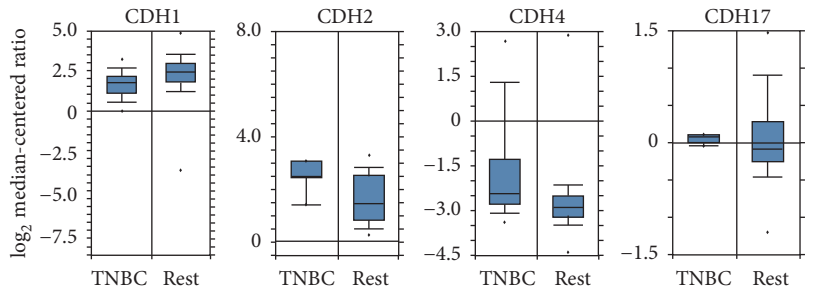
To further examine the effect of CDH2/4/6/17 on CSCs, we performed transcription factor discovery analysis by using DAVID Bioinformatics Resources 6.8. Interestingly, CDH2/4/6/17-enriched genes including two SC-TFs (MCM2 and FOXM1) were mainly grouped into a cluster that can be regulated by Nuclear Transcription Factor Y (NFY) and/or MECOM (EVI1) (Table 3), both of which have been reported to be involved in stem cell regulation [41, 42]. Therefore, our findings suggested that NFYs and MECOM might be the master transcription factors responsible for gene regulation and stem cell maintenance under the alterations of CDH2/4/6/17.

3.4. CDH2/4/6/17-Enriched Stem Cell-Related Transcription Factors Are Upregulated in TNBC. SC-TFs play important roles in the maintenance of breast cancer stem cells as a whole. To gain insights into the changes of SC-TFs in clinical subtypes, especially in TNBC, we first analyzed the correlation of SC-TFs with clinical markers (ESR1, PGR, and HER2). We found that two SC-TFs such as FOXM1, MYC exhibited modest negative correlation with ESR1/PGR or HER2 status (Supplementary Table S4). To further investigate the status of SC-TFs in TNBC, we analyzed the levels of CDH2/4/6/17-associated SC-TFs in the samples and cell lines of TNBC. All 7 SC-TFs (i.e., FOXM1, WWTR1, NFYA, MCM2, SOX9, MECOM, and SNAI1) were found to be amplified and/or upregulated in TCGA TNBC samples; the rates of their alterations were much higher than those in TCGA total breast samples (Figure 3(a) and Supplementary Figure S4). Next, we retrieved the levels of 6 out of 7 SC-TFs (since no available data for MECOM) in breast cancer cell lines [17] from UCSC cancer genomics browser. Most of 6 SC-TFs were consistently upregulated in TNBC cell lines including basal-like and claudin-low (Figure 3(b)). Conversely, these SC-TFs were downregulated in luminal subtype of breast cancer cells (Figure 3(b)). In parallel, the proportion of cancer stem cell population determined by $CD24^{-/low}CD44^{+/high}$ was simultaneously higher in TNBC cell lines than other subtypes of breast cancer cell lines (Table 4). For instance, in claudin-low (TNBC) MDA-MB231 cells, there were 69.6%

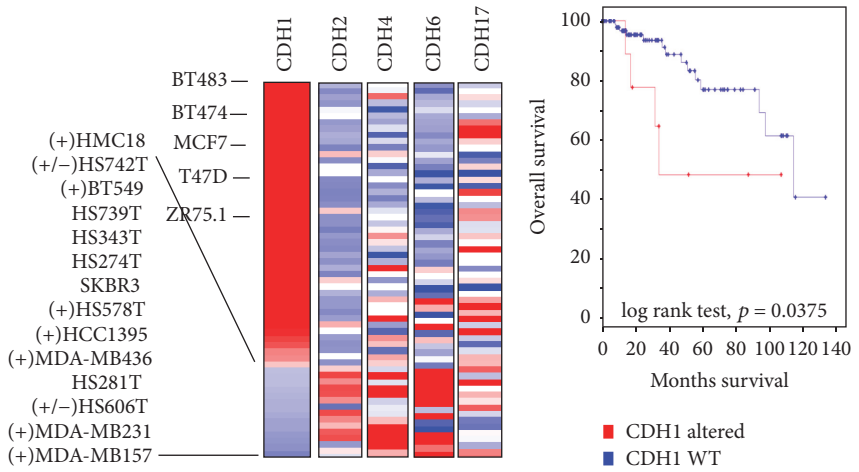


Gene A	Gene B	*p* value	Log odds ratio	Association
CDH4	CDH17	0.016	<−3	Mutually exclusive, significant
CDH4	CDH26	0.022	1.567	Cooccurrence, significant

(a)



(b)



(c) (d)

Figure 1: Alterations of CDHs in triple-negative breast cancer. (a) Alterations of CDH1, 2, 4, 6, 17, and 26 in TNBC samples from TCGA provisional datasets. The alterations here include deletion, amplification, downregulation, upregulation, and mutations. CDH1 was queried with EXP < −2.0 MUT HOMDEL PROT < −2.0 and other CDHs were queried with default parameters. Microarray data were used for mRNA expression level. The data were retrieved from cBioPortal as of December 15, 2016 (TNBC samples, $n = 116$). CDH, cadherin; TNBC, triple-negative breast cancer; TCGA, The Cancer Genome Atlas. EXP, mRNA expression level; MUT, mutation; HOMDEL, homozygous deletion; PROT, protein level as determined by reverse phase protein assay (RPPA). (b) Expression of CDH1, 2, 4, and 17 in TNBC compared to non-TNBC samples. These data were retrieved as of December 15, 2016, from Oncomine. Rest indicates non-TNBC samples. For CDH1 (from partial TCGA dataset), TNBC, $n = 46$; rest, $n = 250$. For CDH2 (from Curtis breast dataset, European Genome-phenome Archive accession number EGAS00000000083), TNBC, $n = 4$; rest, $n = 17$. For CDH4 (from partial TCGA dataset), TNBC, $n = 49$; rest, $n = 300$. For CDH17 (from Zhao breast dataset, GEO accession number GSE3971), TNBC, $n = 5$; rest, $n = 37$. (c) Expression of CDH1, 2, 4, 6, and 17 in CCLE breast cancer cell lines. The expression data (mRNA level) of CDH1, 2, 4, 6, and 17 were retrieved from breast cancer cell lines dataset (GEO accession number GSE36139) by suing CCLE portal according to the instructions. Red represents upregulation; blue means downregulation. The symbol (+) represents triple-negative breast cancer cells, and (+/−) for TNBC-like cells. CCLE, Cancer Cell Line Encyclopedia. (d) Overall survival of TNBC patients with or without CDH1 alterations. TNBC samples ($n = 116$) were obtained from TCGA provisional dataset and retrieved with cBioPortal. For CDH1 altered: $n = 10$ with median months survival = 33.97; for CDH1 WT: $n = 106$ with median months survival = 114.06.

Table 1: Cooccurrence of stem cell-related transcription factors in association with alterations of CDH2, 4, 6, and 17.

Gene A (CDH)[a]	Gene B (SC-TF)[b]	p value[c]	log odds ratio[d]	Association[e]
CDH2	**MCM2**	<0.001	1.340	Tendency for cooccurrence, significant
	WWTR1	<0.001	1.235	Tendency for cooccurrence, significant
	FOXO3	0.003	1.080	Tendency for cooccurrence, significant
	FOXM1	0.019	0.763	Tendency for cooccurrence, significant
	SNAI1	0.022	0.864	Tendency for cooccurrence, significant
	SOX9	0.099	0.553	Tendency for cooccurrence, marginal
CDH4	**FOXM1**	<0.001	1.256	Tendency for cooccurrence, significant
	MCM2	<0.001	1.181	Tendency for cooccurrence, significant
	SNAI1	<0.001	2.421	Tendency for cooccurrence, significant
	SOX9	<0.001	1.088	Tendency for cooccurrence, significant
	NANOG	0.004	1.281	Tendency for cooccurrence, significant
	POU5F1	0.004	0.949	Tendency for cooccurrence, significant
	HMGA1	0.005	0.854	Tendency for cooccurrence, significant
	MYC	0.017	0.494	Tendency for cooccurrence, significant
	WWTR1	0.057	0.472	Tendency for cooccurrence, marginal
	HIF1A	0.063	0.653	Tendency for cooccurrence, marginal
CDH6	GLI2	<0.001	1.532	Tendency for cooccurrence, significant
	WWTR1	<0.001	1.151	Tendency for cooccurrence, significant
	ZEB1	<0.001	1.737	Tendency for cooccurrence, significant
	FOXM1	0.005	0.827	Tendency for cooccurrence, significant
	KLF4	0.028	1.093	Tendency for cooccurrence, significant
	SNAI1	0.039	0.710	Tendency for cooccurrence, significant
	POU5F1	0.043	0.768	Tendency for cooccurrence, significant
	NANOG	0.062	0.968	Tendency for cooccurrence, marginal
	MCM2	0.071	0.593	Tendency for cooccurrence, marginal
CDH17	PRDM14	<0.001	2.551	Tendency for cooccurrence, significant
	LIN28B	<0.001	1.122	Tendency for cooccurrence, significant
	MYC	<0.001	>3	Tendency for cooccurrence, significant
	FOXM1	0.008	0.556	Tendency for cooccurrence, significant
	SNAI1	0.008	0.662	Tendency for cooccurrence, significant
	STAT3	0.009	0.692	Tendency for cooccurrence, significant
	SOX9	0.035	0.466	Tendency for cooccurrence, significant
	LIN28A	0.040	0.842	Tendency for cooccurrence, significant
	KLF4	0.041	−1.276	Tendency towards mutual exclusivity, significant
	GLI2	0.043	−0.842	Tendency towards mutual exclusivity, significant
	MCM2	0.048	0.460	Tendency for cooccurrence, significant
	TBX3	0.069	−0.693	Tendency towards mutual exclusivity, marginal
	FOXO3	0.073	0.418	Tendency for cooccurrence, marginal
	ZEB1	0.086	−0.653	Tendency towards mutual exclusivity, marginal

Note. [a]CDH, cadherin: here including CDH2, 4, 6, and 17. [b]SC-TF, stem cell-related transcription factor. SC-TFs in bold indicate that their occurrence simultaneously happened with the alterations of 3 or 4 CDHs mentioned in a. [c]p values are derived from Fisher's exact test; p values less than 0.1 are included. [d]log odds ratio indicates the likelihood that the events in genes A and B are mutually exclusive or cooccurrent across the selected cases. The value quantifies how strongly the presence or absence of alterations in gene A is associated with the presence or absence of alterations in gene B in the selected cases. [e]log odds ratio > 0: association towards cooccurrence; log odds ratio ≤ 0: association towards mutual exclusivity; significant means p value < 0.05; marginal means p value is 0.05–0.1.

Table 2: Cooccurrence of SC-TFs with CDH2, 4, 6, and 17 by RPPA analysis[a].

SC-TF	Locus	Expression change	p value[b]	q value[c]
FOXM1	12p13	Upregulation	$1.98E-07$	$6.69E-06$
SNAI1	20q13.2	Upregulation	$2.15E-04$	$1.24E-03$
WWTR1	3q23-q24	Upregulation	$3.86E-03$	0.0136
MYC	8q24.21	Upregulation	0.0159	0.0417

Note. [a]SC-TFs, stem cell-related transcription factors; CDH, cadherin; RPPA, reverse phase protein array. [b]p value is derived from Student's t-test. p value < 0.05 indicates significant. [c]q value is derived from Benjamini-Hochberg procedure. q value < 0.05 indicates significant.

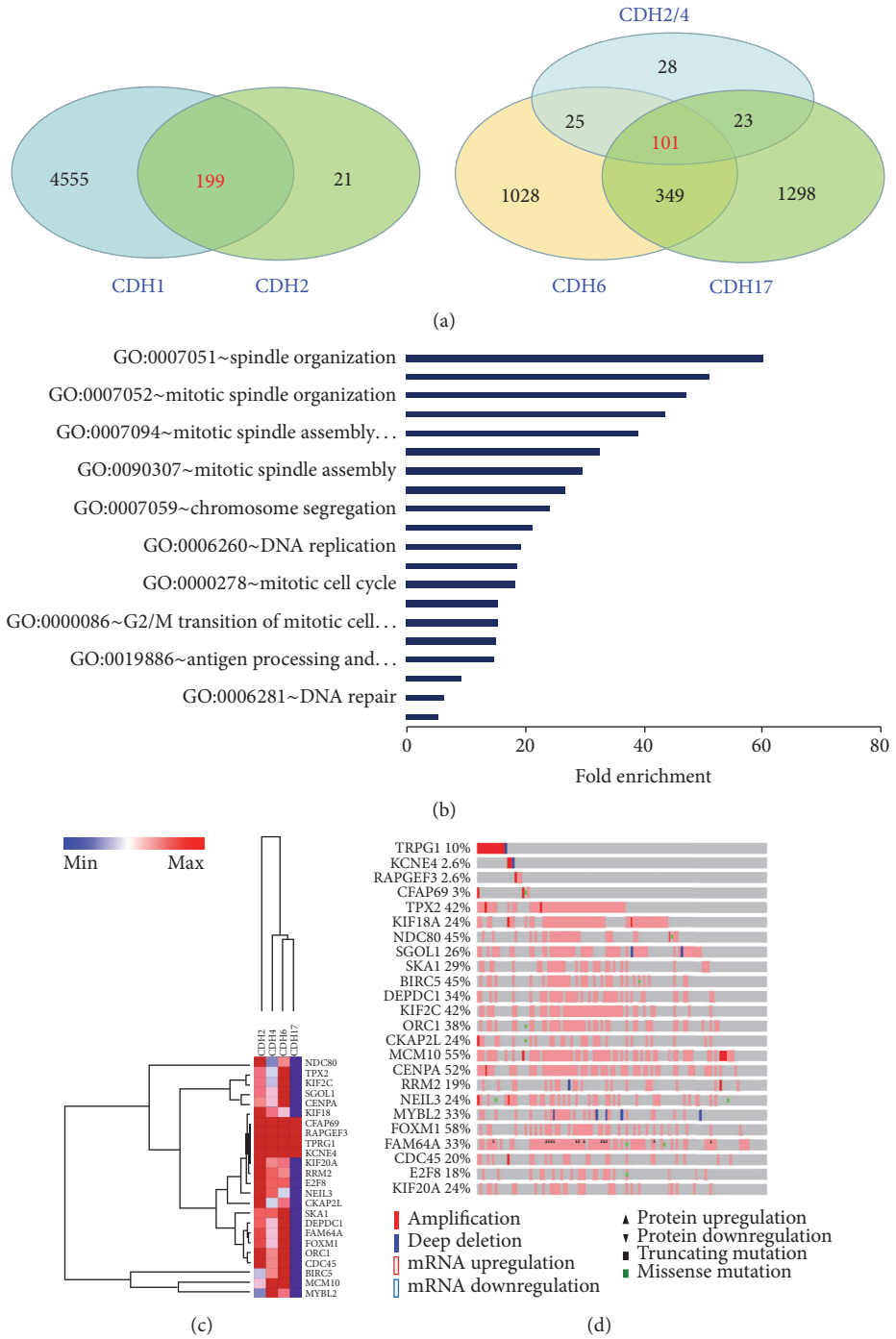


Figure 2: CDH2/4/6/17-enriched genes in breast cancer. (a) Venn diagrams showing the number of the overlaps between CDH1- and CDH2-enriched genes (left panel) and the overlaps between CDH2-, CDH4-, CDH6-, and CDH17-enriched genes (right panel). The enriched genes for each individual altered cadherin were retrieved with cBioPortal enrichments module from TCGA provisional breast datasets (complete tumor group, $n = 960$). CDH1 was queried with EXP < -2.0 MUT HOMDEL PROT < -2.0 and other CDHs were queried with default parameters. The genes were selected with $p < 0.01$ (derived from Student t-test) and $q < 0.01$ (derived from Benjamini-Hochberg procedure). EXP, mRNA expression level; MUT, mutation; HOMDEL, homozygous deletion; PROT, protein level as determined by reverse phase protein assay (RPPA). (b) Gene Ontology (GO) analysis of CDH2/4/6/17-enriched genes with DAVID Bioinformatics Resources 6.8. Fold enrichment indicates the magnitude of enrichment compared to population background regarding a given term. DAVID, Database for Annotation, Visualization, and Integrated Discovery. (c) Clustering analysis of top 24 out of 101 CDH2/4/6/17-enriched genes. Clustering analysis was performed in GenePattern according to the instructions. Min, minimal level; Max, maximal level. (d) Alterations of top 24 from 101 CDH2/4/6/17-enriched genes in TNBC. TNBC samples ($n = 116$) were obtained from TCGA provisional dataset as of December 15, 2016. The data were retrieved from cBioPortal with default parameters for mutations, copy number alterations, mRNA levels, and protein levels.

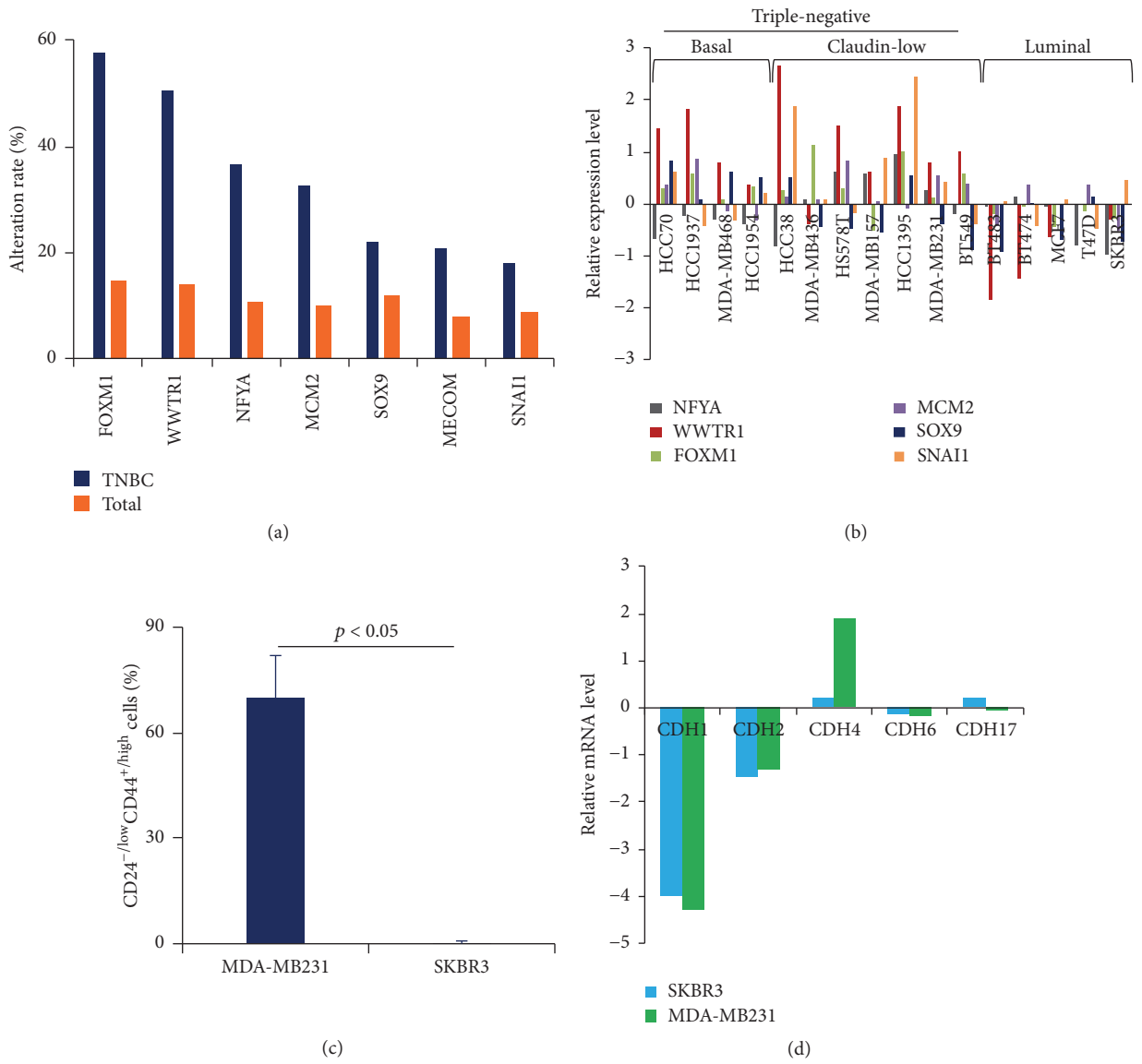


Figure 3: Stem cell-related transcription factors and cancer stem cells are enriched in TNBC cells. (a) The alteration rates of stem cell-related transcription factors (SC-TFs) in TCGA breast cancer samples ($n = 960$) or TCGA TNBC samples ($n = 116$). (b) The expression levels of SC-TFs were elevated in TNBC cells including basal-like and claudin-low. The expression data of the indicated SC-TFs were retrieved from breast cancer cell lines dataset (ArrayExpress accession number E-MTAB-181) by suing UCSC Cancer Genomic Browser according to the instructions. (c) The population of CSCs was higher in basal-like cells MDA-MB231 but lower in luminal subtype of cells SKBR3. The population of CSCs was determined by the staining of stem cell marker $CD24^{-/low}CD44^{+/high}$ followed by flow cytometry. (d) The expression levels of CDH1, 2, 4, 6, and 17 in TNBC claudin-low MDA-MB231 cells and luminal subtype of cells SKBR3. The expression data of CDH1, 2, 4, 6, and 17 in both cell lines were retrieved from the same dataset as described in (b).

of them exhibiting $CD24^{-/low}CD44^{+/high}$, which was dramatically higher than luminal SKBR3 cells (0.21%) (Figure 3(c), Supplementary Figure S5). Furthermore, at least CDH4 (among CDH2/4/6/17) was expressed at a higher level in MDA-MB231 than in SKBR3, while CDH1 was low expressed in both MDA-MB231 and SKBR3 (Figure 3(d)), as reported previously [43–45]. Therefore, these findings strongly suggested that CDH2/4/6/17-SC-TFs axis might play a key role in the enrichment of CSCs in TNBC.

3.5. TNBC Cells with the Alterations of CDH2/4/6/17-SC-TFs Axis May Be Targeted with the Perturbagens Discovered with the Connectivity Map. To evaluate whether TNBC cells with the alterations of CDH2/4/6/17-SC-TFs axis can be targeted, we adopted a web-based resource CMap. The CMap utilizes a pattern-matching algorithm to link the compounds (perturbagens) with physiological or pathological phenotypes by measuring similarities in gene expression, and therefore can be used to predict the affected pathways through the

Table 3: Potential transcription factors predicted using DAVID analysis[a].

Transcription factor[b]	%[c]	Fold enrichment[d]	Statistics	
			p value[e]	Benjamini[f]
NFY	**61.63**	**1.520018153**	**9.59E − 05**	**0.016736215**
EVI1	**86.05**	**1.216541909**	**8.52E − 04**	**0.072283654**[*]
E2F	63.95	1.335510686	0.002616935	0.142494828
MEF2	75.58	1.192911873	0.013046475	0.438881091
MEIS1	50.00	1.305125299	0.023933933	0.573746109
COMP1	52.33	1.270475168	0.031160597	0.604888864

Note. [a]DAVID, Database for Annotation, Visualization, and Integrated Discovery version 6.8. [b]Transcription factors in bold indicate significantly predicted transcription factors. EVI1, also called MECOM. [c]% means the percentage of genes regulated by the indicated transcription factor among the total query genes. [d]Fold enrichment indicates the magnitude of enrichment compared to population background regarding a given term. [e]p value is derived from modified Fisher's exact test (also called EASE score). [f]Benjamini indicates a more conservative method to control family-wide false discovery under certain rate. [*]Marginal significance.

Table 4: Cancer stem cell population in different breast cancer cell lines[a].

Breast cancer cell lines	Stem cell markers[b]		
	$CD24^{-/low}CD44^{+/high}$ (%)	ALDH1 activity	Side population
Basal-like/claudin-low			
MDA-MB157	97.4% [46][c]	14.0 ± 1.8% [47]	
HS578T	65% [48], 85 ± 5% [31], 99.3% [49]		
MDA-MB231	99.9% [46], 85 ± 5 [31], 98.6% [49], most cells [50]	13.0 ± 1.4% [47]	3.40 ± 0.60% [50]
MDA-MB436	72 ± 5% [31]		
HCC1937	17.9% [49]		
BT549	90.3% [46], 16.5% [49]		
Luminal subtype			
MCF-7	0% [31, 46], 0.028% [49], 1% [51]		
MDA-MB453	0% [46]		
T47D	0% [31, 46]		
SKBR3	0% [31, 46]		

Note. [a]The data here are retrieved from the literature. [b]$CD24^{-/low}CD44^{+/high}$ is a more powerful stem cell marker. ALDH1, aldehyde dehydrogenase 1. [c]The numbers in parentheses are the reference numbers.

perturbagens affiliated with known molecular targets and signaling pathways, and discover potential pharmaceutical treatment based on the query signatures [32]. A CMap score from +1 to −1 is assigned based on the degree of similarity or dissimilarity between two signatures [32]. Here, we used the CDH2/4/6/17-associated signature to query the CMap. If a compound with a high CMap score (close to +1), it will have a similar gene pattern to that induced by CDH2/4/6/17 and may act on a parallel pathway induced by CDH2/4/6/17; if the score is close to −1, the perturbagen may counteract the effects induced by the alterations of these CDHs. First, we focused on the perturbagens, which are better related to known molecular targets and signaling pathways. For instance, we found that the CDH2/4/6/17-associated signature was similar to paclitaxel-induced signature (Table 5). Since paclitaxel is a microtubule-damaging agent and functions partially through p70S6K activation via multiple signaling pathways including ERK1/2 MAPK, JNK, PKC, Ca^{++}, PI3K/mTOR [52], it provided evidence to support the finding revealed by analysis of GO/KEGG, as described above (Figure 2(b)). Next, we investigated whether some compounds can be used to target the cases with the CDH2/4/6/17-associated signatures. As shown in Table 6, multiple drugs were identified that had a significantly anticorrelating gene pattern to that induced by CDH2/4/6/17. For instance, resveratrol and its derivatives had been reported to exhibit anticancer activity in TNBC cells possibly through interfering with epigenetic regulation [53, 54]. Thus, our findings provided additional evidence to support the claim that the affected pathways were induced by CDH2/4/6/17 and revealed that the perturbagens predicted here might be used to target TNBC with the alterations of CDH2/4/6/17-SC-TFs axis.

4. Discussion

In this study, we evaluated the status of CDHs in TCGA breast cancer samples, especially in TNBC by using informatics and experimental analyses, and demonstrated that CDH2/4/6/17 themselves and their associated SC-TFs including WWTR1, NFYA, and FOXM1 might be involved in the enrichment of CSCs in TNBC.

CDH1 is an original cadherin and plays a pivotal role in epithelial structure remodeling and maintaining the stemness of stem cells in breast epithelial and cancer cells [5–7, 38–40].

Table 5: Top 10 perturbagens identified through the Connectivity Map that induce the CDH-associated signature.

Pharmaceutical perturbagen	Enrichment CMap score	Rank[a]	n[b]	p value	Description
Trimethobenzamide	0.885	5	5	0.00006	An antiemetic used to prevent nausea and vomiting
Felbinac	0.892	7	4	0.00012	A nonsteroidal anti-inflammatory drug of the arylacetic acid class
Iopamidol	0.886	8	4	0.00018	A radiopaque contrast agent
Diethylstilbestrol	0.738	13	6	0.00085	A synthetic, nonsteroidal estrogen, and a potent agonist of estrogen receptors
Adiphenine	0.789	14	5	0.00092	An inhibitor of nicotinic receptors
Paclitaxel	0.728	17	6	0.00107	A microtubule-damaging agent, affecting mitosis
Thioperamide	0.754	20	5	0.00216	A potent antagonist of histamine receptor H3/H4
Cinchonine	0.813	21	4	0.00237	A multidrug resistance-reversing agent
Diphenhydramine	0.737	25	5	0.00290	An antihistamine agent
Vinburnine	0.796	28	4	0.00334	A vinca alkaloid acting as a vasodilator

Note. [a]Rank by p value. [b]n indicates the number of instances related to this perturbagen tested in the Connectivity Map.

Table 6: Top 10 perturbagens identified through the Connectivity Map that anticorrelated with the CDH-associated signature.

Pharmaceutical perturbagen	Enrichment CMap score	Rank[a]	n[b]	p value	Description[c]
Resveratrol	−0.765	1	9	<0.00001	A stilbenoid, a type of natural phenol
Trifluoperazine	−0.553	2	16	<0.00001	A blocker of dopamine D1/D2 receptor
0297417-0002B	−0.981	3	3	0.00004	N.D.
MG-262	−0.939	9	3	0.00032	A proteasome inhibitor
Apigenin	−0.866	10	4	0.00062	A potent inhibitor of CYP2C9; a monoamine transporter activator; a ligand for central benzodiazepine receptors
Pyrvinium	−0.740	11	6	0.00066	An antinematodal agent
Methotrexate	−0.654	12	8	0.00078	An inhibitor of dihydrofolate reductase (DHFR), participating in DNA repair; a suppressant of immunology
Amiodarone	−0.777	15	5	0.00096	A calcium channel blocker
Piperidolate	−0.908	19	3	0.00142	An antimuscarinic
Acepromazine	−0.811	24	4	0.00251	A phenothiazine derivative antipsychotic drug

Note. [a]Rank by p value. [b]n indicates the number of instances related to these perturbagens tested in the Connectivity Map. [c]N.D., not determined.

However, CDH1 was low expressed in TNBC (Figure 1) [36, 37], and CDH1 alteration events were mutually exclusive with high-level alterations (amplification or upregulation) of most of SC-TFs used in this study in breast cancers (Supplementary Table S2). As such, CDH1 may be not a key protein in the enrichment of CSCs in TNBC. Therefore, it raised a challenging question as to whether other CDHs have an effect on CSCs when CDH1 is low expressed or mutated

in TNBC. Here, we shed lights on the CDHs and provided insights into the potential roles of CDH2, 4, 6, 17 in CSCs of TNBC. We clearly demonstrated that CDH2/4/6/17 alterations including amplifications and upregulation happened at a higher frequency (47%) in TNBC samples (Figure 1), which was correlated with the elevated expression of SC-TFs (Table 1), and also the enrichment of CSCs in TNBC (Figure 3 and Table 4), indicating CDH2/4/6/17 might have a potential role in the accumulation of CSCs in TNBC. As previously reported, CDH2 was often upregulated in cancers including breast cancers and acts as a promoting factor of cancer invasion and metastasis [10, 55, 56]. Moreover, CDH2 can regulate an EMT-like behavior [10], which is one of the properties belonging to the CSCs, and can even directly regulate stem cell fate decision [11, 12]. As for CDH4, CDH6, and CDH17, although there was rare emerging evidence to demonstrate their possible direct roles in the CSCs, a few reports demonstrated their functional roles in epithelial structure and even in regulation of EMT-like activity [57–60]. These studies are in consistency with our results and therefore support our notion here that CDH2, 4, 6, and 17 may have an implication in the CSCs in TNBC.

Our study here demonstrated that SC-TFs such as WWTR1, FOXM1, NFYA, and SOX9 might be involved in the enrichment of CSCs in TNBC (Figure 3, Tables 1 and 4). Consistently, these SC-TFs have been identified to be involved in stem cell regulation [22, 61–66]. For example, WWTR1 (also called TAZ), together with Yes-associated protein (YAP), is a key transcription regulator in Hippo-YAP signaling pathway, which is crucial for self-renewal of stem cells [28, 66–69]. Recently, several studies demonstrated that WWTR1/TAZ was strongly associated with triple-negative phenotype, and can sustain self-renewal potential of breast CSCs and increase the population of CSCs in TNBC [70–73]. When we prepared this manuscript, a report demonstrated that CDH2 indeed modulates mesenchymal cancer stem cells through WWTR1/TAZ [74], supporting our result of WWTR1's potential role in the enrichment of CSCs in TNBC induced by CDH2 in combination with CDH4, 6, and 17. Another important SC-TF is FOXM1. Likely, FOXM1 has also been reported to be involved in the EMT remodeling, self-renewal and the maintenance of stemness of stem cells [64, 75–77]. FOXM1 was highly upregulated in TNBC [78], confirming its role in the accumulation of CSCs in TNBC. Therefore, together with our results that CDH2/4/6/17 alterations were accompanied with elevated expression of SC-TFs (Tables 1 and 2), and TNBC are enriched in stem cell population (Figure 3, Table 4) [4, 79], this may provide a novel insight into the enrichment of CSCs in TNBC. In other words, CDH2/4/6/17 overexpressed individually or in combination might strengthen the inappropriate signals outside in, which may in turn trigger CDH2/4/6/17-related signal pathways to enhance the activity of SC-TFs such as WWTR1 and FOXM1, etc. The latter may then maintain the stem cell properties and increase the reservoir of CSCs in TNBC. Although this signal cascade remains to be investigated, our data provide a meaningful clue to further understand the mechanism underlying the maintenance of CSC in TNBC where CDH1 is often lost or mutated.

Although CDH1 is key for the maintenance of epithelial stem cells [5, 7, 80], many reports reveal that CDH1 loss is associated with EMT [81, 82], a cancer stem cell-like behavior [39], and its deficiency is characteristic of invasive breast lobular carcinomas, especially in TNBC [36, 37]. Our study showed that the level of CDH1 had a positive correlation with the status of ESR1 and HER2 (Supplementary Table S1), and CDH1 was low expressed/mutated in TNBC (Figure 1). Together with our notion that CDH2/4/6/17 contributed to the enrichment of CSCs in TNBC, it raised a question that which part (CDH1 low or CDH2/4/6/17 high or in combination) was more important for the enrichment of CSCs in TNBC. Frankly speaking, it is still an open issue. However, our results here supported the hypothesis that the elevated level of CDH2/4/6/17 may have a prevailing effect on the accumulation of CSCs than the decreased level of CDH1 in TNBC. The high level of CDH-associated SC-TFs such as FOXM1, MCM2, SOX9, and SNAI1 were concurrent with the overexpression of CDH2, 4, 6, and 17 (Table 1), but mutually exclusive with the decrease of CDH1 (Supplementary Table S2), strongly suggested that the enrichment of CSCs was more closely related to the high of CDH2, 4, 6, and 17, but not to the low of CDH1. Consistently, CDH2 has been identified as an indicator of EMT, and strongly correlated with CD133, one of the stem cell markers [11, 12, 83]. Moreover, CDH2 has a role in cell migration and metastasis over CDH1. For instance, CDH2 promotes cell motility in breast cancer cells regardless of the expression of CDH1 [84]. Also, CDH2 positivity in IHC staining was correlated to lymph node metastasis of TNBC, but CDH1 was not [85]. Altogether, our data support the hypothesis here that CDH2/4/6/17 may have a direct effect on the enrichment of CSCs in TNBC, which may have an implication in targeted therapy for TNBC in the future.

5. Conclusion

In this study, we demonstrated that CDH2/4/6/17 were amplified/overexpressed in TNBC, where the expression of CDH2/4/6/17-enriched SC-TFs was also increased. In parallel, these SC-TFs were strongly associated with the accumulation of CSCs in TNBC cells. Thus, we concluded that one mechanism through which the stemness is maintained in TNBC when E-cadherin is downregulated or lost is by way of upregulating the expression of other CDH family members such as CDH2/4/6/17 along with increased expression of SC-TFs. Moreover, we demonstrated that small compounds might be used to target TNBC based on alterations of CDH2/4/6/17 and SC-TFs. Although further studies have to be conducted on the mechanisms for regulation of SC-TFs by CDH2/4/6/17, all these observations highlight the potential functions of CDH2/4/6/17 on the reservoir of CSCs in TNBC with low expression or mutation of CDH1 and provide evidence with implications in targeted therapy in TNBC.

Competing Interests

The authors declare no financial conflict of interests.

Acknowledgments

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Review Article

Mesenchymal Stem Cell Administration in Patients with Chronic Obstructive Pulmonary Disease: State of the Science

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Patients with chronic obstructive pulmonary disease (COPD) have chronic, irreversible airway inflammation; currently, there is no effective or curative treatment and the main goals of COPD management are to mitigate symptoms and improve patients' quality of life. Stem cell based therapy offers a promising therapeutic approach that has shown potential in diverse degenerative lung diseases. Preclinical studies have demonstrated encouraging outcomes of mesenchymal stem/stromal cells (MSCs) therapy for lung disorders including emphysema, bronchopulmonary dysplasia, fibrosis, and acute respiratory distress syndrome. This review summarizes available data on 15 studies currently registered by the ClinicalTrials.gov repository, which used different stem cell therapy protocols for COPD; these included bone marrow mononuclear cells (BMMCs), bone marrow-derived MSCs, adipose-derived stem/stromal cells (ADSCs), and adipose-derived MSCs. Published results of three trials indicate that administering BMMCs or MSCs in the setting of degenerative lung disease is safe and may improve patients' condition and quality of life; however, larger-scale studies are needed to evaluate efficacy. Results of another completed trial (NCT01872624) are not yet published, and eleven other studies are ongoing; these include MSCs therapy in emphysema, several studies of ADSCs in COPD, another in idiopathic pulmonary fibrosis, and plerixafor mobilization of CD117 stem cells to peripheral blood.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the world and a major cause of chronic morbidity and mortality. Consequently, COPD imposes substantial and increasing economic and social burdens, which are expected to grow in coming decades due to accumulating exposure to COPD risk factors and population aging [1].

Subcategories of COPD include chronic bronchitis (the obstruction of small airways) and pulmonary emphysema. Chronic bronchitis is characterized by hypersecretion of mucus accompanied by a chronic (more than 3 months in 2 consecutive years) productive cough; infectious agents are a major cause. The main feature of pulmonary emphysema is airflow obstruction due to destruction of the alveolar walls distal to the terminal bronchiole, without significant pulmonary fibrosis. Most COPD patients have chronic bronchitis and pulmonary emphysema, together with mucus plugging; however, the severity varies between individuals [2]. Until now, no effective or curative treatment has been devised; thus, the goal of COPD management is to relieve symptoms and to improve quality of life. Experimental models have aimed to investigate the pathophysiology of pulmonary emphysema and to discover new treatment approaches. Stem cell based therapy is a promising approach with great potential in degenerative lung diseases.

Mesenchymal stem cells (MSCs) are known to give rise to several cell types, including osteoblasts, chondrocytes, adipocytes, stroma cells, and skeletal myoblasts; they can differentiate into endothelial cell types. In preclinical studies, MSC treatment has shown promising results in diverse lung disorders, including emphysema, bronchopulmonary dysplasia, fibrosis, and acute respiratory distress syndrome.

Intratracheal administration of MSCs has been shown to reduce tissue destruction in elastase-induced emphysema, through secretion of paracrine factors such as epidermal growth factor [3].

MSCs have been proposed to interfere with inflammation responses and exert immunomodulatory effects [4–6]. MSCs have been found to show profound suppressive effects on immune cells and pathways and recent researches have demonstrated that MSCs suppress lung injury and inflammation in several mouse models of inflammatory and immune-mediated lung diseases [7]. Moreover, MSCs have antifibrotic activity and hold great therapeutic potential for treating pulmonary fibrosis. The most widely studied cell types are bone marrow-derived MSCs (BM-MSCs) and adipose-derived MSCs (AD-MSCs).

The first animal model of rodent MSC administration in COPD was published by Shigemura et al. in 2006 [8, 9]. More and more studies on COPD with MSC therapy were published after 2014. MSC sources included humans, rabbits, rats, guinea pigs, or mice [10]. Two principle delivery routes were systemic delivery and local [11]. In regard to cell based therapy in lung diseases, systemic delivery is usually through vascular route, such as intravenous (IV) infusion. Local delivery introduces cells into the lung via intratracheal (IT) or intrabronchial (IB) instillation. The most used route of administration for COPD therapy is IV infusion. No clinical trials of IT delivery of cells for adult COPD patients are listed on ClinicalTrials.gov (https://www.clinicaltrials.gov) until now. IV infusion provides broader biodistribution and is easy to perform; thus, it is the major route of administration in preclinical and clinical studies for the delivery of various cell types (Hicks and Jolkkonen, 2009). Studies on rodent animal models of COPD have proved that IV injection or IT instillation of rodent BM-MSCs or AD-MSCs were safe and effective in attenuating airway injury by ameliorating airway inflammation and apoptosis and IT instillation of BM-MSCs appeared to be superior to IV injection in reducing alveolar hyperinflation and collagen fiber content in the elastase-induced emphysema models (Antunes et al., 2014; Guan et al., 2013; Katsha et al., 2011; Liu et al., 2016). Findings from these animal studies suggested that IT or IB instillation is a preferred and safer way of MSC administration for the treatment of airway diseases. However, the optimal method of delivery will depend on which mechanism of action of the MSC is being utilized.

In 2006, the International Society for Cellular Therapy (ISCT) published a position paper on defining the minimal criteria for multipotent MSC [12]. In addition, ISCT has published a sequence of MSC review papers in *Cytotherapy* focusing on the issue of immunotherapy, controversies, and safety as well [13–18]. Recently, stem cell-mediated therapeutic strategies and lung regeneration have been thoroughly reviewed by Akram et al. [19]. Important issues of MSC therapy in pulmonary disease, including pathogenesis, mechanisms of action, paracrine effects, plasticity and heterogeneity of MSCs, reactive oxygen species in MSC aging, respiratory tissue engineering, and clinical application, have been reviewed [20–29]. A systematic review and a meta-analysis on the published preclinical studies of MSC administration in the treatment of COPD in animal models have been conducted by Liu et al. [10] and suggest a promising role for MSC administration in COPD treatment. Here, we review the findings of published trials to date and the protocol used by the registered trials, including study design, treatment method (cell type, dose, and delivery route), and what kind of outcome measurements are used.

2. Review Protocol and Results

2.1. Databases Searches. The Cochrane Central Register of Controlled Trials (CENTRAL), PubMed, Medline (Ovid), EMBASE, and ClinicalTrials.gov were searched up until September 2015. Searches were limited to articles in English and used the terms listed in Table 1. Studies were included if (1) they examined the effects of MSCs on COPD or emphysema in human clinical trials and (2) they were available as full text articles. Relevant studies were also identified from among references of included articles.

2.2. Search Results. After excluding duplicates and irrelevant studies, only four published studies fit the inclusion criteria. ClinicalTrials.gov currently lists 15 studies of different MSC preparations for COPD treatment; these include three with published results (NCT01110252, NCT00683722, and NCT01306513) (Table 2), which matched the database search results, one completed trial without published results (NCT01872624), and eleven ongoing (NCT02041000, NCT01559051, NCT02161744, NCT02216630, NCT02135380, NCT02645305, NCT02348060, NCT01758055, NCT01849159, NCT02412332, and NCT01916577).

3. Discussion

3.1. Published Trials of Stem Cell Therapies in COPD (Table 2)

3.1.1. Bone Marrow Mononuclear Cells in Emphysema (NCT01110252). Bone marrow-derived-mononuclear cells (BMMCs) have displayed beneficial effects for the treatment of various diseases due to their multipotent effects [28] and the easiness of obtaining them for autologous transplantation. BMMCs can be used on the same day of harvesting with low cost and are not at risk of cell rejection (graft-versus-host disease) [30].

Ribeiro-Paes et al. (2011) conducted the first study in COPD to evaluate the safety of the cell therapy with BMMCs in patients with advanced-stage (stage IV dyspnea) pulmonary emphysema [31]. Stem cell stimulation was achieved by subcutaneous injection of 5 μg/kg of granulocyte colony stimulating factor (G-CSF) 3 days before autologous BMMCs were infused into a medial brachial peripheral vein. G-CSF, a hematopoietic growth factor which stimulates the proliferation and mobilization of bone marrow hematopoietic cells to the peripheral blood, [32–36] has been used in cell therapy procedures in both animal and human models [37–42]. The rationale for the use of G-CSF was according to previous results showing that this drug results in an increase in myeloid progenitor cells and CD34+ cells in the bone marrow [32, 43].

Table 1: Search strategies used in each database.

Database	Search strategy	Results
CENTRAL	MeSH descriptor: [Lung Diseases, Obstructive] explode all trees AND MeSH descriptor: [Stem Cell Transplantation] explode all trees	4
PubMed	"Pulmonary Disease, Chronic Obstructive"[Mesh] AND "Stem Cell Transplantation"[Mesh] AND "English"[Language] AND "clinical trial"[Publication Type]	4
Medline (Ovid)	exp *Pulmonary Disease, Chronic Obstructive/AND exp *Mesenchymal Stem Cell Transplantation/AND "Clinical Trial" [Publication Type]	16
Embase	obstructive airway disease'/exp/mj AND 'stem cell transplantation'/exp/mj AND ([controlled clinical trial]/lim OR [randomized controlled trial]/lim) AND [English]/lim	9
ClinicalTrial.gov	Advanced search with "Lung Diseases, Obstructive" for Conditions and "stem cell" for Interventions	34
Author search	From references of included articles	1*

* Krampera et al., 2006 [6].

Primary outcome measures were evaluated by pulmonary function tests, which included forced vital capacity (FVC), forced expiratory volume in one second (FEV1), and vital capacity (VC). Secondary outcomes included arterial blood gases test of partial pressure of oxygen (PaO_2) and partial pressure of carbon dioxide ($PaCO_2$). Outcomes measures were evaluated at baseline and 30 days after the procedure.

At 1-year follow-up, no adverse effects of BMMC therapy were observed, and the four advanced COPD patients showed significantly improved quality of life (QoL) as well as a more clinical stable condition. Spirometry results of all patients showed a slight improvement in the first 30 days after the cell therapy procedure. However, after 30 days through 90 days, indicators of pulmonary function tended to decrease, although not to preprocedure baseline values, indicating that the BMMC cell therapy may be beneficial for long-term stimulation of pulmonary regeneration.

Besides the lung function results, analysis of cell concentration and markers from mononuclear cells pool before and after infusion showed that $CD133^+$ cells were only evident in patients with the highest number of nuclear cells (NC) and best BMMC recovery. These findings suggest that the bone marrow volume to be collected for the BMMC therapy protocol could be increased.

The use of G-CSF is worth further investigation. In the Ribeiro-Paes et al. (2011) study, G-CSF did not show an effect on the proliferation of cell lines $CD34^+$ and $CD133^+$, which are possibly involved in the regeneration of damaged tissues [44–46]. Therefore, the authors suggest that since there is no consensus on the possible advantage of G-CSF when cells are attained with direct bone marrow puncture [47], G-CSF can be omitted when adopting a direct bone marrow puncture.

Stessuk et al. (2013) included the same participants and adopted the same protocol of Ribeiro-Paes et al. (2011) but with a longer follow-up period of 3 years [48], which corroborated their earlier study. BMMC therapy seems to be safe and no significant adverse effects were reported. Two in four patients showed improved lung function, with predicted FVC increases from 21% to 36.5% and 34% to 58%, respectively. All patients reported significant improvements in their emotional state and physical abilities.

Improved lung function in these two advanced pulmonary emphysema patients could be explained by paracrine effects and diminished plasma levels of inflammation-associated proteins after BMMC infusion. However, the sample size of this study was too small to draw firm conclusions; therefore, no conclusions can be drawn from these studies except no obvious adverse effects during infusion and no obvious safety issues during a 3-year follow-up. However, the safety issue is still questionable as one of four patients died and no information is presented on this. Larger-scale multicenter studies would be needed to evaluate both the safety and efficacy of BMMC therapy in COPD.

3.1.2. Mesenchymal Stem Cells in COPD (NCT00683722). Weiss et al. (2013) conducted a Phase II, multicenter, randomized, double-blind placebo-controlled study in the United States, to evaluate the safety and efficacy of intravenous allogeneic MSCs (PROCHYMAL®; Osiris Therapeutics Inc.) for treating moderate to severe COPD [49]. The study randomized 62 patients to either MSC ($n = 30$) or placebo ($n = 32$). Patients received four monthly infusions of MSCs (100×10^6 cells/infusion) with a 2-year follow-up. The primary endpoint was safety, assessed by occurrence of adverse events (AEs), electrocardiography (ECG), and COPD exacerbations; efficacy endpoints included pulmonary function, QoL, COPD exacerbation assessments, and markers of systemic inflammation.

No AEs and nor increase in the frequency of exacerbations was observed during the study. Safety analysis indicated that most AEs were mild to moderate in intensity for both MSC and placebo groups [MSC Group, 56.6%; Placebo Group, 65.6%] and a majority were reported as being unlikely related to the MSC treatment [MSC Group, 19 (63.3%); Placebo Group, 22 (68.8%)]. Concerning efficacy, no differences in pulmonary function tests were detected between the groups. The results demonstrate that the administration of MSCs in patients with moderate to severe COPD appears to be safe. However, MSC treatment did not show beneficial effects in terms of either pulmonary function or QoL. A more effective dosage and treatment schedule may be necessary to evaluate efficacy more accurately.

Table 2: Published trials of stem cell therapies in COPD.

Registry code	NCT01110252	NCT00683722	NCT01306513
Patients (n)	Stage III/IV COPD, advanced emphysema ($n = 4$)	Stage II/III COPD ($n = 62$: 30 MSC; 32 placebo)	Stage III COPD, severe emphysema, eligible for LVRS ($n = 10$)
Design	Single center, single arm, open-label, safety study	Multicenter, placebo-controlled, randomized, double-blind, Phase II safety & efficacy study	Single arm, open-label, safety study
Treatment (cells, dose & delivery route)	Autologous BMMC 1×10^8/ml, one IV dose (brachial)	Allogenic MSC 1×10^8/ml, four IV infusions monthly	Autologous BM-MSC 1-2 $\times 10^6$ cells/kg, two IV infusions 1 week apart, 4 & 3 weeks before 2nd LVRS
Study year (follow-up)	May 2009 (1 and 3 years)	April 2008 (2 years)	October 2010 (1 year)
Primary outcomes	*Lung function*: FVC; FEV1; VC	*AEs* during infusion or by physician/lab assessment *ECGs* during study & follow-up *COPD exacerbations*	*Safety*: AEs ≤ 3 weeks after infusion (WHO criteria) *Feasibility*: quantities of expanded MSCs versus BM collected; passages needed & time to reach target dose
Secondary outcomes	*Arterial blood gases*: PaO_2; $PaCO_2$	*Lung function*: FEV1, FVC, FEV1/FVC, total capacity, DLCO, 6MWT, dyspnea (Borg scale) *QoL*: SGRQ & global assessment *Exacerbations*: time to 1st exacerbation; exacerbation rate ratio between study arms *Inflammation markers*: TNF-α, IFN-γ, IL-2, TGF-β, IL-4, IL-5, IL-10, and CRP	Difference (days) between post-LVRS transpleural air leak after 1st versus 2nd LVRS Immunohistochemistry of markers of inflammation, fibrosis, and repair in resected lung tissue
Other markers	*Cell concentration & markers*: NC, BMMC, CD34+, and CD133+	SaO_2 (before & after 6MWT), CRP, and TGF-β	*Clinical*: spirometry, gas transfer, lung volumes, and CT-derived lung densitometry at baseline & 1 year *Immunohistochemistry*: CD3, CD4, CD8, CD31, CD68, Ki-67, or SP-C *Gene expression*: growth factors, immune mediators, proliferation markers, and lung cell markers
Safety results	Safe, no significant AEs	AEs mostly mild to moderate (MSC 56.6%; placebo 65.6%) and unlikely to be procedure-related (MSC 63.3%; placebo 68.8%)	*Safety*: stable vital functions and no change in WHO-toxicity; no infusion-related symptoms *Feasibility*: 7/10 patients completed the study; BM could be aspirated from nine (mean 158 ± 64 ml); target MSC number was obtained with 3 expansion cycles in eight

Table 2: Continued.

Registry code	NCT01110252	NCT00683722	NCT01306513
Efficacy results	Slightly improved lung function ≤30 days after infusion, declined thereafter, but not to baseline Three-year expiratory tests in two patients predicted FVC increase from 21% to 36.5% and 34% to 58%; all patients reported significantly improved emotional and physical status	*COPD exacerbations*: MSC 66.7% versus placebo 46.9% *Median time to 1st exacerbation*: MSC 6.7 months versus placebo not estimated (too few events) *Exacerbation-free at 1 and 2 years*: MSC 46.0% & 31.9% versus placebo 56.3% & 52.7%	*Clinical*: FEV1 rose by 390 ± 240 ml from baseline at 1-year follow-up ($P = 0.03$) Patients' weight significantly increased: mean 4.6 kg (range 1–10 kg; $P = 0.016$) *Immunohistochemistry*: alveolar septa showed tripled expression of CD31 ($P = 0.016$) Significantly higher CD3+ T cell count in alveolar septa after LVRS + BM-MSC versus before ($P = 0.016$); CD4+ T cell count in alveolar septa increased in all but one patient after LVRS + BM-MSC ($P = 0.30$; fold change $P = 0.047$) *Gene expression*: higher mRNA expression of IL10 and TSG6 in biopsy tissue after versus before LVRS + BM-MSC ($P = 0.06$)
Source(s) [reference(s)]	Gupta et al., 2007 [4] Krampera et al., 2006 [6]	Weiss et al., 2008 [7]	Shigemura et al., 2006 [8]

COPD, chronic obstructive pulmonary disease; BMMC, bone marrow mononuclear cell; IV, intravenous; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; VC, vital capacity; PaO_2, partial pressure of oxygen; $PaCO_2$, partial pressure of carbon dioxide; NC, nuclear cells; CD, cluster of differentiation; MSC, mesenchymal stem/stromal cell; AE, adverse event; ECG, electrocardiography; DLCO, diffusing lung capacity for carbon monoxide; 6MWT, six-minute walk test; QoL, quality of life; SGRQ, St. George Respiratory Questionnaire; TNF, tumor necrosis factor; IFN-γ, interferon γ; IL, interleukin; TGF-β, transforming growth factor β; CRP, C-reactive protein; SaO_2, peripheral oxygen saturation; LVRS, Lung Volume Reduction Surgery; BM-MSC, bone marrow-derived MSCs; CT, computed tomography; SP-C, surfactant protein-C; WHO, World Health Organization; TSG-6, TNF-α stimulated gene/protein 6.

Interestingly, post hoc analyses of systemic inflammation markers showed a decrease in circulating C-reactive protein (CRP) levels in MSC-treated patients at 1 month after the first infusion in patients with elevated CRP levels at baseline (>4.0 mg/L in 29 of 62 patients; 14 in the MSC group, 15 in the placebo group). This trend persisted throughout the study, suggesting that MSCs might inhibit systemic inflammation in COPD.

3.1.3. Bone Marrow-Derived MSC in Emphysema before and after LVRS (NCT01306513). Currently, the only treatment available for severe emphysema is lung volume reduction surgery (LVRS) to remove the most destroyed parts of the lungs. LVRS is generally performed in two separate sessions, one for each lung, 10–12 weeks apart. LVRS allows improved ventilation in the less affected areas of the lungs that remain, as demonstrated by postsurgical clinical improvement of lung function and increased survival rates. Delayed wound healing after LVRS is an important clinical issue; patients are at high risk for prolonged air leakage, which may be related to the inflammatory sequelae of emphysema.

Stolk et al. (2016) conducted a Phase I, nonrandomized, nonblinded, prospective study in the Netherlands, to test the safety and feasibility of administering bone marrow MSCs (BM-MSCs) before and after LVRS for severe emphysema [50]. The intervention consisted of two BM-MSCs infusions in 10 patients 1 week apart, 4 and 3 weeks prior to the second LVRS, respectively. The primary endpoints were safety and feasibility: safety was assessed by the occurrence of AEs during the first 3 weeks after infusion, according to World Health Organization (WHO) toxicity criteria; feasibility was determined by the number of expanded MSCs in relation to the amount of autologous bone marrow collected, number of passages required, and time to reach study target dose. Secondary endpoints included the difference in days between postsurgical transpleural air leak in each patient after the first and second surgical intervention, and histological responses in resected lung tissue, which were assessed by immunohistochemistry of inflammatory markers, fibrosis, and repair.

All patients showed stable vital functions in the first 48 hours after both BM-MSC infusions, and no toxicity or symptoms related to the infusions were observed in the first 48 hours and at 3 weeks after the second infusion, the day before the second LVRS. Regarding feasibility, seven patients completed the study protocol. Bone marrow could be aspirated from nine, with a mean volume of 158 ml ± 64 ml, and in eight patients the targeted total MSC number was obtained after three expansion cycles. One patient's bone marrow could not be aspirated; one had very poor expansion of MSCs and withdrew from the protocol; one could not be evaluated histologically and the second surgical procedure could not be conducted due to a persistent air leak after the first LVRS.

Clinically, FEV1 had increased by 390 ml ± 240 ml (P = 0.03) from baseline at 12-month follow-up. The body weight of all patients increased significantly, by a mean of 4.6 kg (range 1–10 kg; P = 0.016). According to immunohistochemistry, alveolar septa showed a threefold increased expression of the endothelial marker CD31 (P = 0.016). Besides, significantly increased $CD3^+$ and $CD4^+$ T cell counts were observed in randomly selected parenchymal tissue sections. Gene expression analysis showed a trend towards higher mRNA expression of interleukin- (IL-) 10 and tumor necrosis factor-α (TNF-α) stimulated gene/protein 6 (TSG-6) in biopsy tissue after LVRS and MSC treatment.

These results demonstrate that autologous MSC treatment in severe emphysema is feasible and safe. Increased CD31 expression suggests that the BM-MSC treatment might stimulate microvascular endothelial cells in the most severely affected parts of the lung. Therefore, MSC therapy could be a promising treatment approach for emphysema.

3.2. Ongoing Trials of Stem Cell Therapies in COPD

3.2.1. Adipose-Derived Stem Cells Therapy (Table 3). Among the different types of MSCs, adipose-derived MSCs (ADSCs) are a particularly attractive autologous cell source for various therapeutic purposes. First, ADSCs are easy to isolate and relatively abundant. Besides, ADSCs retain a high proliferation capacity in vitro and have the ability to undergo extensive differentiation into multiple cell lineages. Moreover, ADSCs secrete a wide range of growth factors that can stimulate tissue regeneration. Therefore, the clinical use of ASCs is feasible [51].

Current treatment options for COPD are not able to reverse airflow obstruction and accelerated loss of lung function [Celli, 2004]. ADSCs have high immunomodulating capacity and can ameliorate lung injury by secreting several factors with paracrine effects [52]. It has been proven that ADSCs have beneficial effects in animal models of pulmonary diseases [3, 4, 8, 52].

The stromal vascular fraction (SVF) comprises stromal cells isolated from total fat via ex vivo enzymatic digestion of adipose tissue harvested from the patient's abdomen or another applicable area by tumescent syringe liposuction under local anesthesia. SVF cells are not cultured but are isolated from adipose tissue using a sterile process, including a saline rinse to remove red blood cells, draining, and collagenase digestion, which isolates endothelial cells from adipose tissue. Adipose-derived SVF (AD-SVF) harvested from autologous adipose tissue will be delivered via intravenous infusion and inhalation. The number of cells available for infusion varies depending on the amount of tissue processed and the number of cells obtained. SVF contains multiple cellular components, including stem cells, with both regenerative and anti-inflammatory properties. SVF therapy has shown promise for ameliorating the symptoms of COPD. Preclinical studies have found AD-SVF and ADSCs to be safe and effective treatments for COPD. To our knowledge, there has been no published results of ADSC therapy in COPD patients until now.

Though ADSCs hold great potential for use in stem cell therapy, after transplantation, a complex and hostile environment with local hypoxia, oxidative stress, and inflammation may result in a large amount of cell loss or death of ADSCs. In addition, the stemness properties of ADSCs are influenced

Table 3: Ongoing clinical trials with ADSC administration in COPD.

Registry code	NCT02041000	NCT01559051	NCT02161744	NCT02216630	NCT02135380	NCT02645305	NCT02348060
Country	USA	Mexico	USA	USA	India	Vietnam	USA
Start year (follow-up)	January 2014 (6 months)	March 2014 (6 months)	May 2014 (1 year)	August 2014 (1 year)	August 2014 (9 months)	June 2015 (1 year)	November 2015 (1 year)
Status	Recruiting	Recruiting	Recruiting	Recruiting	Unknown	Recruiting	Recruiting
Patients (n)	Stage III/IV COPD; age 18–85 years (n = 100)	Stage III/IV COPD; age 18–80 years (n = 100)	COPD; age ≥ 18 years (n = 60)	Stage IIa/III/IV COPD; age 18–85 years (n = 200)	IPF, COPD; age 30–70 years (n = 60)	Stage IIa/III/IV COPD, age 40–80 years (n = 20)	COPD; age ≥ 18 years (n = 75)
Design	Multicenter, open-label, nonrandomized safety/efficacy study	Multicenter, open-label, nonrandomized, Phase I/II safety/efficacy study	Multicenter, open-label, nonrandomized, Phase I safety/efficacy study	Open-label, nonrandomized, Phase I/II, safety/efficacy study	Multicenter, open-label, randomized, Phase I/II safety/efficacy study	Open-label, safety/efficacy study	Prospective observational cohort study
Treatment (cells, dose & delivery route)	ADSC	Autologous AD-SVF; IV infusion & inhaled	Autologous AD-SVF; single IV injection	Autologous AD-SVF; IV injection	Autologous AD-SVF/AD-MSC; IV injection	Autologous AD-SVF & PRP; IV injection	Autologous AD-SVF; Single IV injection
Study arm(s) (procedure)	Single arm: ADSC therapy	Single arm: ADSC therapy (lipoaspiration with local anesthesia)	Single arm: ADSC therapy (lipoaspiration & IV SVF saline suspension)	Single arm: ADSC therapy (lipoaspiration)	Randomized: SVF; AD-MSCs; standard therapy (control)	Single arm: ADSC & PRP	Single arm: ADSC therapy (lipoaspiration)
Primary outcomes	*Safety*: AEs occurrence/frequency at follow-up *Efficacy*: SGRQ QoL at follow-up	*Efficacy*: 6MWT at 3 & 6 months *Safety*: number of AEs at 3 & 6 months	*Safety*: frequency of AEs and SAEs at follow-up	*Efficacy*: FEV1 decline of ≤30 mL at follow-up *Safety*: number of AEs at follow-up	*Safety*: treatment emergent AE rates at follow-up	*Safety*: SGOT, SGPT at 1 month	*Efficacy*: CRQ-SAI at 1 year
Secondary outcomes	*Efficacy*: GOLD-classified airflow obstruction & 6MWT at follow-up	*Efficacy*: SGRQ at 3 & 6 months	*Efficacy*: less decrease of FEV1 (ml), FEV1/FVC (%), DLCO (%) & 6MWT (mm) at 6 weeks to 1 year	*Efficacy*: decrease in 6MWD of <5% over 1 year	*Efficacy*: change in predicted FVC (%) & DLCO (%), changes in 6MWT & disease extent and severity (HRCT) at follow-up	*Efficacy*: respiration rate, 6MWT, panic attack rate, and CRP concentration at 1, 6 & 12 months	*Efficacy*: CRQ-SAI dyspnea, fatigue, emotional function, and mastery subscales at 12 months

COPD, chronic obstructive pulmonary disease; ADSC, adipose-derived stem/stromal cell; AE, adverse event; SGRQ, St. George Respiratory Questionnaire; QoL, quality of life; 6MWT, six-minute walk test; IV, intravenous; AD-SVF, adipose-derived stromal vascular fraction; SVF, stromal vascular fraction; SAE, severe adverse event; FEV1, forced expiratory volume in 1 second; DLCO, diffusing lung capacity for carbon monoxide; IPF, idiopathic pulmonary fibrosis; AD-MSC, adipose-derived mesenchymal stem/stromal cell; FVC, forced vital capacity; HRCT, high-resolution computed tomography; PRP, platelet-rich plasma; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; CRP, C-reactive protein; CRQ-SAI, Chronic Respiratory Disease Questionnaire-Self-Administered Individualized.

by the disease state of the donor [53]. The therapeutic effects of ADSCs may massively decrease as a result of insufficient retention and survival of transplanted cells [54]. Thus, tissue engineering approaches need to be dramatically improved by the addition of adjuncts that increase the proliferation and differentiation of ADSCs. Platelet-rich plasma (PRP), which contains high levels of diverse growth factors that can stimulate stem cell proliferation and cell differentiation in the context of tissue regeneration, has recently been identified as a biological material that could be applied to tissue regeneration [53]. The novel approach of adding PRP to ASCs was shown to have promising benefits in regenerative medicine from preclinical and clinical studies.

Ongoing clinical trials evaluating the safety and efficacy of therapy with autologous AD-SVF, ADSCs, cotransplantation of ADSCs, and PRP in COPD registered on ClinicalTrials.gov website include NCT02041000, NCT01559051, NCT02161744, NCT02216630, NCT02135380, NCT02645305, and NCT02348060. Table 3 summarizes the details of ongoing trials with the ADSC therapy.

(1) NCT02041000 (Recruiting). USA investigators are conducting this study to ascertain whether ADSC treatment is safe and effective in improving the disease pathology of COPD. Safety will be assessed as the occurrence and frequency of AEs during the study procedures and at 6-month follow-up. The St. George Respiratory Questionnaire (SGRQ) is an index developed to rate patients QoL by measuring and quantifying health-related variables of patients with airflow obstruction. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) staging system classifies COPD severity based on patients' degree of airflow limitation according to pulmonary function tests. Exercise capacity is measured as the distance a patient can walk in 6 minutes, six-minute walk test (6MWT).

(2) NCT01559051 (Recruiting). NCT01559051 is a Phase I/II, open-label, nonrandomized, multicenter study to evaluate the safety and efficacy of autologous ADSC transplantation in Mexican GOLD III/IV patients. Efficacy is being accessed by whether the therapy improves functional capacity and QoL at 3-month and 6-month follow-up. Safety will be determined by AEs at 6-month follow-up.

(3) NCT02161744 (Recruiting). Another Phase I, open-label, nonrandomized, multicenter study, NCT02161744, conducted in USA, aims to assess the safety, tolerability, and efficacy of ADSC therapy. Efficacy is to be evaluated by whether ADSC results in less decrease from baseline in lung function parameters (FEV1, FEV1/FVC, diffusing lung capacity for carbon monoxide, and 6MWT). Patients will be followed for 12 months after receiving a single intravenous SVF infusion.

Standard COPD therapy will not be interrupted during the study. In the lipoaspiration procedure, 100–240 ml of lipoaspirate will be extracted, and SVF was isolated with minimal manipulation and reconstituted in saline solution for intravenous administration.

(4) NCT02216630 (Recruiting). Similar to NCT01559051 and NCT02161744, NCT02216630 is an open-label, nonrandomized, multicenter study of safety and effects of intravenous liposuction-derived autologous ADSCs in COPD; proposed specific aim is to investigate the immunosuppressive potential of nonmanipulated noncultured SVFs obtained via liposuction. Following liposuction extraction of 100 ml of fat, ADSCs will be isolated and injected intravenously. Safety assessment is by number of AEs and efficacy endpoints comprise an FEV1 decline of ≤30 ml at 12-month follow-up (primary endpoint) and decrease in 6MWD of less than 5% over 1 year (secondary endpoint).

(5) NCT02135380 (Unknown Status). This Phase I/II, open-label, randomized, multicenter study in India is evaluating the safety and efficacy of ADSC for treating idiopathic pulmonary fibrosis (IPF) and COPD. Despite intense research efforts and numerous clinical trials, there is still no effective alternative to lung transplantation to prolong survival of patients with IPF; however, not all IPF patients are eligible for lung transplantation and a large proportion of patients died while awaiting one. Conventional therapies include combinations of corticosteroids, antioxidants, immunosuppressants, and immunomodulatory antifibrotic agents, which will be discontinued for 20 days before screening. Therefore, a safe, effective, and affordable treatment option is needed urgently and the potential application of ADSC as a safe and novel therapeutic agent in lung diseases including COPD and IPF is of great interest. MSCs with antifibrotic actions offer an excellent resource to treat pulmonary fibrosis. Given the limited clinical information regarding the use of SVF and MSC in IPF, this placebo-controlled comparative study will explore the tolerability and effectiveness of SVF for IPF patients in one treatment arm and ADSC in another. Subjects in the SVF arm will receive a single intravenous dose of autologous AD-SVF and those in the MSC arm three intravenous doses of two million ADSCs per kg body weight, given at weekly intervals. Control subjects will receive standard therapy comprising prednisolone ≤10 mg/day or ≤20 mg on alternate days, immunosuppressants such as cyclophosphamide or azathioprine at a dose of 2 mg/kg/day not exceeding 150 mg/day, antioxidants such as N-acetylcysteine at a dose up to 1800 mg/day, and pirfenidone at dose up to 1200 to 1800 mg/day.

(6) NCT02645305 (Recruiting). Preclinical data show that COPD is closely related to chronic inflammation. In Vietnam, NCT02645305 aims to use ADSC in the form of nonexpanded culture, which is SVF combined with activated platelet-rich plasma (PRP) used to treat COPD. Both SVF and PRP are autologous sources, obtained from adipose tissue and peripheral blood, respectively. This mixture will be intravenously transfused into 20 COPD patients. The primary endpoint includes the blood serum glutamic oxaloacetic transaminase level and glutamic pyruvic transaminase level, which are elevated with liver damage. The secondary endpoints comprise respiration rate, 6MWT, panic attack rates, and the CRP concentration, which will be evaluated before and 6 and 12 months after treatment.

(7) NCT02348060 (Recruiting). In USA, NCT02348060 will evaluate the effect of SVF treatment on QoL in individuals with COPD for up to 12 months following SVF treatment. The primary outcome is change from baseline over the course of 12 months using participants' assessment of overall QoL assessed by the Chronic Respiratory Disease Questionnaire-Self-Administered Individualized (CRQ-SAI), which is a seven-point Likert response scale. Mean scores are used for baseline and all interviews up to the 12-month follow-up. Secondary outcomes include participants' assessment of breathing comfort, fatigue, emotional function, and mastery (the ability to control feelings of disease-related fear or panic) from baseline to month 12 using the CRQ-SAI dyspnea subscale, CRQ-SAI fatigue subscale, CRQ-SAI emotional function subscale, and CRQ-SAI mastery subscale, respectively.

3.3. Bone Marrow-Derived Mesenchymal Stem Cells (Table 4)

3.3.1. NCT01758055 (Unknown Status). In Iran, NCT01758055 will evaluate the safety of endobronchial transplantation of autologous bone marrow-derived MSC (BM-MSC) in patients with emphysema. The primary endpoint is accessed by pulmonary function, including FVC, FEV1, and FEV1/FVC at baseline and 1-year follow-up. The secondary endpoint is 6MWT. Other outcome measures include oxygen saturation by oximeter, QoL according to the Medical Outcomes Study Questionnaire Short Form 36 Health Survey (SF-36), diffusing lung capacity for carbon monoxide by body-box device, changes of computed tomography scan, dyspnea score with modified Medical Research Council scale, PaO_2 and $PaCO_2$ from the atrial blood gases test, and infection evaluated by a complete blood count. Patients are given spinal anesthesia. About 120 ml of bone marrow is aspirated from each puncture, and after preparation a single dose of ~60 million autologous MSCs are transplanted by bronchoscopy into the endobronchial lumen of these patients.

3.3.2. NCT01872624 (Completed). This Phase I, nonrandomized, open-label study recruited patients in Brazil with severe heterogeneous emphysema to evaluate the safety of one-way endobronchial valves combined with BM-MSCs and to investigate the potential of MSC administration to decrease local inflammation related to the one-way valve placements. This study determined the effect on QoL and the systemic inflammatory potential of cell therapy, measured by CRP levels, erythrocyte sedimentation rate, and complete peripheral blood count. Moreover, the study investigated whether this treatment modality modulates other markers of inflammatory response and remodeling.

In the treatment group ($n = 5$), BM-MSC was delivered immediately preceding insertion of one-way endobronchial valves by bronchoscopy. In the placebo group ($n = 5$), patients received treatment with one-way endobronchial valves only, with saline injected prior to valve insertion. This study had a 4-month follow-up period to assess safety, QoL, pulmonary function, and inflammatory status (blood samples for CRP and ESR, CBC in peripheral blood).

3.3.3. NCT01849159 (Recruiting). A problem with MSC transplants in patients with respiratory failure is accelerated apoptosis of transplanted cells under the influence of proinflammatory cytokines and oxidative stress. Since it is known that preconditioning MSCs under hypoxia increases their survival in hypoxic conditions and the expression of growth factors and anti-inflammatory cytokines, this study will investigate whether growing MSCs in hypoxic medium may be beneficial. A Phase I/II, randomized, placebo-controlled study has been designed in Russia to evaluate the safety and efficacy of intravenous infusions of allogeneic BM-MSCs. The BM-MSC treatment group will receive an intravenous MSC suspension of 200×10^6 cells per 400 ml of physiological saline solution, preconditioned under 1% oxygen. Control patients will receive only 400 ml 0.9% physiological saline solution. Infusions will be performed every 2 months for 1 year. The primary endpoint is safety versus placebo, including mortality, AEs and treatment reactions, and vital signs (pulse rate, arterial blood pressure) at baseline and 2-year follow-up. Efficacy endpoints include lung tissue density measured by computed tomography-densitometry, pulmonary function, and diffusion capacity at 6, 12, and 24 months.

3.4. BMMC versus ADSC (Table 5)

3.4.1. NCT02412332 (Enrolling Participants by Invitation). The main goal is to evaluate the safety of infusing BMMC and/or ADSC, separately or concomitantly in COPD patients. The study cohort comprises 20 patients with GOLD grade 3 COPD, divided into four groups: (1) control; (2) BMMC; (3) ADSC; and (4) BMMC + ADSC, with 5 patients per group. Therapeutic stem cells will be obtained from each patient's own bone marrow or adipose tissue, for infusion via a peripheral vein after preparation, separation, expansion, and quality control. Patients will be followed for 12 months. It is expected that this study will extend knowledge about cell therapy in pulmonary diseases and may represent a significant step towards establishing new therapeutic approaches in COPD treatment.

Control subjects will receive no interventions besides conventional (in-course) treatment. BMMC group patients will undergo bone marrow harvesting surgery to obtain approximately 200 ml of bone marrow from the iliac crest under spinal anesthesia. BMMC will be obtained by Ficoll separation and returned by systemic infusion (1×10^8 BMMC in 30 ml saline). In the ADSC group, liposuction will harvest approximately 50 ml of adipose tissue from the abdominal region under spinal anesthesia. Fat tissue will be cultivated for 3 weeks and ADSC returned by systemic infusion (1×10^8 ADSC in 30 ml saline). In the BMMC + ADSC group, BMMC and ADSC will be returned to patients by systemic infusion (5×10^7 ADSC + 5×10^7 BMMC in 30 ml saline). Three patients in each treatment group will have lung perfusion scintigraphy with technetium to evaluate cell engraftment. The primary outcome is total pulmonary capacity, assessed

Table 4: Ongoing clinical trials with BM-MSC administration in COPD.

Registry code	NCT01758055	NCT01872624	NCT01849159
Country	Iran	Brazil	Russia
Start year (follow-up)	December 2012 (1 year)	May 2013 (4 months)	March 2014 (2 years)
Status	Unknown	Completed (March 2015)	Recruiting
Patients (n)	Moderate to severe emphysema; age 16–70 years ($n = 12$)	Pulmonary emphysema (severe heterogeneous emphysema); age ≥ 18 years ($n = 10$)	Stage III/IV pulmonary emphysema ($n = 30$)
Design	Single arm, open-label, Phase I safety study	Nonrandomized, open-label, Phase I safety study	Randomized, open-label, Phase I/II safety/efficacy study
Treatment	Autologous BM-MSC; single IB injection of 6×10^6 cells by bronchoscopy	Autologous BM-MSC; IB injection by bronchoscopy	Allogeneic BM-MSC; 2×10^8 cells (hypoxic-preconditioned in 1% oxygen); IV infusion every 2 months for 1-year
Study arm(s)	BM-MSC therapy	Valves + BM-MSC treatment ($n = 5$) versus valves + saline controls ($n = 5$)	BM-MSC suspension versus placebo (400 ml 0.9% saline)
Primary outcomes	*Safety*: FVC, FEV1, and FEV1/FVC at 1 year	*Safety*: absence of lung deficits during the procedure and/or follow-up	*Safety*: mortality, AEs & treatment reactions, and vital signs (pulse, arterial blood pressure) at 1 year
Secondary outcomes	*Safety*: 6MWT at 1 year *Others*: oxygen saturation: oximeter test, SF-36 QoL, DLCO, and CT scan; MMRC dyspnea score, blood gases PaO_2 & $PaCO_2$, CBC test, at baseline and 1 year	*Safety*: SGRQ *QoL*; *Pulmonary function*: spirometry, flow-volume curve, postbronchodilator test, RV, airway resistance by plethysmography, DLCO & 6MWT *Inflammation*: serum CRP, erythrocyte at 4 months	*Lung tissue density*: CT-densitometry *Pulmonary function*: DLCO, FEV1, TLC, RV, and FEV1/FVC *Physical capacity*: 6MWT *Blood gases*: PaO_2, $PaCO_2$ *Serum* IL-6, TNF-α, leptin *QoL*: SF-36 *Number and frequency of exacerbations* *Body mass index* at follow-up

BM-MSC, bone marrow-derived mesenchymal stem/stromal cell; COPD, chronic obstructive pulmonary disease; IB, intrabronchial; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; 6MWT, six-minute walk test; SF-36, Medical Outcomes Study Questionnaire Short Form 36 Health Survey; QoL, quality of life; DLCO, diffusing lung capacity for carbon monoxide; CT, computerized tomography; MMRC, modified Medical Research Council; PaO_2, partial pressure of oxygen; $PaCO_2$, partial pressure of carbon dioxide; CBC, complete blood count; SGRQ, St. George Respiratory Questionnaire; CRP, C-reactive protein; IV, intravenous; AE, adverse event; TLC, total lung capacity; RV, residual volume.

Table 5: Ongoing clinical trials comparing BMMC and ADSC therapy in COPD.

Registry code	NCT02412332
Country	Brazil
Start year (follow-up)	April 2015 (1 year)
Status	Enrolling by invitation
Patients (n)	Stage II/III COPD; age 40–70 years ($n = 20$)
Design	Randomized, open-label, Phase I/II safety/efficacy study
Treatment	Autologous BMMC/ADSC 1×10^8 in 30 ml saline by IV injection
Study arms & procedures	(1) *Control* ($n = 5$) (2) *BMMC* ($n = 5$): ~200 ml bone marrow will be surgically extracted from the pelvis under spinal anesthesia (3) *ADSC* ($n = 5$): 50 ml of adipose tissue will be extracted by abdominal liposuction under spinal anesthesia (4) *BMMC + ADSC* ($n = 5$): 5×10^7 ADSC + 5×10^7 BMMC in 30 ml saline Three patients in each active treatment group will receive lung perfusion scintigraphy with technetium to evaluate cell engraftment
Primary outcomes	*Efficacy:* total pulmonary capacity by whole body plethysmography at 1 year
Secondary outcomes	*Efficacy*: pulmonary morphology (by chest X-ray) and pulmonary function (composite TLC, FVC, FEV1, FEV1/FVC, FEF 25–75, RV, TLC/RV, and airway resistance) at 9 months

BMMC, bone marrow mononuclear cell; ADSC, adipose-derived stem/stromal cell; COPD, chronic obstructive pulmonary disease; IV, intravenous; TLC, total lung capacity; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; FEF 25–75, forced expiratory flow at 25–75% of forced vital capacity; RV, residual volume.

Table 6: Ongoing clinical trial of plerixafor mobilization of stem cells for COPD therapy.

Registry code	NCT01916577
Country	USA
Start year (follow-up)	August 2013 (1 year)
Status	Recruiting
Patient (*n*)	COPD, cystic fibrosis, pulmonary fibrosis; age 18–70 years; awaiting lung transplant ($n = 20$)
Design	Randomized, open-label, Phase I safety study
Treatment	Autologous CD117+ progenitor cell mobilization; one dose of 240 μg/kg by IV infusion
Study arms & procedures	(1) *Treatment* ($n = 15$): COPD ($n = 5$), cystic fibrosis ($n = 5$), pulmonary fibrosis ($n = 5$) (2) Control ($n = 5$) Blood flow cytometric analysis of CD117+ peripheral blood cells will be collected just before the dose of plerixafor (time zero), and at 8 hours posttreatment
Primary outcomes	*Efficacy*: change from baseline in peripheral blood CD117+ cells per ml at 8 hours posttreatment
Secondary outcomes	*Safety*: number of plerixafor-related AEs/SAEs and number of patients with plerixafor-related AEs/SAEs at 30 minutes, 1 week, and 1 year posttreatment

COPD, chronic obstructive pulmonary disease; IV, intravenous; AE, adverse event; SAE, severe adverse event.

by whole body plethysmography; the secondary outcome is pulmonary morphology, evaluated by the chest X-ray.

Regarding the adipose cultivation, MSCs with preadipocyte characteristics can be isolated from adipose tissue, propagated in vitro, and induced to differentiate in vitro into multiple lineages when treated with established lineage-specific factors. Sharifi et al. (2012) isolated stem cells from human adipose tissue and cultured, expanded, and examined their stemness by determining their surface CD markers and their ability to differentiate into adipocyte lineage. In Sharifi et al.'s study, adhered cells were cultured for 2-3 weeks and the ADSCs were isolated, cultured, and expanded [55]. Thus, the fat being cultivated for 3 weeks seems to be reasonable in this study.

3.5. Plerixafor Mobilization of Autologous CD117 Stem Cells (Table 6)

3.5.1. NCT01916577 (Recruiting). Plerixafor, an CXCR4-SDF1 antagonist, has been found to be a strong inducer of mobilization of hematopoietic stem cells from the bone marrow to the bloodstream as peripheral blood stem cells [56]. Plerixafor in combination with G-CSF is usually being used for mobilization of hematopoietic progenitor cells. Mobilization is the process by which progenitors are made to migrate from the bone marrow into the bloodstream, thus increasing their numbers in the blood. Mobilization is used clinically as a source of hematopoietic stem cells for hematopoietic stem cell transplantation. Combination of G-CSF with plerixafor increases the percentage of persons that respond to the therapy and produce enough stem cells for transplantation but is ineffective in around 15 to 20% of patients.

CD117, a stem cell growth factor receptor, also known as protooncogene c-Kit or tyrosine-protein kinase Kit, is a cytokine receptor expressed on the surface of hematopoietic stem cells as well as other cell types. Altered forms of this receptor may be associated with some types of cancer [57]. CD117 is a receptor tyrosine kinase type III, which binds to stem cell factor (SCF), a substance that causes certain types of cells to grow. Signalling through CD117 plays a role in cell survival, proliferation, and differentiation. Hematopoietic progenitor cells are normally present in the blood at low levels. Signalling through CD117 has been implicated in mobilization. G-CSF indirectly activates CD117. Direct CD117 agonists are currently being developed as mobilization agents.

NCT01916577, a Phase I, open-label, placebo-controlled, randomized study will investigate whether the drug plerixafor (Mozobil®, Sanofi-Aventis U.S. LLC) will lead to significant mobilization of CD117+ stem cells to the peripheral blood. The study cohorts comprise five healthy controls and 15 patients awaiting lung transplantation, five each with COPD, cystic fibrosis, and pulmonary fibrosis. Plerixafor will be given once, at 240 μg/kg subcutaneously, to all the patients, with blood for flow cytometric analysis for CD117+ peripheral blood cells collected just before the dose and 8 hours afterwards. The efficacy endpoint is the number of circulating CD117+ cells per ml of peripheral blood at baseline and change in peripheral blood CD117+ cells per ml following plerixafor treatment. The safety endpoint comprises the number and the incidence of plerixafor-related AEs and severe AEs for 30 minutes after administration, at 1 week and at 1 year posttreatment.

4. Conclusion

The first safety study to evaluate cell therapy with BMMCs in COPD was from Ribeiro-Paes et al. (2011) [31]. Their finding that CD34+ and CD133+, which might relate to the regeneration of damaged tissues, did not show proliferation induced by G-CSF suggests that the application of G-CSF can be omitted with the treatment of BMMCs. Weiss et al. (2013) [49] demonstrated that the MSC treatment did not show beneficial effect in either pulmonary function or QoL, indicating that a more effective dosage and treatment schedule may be needed. A decrease in circulating CRP levels in MSC-treated

patients 1 month after the first infusion only in patients with elevated CRP levels at baseline suggests that MSCs might inhibit systemic inflammation in COPD. Stolk et al. (2016) [50] demonstrated that autologous administration of BM-MSCs before and after LVRS for severe emphysema patients is feasible and safe; increased CD31 expression indicates that the BM-MSC treatment may stimulate microvascular endothelial cells in the most severely affected parts of the lung and may therefore be a promising treatment for emphysema.

Ongoing clinical trials of stem cell based therapies in COPD include the ASDC, BM-MSC, and plerixafor mobilization of CD117 stem cells to peripheral blood; ASDC transplantation in COPD seems to be a favored treatment modality (7 of 11 ongoing trials) with considerable therapeutic potential. Although no results of ADSC therapy in COPD are yet published, these ongoing trials will expand our limited knowledge of ADSC for COPD patients. In addition to ADSC, BM-MSC, the most studied therapy in preclinical studies, is being examined in two ongoing clinical trials. These efforts will help to elucidate applications of cell based therapies for COPD and degenerative lung diseases.

Taken together, MSCs are currently being used in clinical trials for the treatment of COPD with varying inclusion criteria (different stages of COPD), route of administration (locally or systemically injected), types of cells (autologous or allogeneic), cell dosage, schedule of transplantation, and outcome measurements (pulmonary function, physical capacity, frequency of exacerbations, and mortality). IV delivery is the most common mode of introduction. The current cell dosage has not elicited a long-term therapeutic effect. Further evaluation of efficacy and safety of systemic and local administration of autologous or allogeneic MSCs in the treatment of COPD will be needed to optimize the timing, dosage, and the route of delivery.

Competing Interests

The authors declare that they have no potential conflict of interests.

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Research Article

β-Arrestin1/miR-326 Transcription Unit Is Epigenetically Regulated in Neural Stem Cells Where It Controls Stemness and Growth Arrest

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Cell development is regulated by a complex network of mRNA-encoded proteins and microRNAs, all funnelling onto the modulation of self-renewal or differentiation genes. How intragenic microRNAs and their host genes are transcriptionally coregulated and their functional relationships for the control of neural stem cells (NSCs) are poorly understood. We propose here the intragenic miR-326 and its host gene β-arrestin1 as novel players whose epigenetic silencing maintains stemness in normal cerebellar stem cells. Such a regulation is mediated by CpG islands methylation of the common promoter. Epigenetic derepression of β-arrestin1/miR-326 by differentiation signals or demethylating agents leads to suppression of stemness features and cell growth and promotes cell differentiation. β-Arrestin1 inhibits cell proliferation by enhancing the nuclear expression of the cyclin-dependent kinase inhibitor p27. Therefore, we propose a new mechanism for the control of cerebellar NSCs where a coordinated epigenetic mechanism finely regulates β-arrestin1/miR-326 expression and consequently NSCs stemness and cell growth.

1. Introduction

Neural stem cells (NSCs) are believed to foster a hierarchical developmental program in which self-renewal and pluri/multipotency are responsible for the expansion and/or the maintenance of an uncommitted cell population pool. NSCs are ready to undergo the cascade of lineage restriction and subsequent terminal differentiation under the action of regulatory morphogenic cues [1]. The early postnatal murine cerebellum contains multipotent NSCs, which can be isolated and expanded in vitro. NSCs maintain their undifferentiated phenotype under mitogenic signals and are able to differentiate into the different kind of neurons [2].

Recent evidences have highlighted the crucial role of microRNAs (miRNAs) in conferring neural cell identities during neural induction, neuronal differentiation, and subtype specification [3]. MiRNAs are widespread throughout the genome, where they can be found in either intergenic or intragenic (especially intronic) regions [4]. Transcription of both intergenic and intragenic miRNAs may be regulated by their own promoters, whether some intragenic miRNAs share promoters with their host genes that generate pre-miRNA and mRNA, both arising from the same transcript [5, 6]. The consequent spatial and temporal coexpression implies a functional relationship between the intragenic miRNA and its host gene.

We previously identified miR-326 as a miRNA that is required to differentiate cerebellar granule cell progenitors (GCPs) to mature granule cells [7]. This miRNA is embedded within the first intron of *β-arrestin1 (βarr-1)* gene and shares with its host gene the same regulatory sequences [8, 9]. Since stem cell commitment to GCPs is a crucial event in cerebellar development [2, 10] we raised the question of miR-326 expression and regulation in cerebellar NSC. We report here that miR-326 expression is further downregulated in NSC prior to progenitor commitment, along with the downregulation of its host gene. Therefore, we investigated the expression, functions, and regulation of *miR-326/βarr-1* transcription unit in NSCs.

2. Materials and Methods

Unless otherwise indicated, media and supplements were purchased from Gibco-Invitrogen (Carlsbad, CA), chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and commercial products were used according to the manufacturers' instructions/protocols.

2.1. Cell Culture

2.1.1. Neural Stem Cells Culture. Mouse cerebella were obtained from postnatal 4-day-old wild-type BL6 mice (Charles River) with the approval of Institutional Review Board [8, 9]. Briefly, tissues were collected in HBSS supplemented with 0.5% glucose and penicillin-streptomycin, grossly triturated with serological pipette and treated with DNAse I to a final concentration of 0.04% for 20 minutes. Cells were mechanically dissociated using pipettes of decreasing bore size to obtain a single-cell suspension. Neural stem cells (NSCs) were derived and enriched through selective medium (SM): DMEM/F12 supplemented with 0.6% glucose, 25 mg/mL insulin, 60 mg/mL N-acetyl-L-cysteine, 2 mg/mL heparin, 20 ng/mL EGF, 20 ng/mL bFGF (Peprotech, Rocky Hill, NJ), penicillin-streptomycin, and B27 supplement without vitamin A. For clonogenicity assay cells were plated at clonal density (1-2 cells/mm^2) into 96-well plate. To induce differentiation, NSCs were plated onto D-poly-lysine-coated dishes in differentiation medium (DFM: DMEM/F12 with N2 supplement and 2 mg/mL heparin, 0.6% glucose, and 60 mg/mL N-acetyl-L-cysteine, containing 1% Calf Serum and PDGF 10 ng/mL (Sigma, P3076) or RA 2 mM (Sigma, R2625), for 48 hours).

2.1.2. Overexpression Studies. Amaxa nucleofector (Lonza) was used to transfect plasmids according to manufacturer's procedure. pcDNA3 β-arrestin1 HA was a gift from Robert Lefkowitz (Addgene plasmid # 14693): [11]; miR-326 vector and its negative control were purchased from GeneCopoeia (MmiR3333-MR01).

2.1.3. Knockdown Experiments. Silencing of β-arrestin1 was performed using ON-TARGETplus SMARTpool (L40976-00-005) from Thermo Scientific, after testing each single siRNA of the pool, alone or in combination, for its specificity to avoid OFF-target effects.

2.1.4. Cell Proliferation. Cell proliferation was evaluated by BrdU incorporation as previously described [2]. More in detail, after 24-hour pulse with BrdU, NSCs were plated on poly-lysine-coated Lab-Tek chamber slides (coverslips) and allowed to adhere for 3 hours. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and BrdU detection (Roche) was performed. Nuclei were counterstained with the Hoechst reagent. Cells were counted in triplicate and the number of BrdU-positive nuclei was annotated.

NSC growth was measured by MTT assay (Promega) according to manufacturer's instructions. Each sample was measured in triplicate and repeated at least three times.

For methylation analysis, NSCs were treated with 10 μM 5-azacytidine (5-AZA) for four days.

2.2. RNA Isolation and Gene Expression Analysis. Total RNA was purified and reverse transcribed as previously described [12]. Quantitative RT-PCR (qRT-PCR) analysis was performed using the ABI Prism 7900HT Sequence Detection System (Thermo Scientific), using best coverage TaqMan gene expression assays, specific for each analyzed mRNA. Each amplification was performed in triplicate and the average of the three threshold cycles was used to calculate the amount of transcripts (Thermo Scientific). mRNA quantification was expressed, in arbitrary units, as previously described [13]. mRNA quantification was expressed, in arbitrary units, as the ratio of the sample quantity to the calibrator or to the mean values of control samples. All values were normalized to three endogenous controls, β-actin, β2-microglobulin, and Hprt. miRNA expression levels were evaluated on RNA samples using specific stem-loop primers to achieve retrotranscription (Thermo Scientific) and was followed by a quantitative RT-PCR using miRNA-specific TaqMan probes (Thermo Scientific) in a ABI Prism 7900HT Sequence Detection System (Thermo Scientific). miRNA expression levels were normalized to RNAU6B [14].

2.3. Western Blot Assay. Cells were lysed using RIPA buffer (Tris-HCl pH 7.6 50 mM, deoxycholic acid sodium salt 0.5%, NaCl 140 mM, NP40 1%, EDTA 5 mM, NaF 100 mM, sodium pyrophosphate 2 mM, and protease inhibitors). Lysates were separated on 8% acrylamide gel and immunoblotted using standard procedures. The following antibodies were used: anti-β-arrestin1 K-16 (sc-8182; Santa Cruz Biotechnology, CA), anti-mouse Nanog (Cosmo Bio Co, Japan), anti-mouse βIII-tubulin (MAB 1637 Millipore), anti-actin I-19 (sc-1616; Santa Cruz Biotechnology, CA), anti-GAPDH (ab8245; Abcam), anti-Sp1 1C6 (sc-420X; Santa Cruz Biotechnology, CA), anti-p27 (F-8) (sc-1641; Santa Cruz Biotechnology), anti-Sox2 (MAB4343 Millipore Billerica, MA), anti-Oct4 (ab19857; Abcam), anti-mouse βIII Tubulin (MAB 1637 Millipore Billerica, MA), anti-GFAP MAB360 (Millipore Billerica, MA), and anti-PARP p85 fragment (G7342 Promega) (anti-parp-C). HRP-conjugated secondary antibodies (Santa

Cruz Biotechnology, CA) were used in combination with enhanced chemiluminescence (ECL Amersham, Amersham, UK). For nucleus/cytoplasmic fractionation, cells were lysed on ice with buffer containing 20 mM Hepes, pH 7.4, 20% glycerol, 50 mM KCl, 1 mM EDTA, 5% NP 40, and protease inhibitors. After centrifugation the cytoplasmic fractions were collected (supernatant). The pelleted nuclear fraction was lysed with buffer containing 20 mM Hepes, pH 7.4, 25% glycerol, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitors.

2.4. In Situ Hybridization and Immunofluorescence Assay. All reagents used prior to probe hybridization were prepared with DEPC-treated water (diethyl pyrocarbonate, Sigma D5758) to prevent RNAse contamination.

Cells were fixed with 4% PFA for 10 minutes at room temperature. After 3 washes with PBS, cells were incubated for 2 minutes with 10 μg/mL proteinase K (Sigma P2308) at 37°C.

Acetylation was then performed to enhance signal to background ratio, using 1.2% triethanolamine (Sigma 90279), 0.0018 N HCl, and 0.25% acetic anhydride (Sigma A6404) with constant stirring for 10 minutes at room temperature. Cells were washed 3 times with PBS and prehybridization was performed using 50% formamide (Sigma F9037), 5XSSC buffer (FLUKA cat. S6639-1L), 0,1% Tween-20, 9,2 mM citric acid (Sigma C1909), 50 μg/mL heparin (Sigma H4784), and 500 μg/mL yeast RNA (Sigma R6750) for 3 hours at 62°C. Hybridization probe (double DIG-labeled, Exiqon) was diluted in hybridization buffer at a final concentration of 25 nM, denaturated at 85°C for 5 minutes, cooled on ice, and then incubated on cells at 62°C for 16 hours.

Samples were incubated with 0.1x SSC buffer for 3 hours at 67°C and washed 3 times with 0.1 M Tris-HCl pH 75 and 0.15 M NaCl. Nonspecific antibody binding was performed with 0.5% blocking reagent (Roche 11096176001), 5% Sheep Serum (Sigma S3772), in 50 mM Tris-HCl pH 7.5, and 5 mM EDTA for 2 hours at room temperature in a humidified chamber. Fluorescein conjugated anti-DIG was incubated at the dilution of 1 : 200 in blocking buffer for 16 hours at 4°C. After antibody incubation, samples were washed 3 times with 0.1 M Tris-HCl pH 75 and 0.15 M NaCl and coverslips were mounted using fluorescent mounting (DAKO S3023).

Immunofluorescence was performed as previously described [13]. More in detail, NSCs were cultured in Lab-Tek chamber slides fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% Triton X-100 cells, and incubated in blocking buffer (PBS with 1% BSA) for 30 min and then with anti-HA (sc-7392 Santa Cruz) overnight in blocking solution at 4°C. 488-conjugated anti-rabbit secondary antibody was purchased from Molecular Probes (Invitrogen). Nuclei were counterstained with Hoechst (H6024 Sigma). At least 300 nuclei were counted in triplicate. Fluorescence was visualized and images were acquired with Carl Zeiss microscope (Axio Observer Z1) using ApoTome technology and AxioVision Digital Image Processing Software.

2.5. Analysis of CpG Islands Methylation. miR-326/βarr-1 regulatory region was retrieved by Rulai database (http://rulai.cshl.edu/TRED/).

CpG islands were identified in the regulatory regions of *miR-326/βarr-1* by using database of CpG islands and Analytical Tool (DBCAT) software.

The primers for methylation specific PCR were designed by using Methyl Primer Express Software v1.0, Thermo Fisher Scientific. Primers were first tested and validated using mouse Universal Methylated Mouse DNA Standard (Zymo Research).

2.5.1. Bisulphite Treatment and PCR Amplification. DNA extraction was performed using the Qiamp DNA mini kit (Qiagen). The obtained DNA was quantified using Nanodrop spectrophotometer (Thermo Scientific) and treated with Epi-Tect Bisulfite kit (Qiagen). The converted DNA was used to PCR-amplify the βarr-1 regulatory region with the following primers:

MeFw: TTTTTATTTTTTGGGCGCGTATAC

MeRw: GTCCAAACTAAAAAATCCCCGAC

UnFw: TTTTTATTTTTTGGGTGTGTATATGT

UnRw: CATCCAAACTAAAAAATCCCCAAC

As positive control we used mouse Universal Methylated Mouse DNA Standard (Zymo Research).

2.6. Statistical Analysis. Statistical analysis of cellular experimental triplicates was performed using StatView 4.1 software (Abacus Concepts, Berkeley, CA). Statistical differences were analyzed by Mann–Whitney U test for nonparametric values and a p value of ≤ 0.05 was considered significant. The results are expressed as mean ± SD from at least three experiments.

3. Results

3.1. miR-326 and βarr-1 Expression Inversely Correlates with Stemness. First we evaluated expression levels of miR-326 and of its host gene *βarr-1* in NSCs (Figures 1(a)–1(d)). NSCs displayed, as compared to the bulk preneurosphere cell population (T0), very low levels of both mature and precursor miR-326 forms and of *βarr-1* (Figures 1(a)–1(d)), together with an enhanced expression of the Nanog stemness marker (Figure 1(d)).

These results suggest that loss of miR-326/*βarr-1* locus expression is associated with a "stem-like phenotype." This notion was further supported by the observation that the expression of miR-326 and *βarr-1* increased in NSCs exposed to differentiation stimuli, for example, retinoic acid (RA) or platelet-derived growth factor (PDGF) (Figures 2(a)–2(d)). Under these conditions NSCs were able to differentiate into distinct lineages as indicated by the increased expression of both neuronal differentiation markers, βIII-tubulin (βIII-tub) and NeuN, and astrocytic differentiation markers Gfap and S100 (Figures 2(c) and 2(d)) and downmodulated the expression of stemness related markers Nanog, Oct4, and Sox2 (Figures 2(c) and 2(d)).

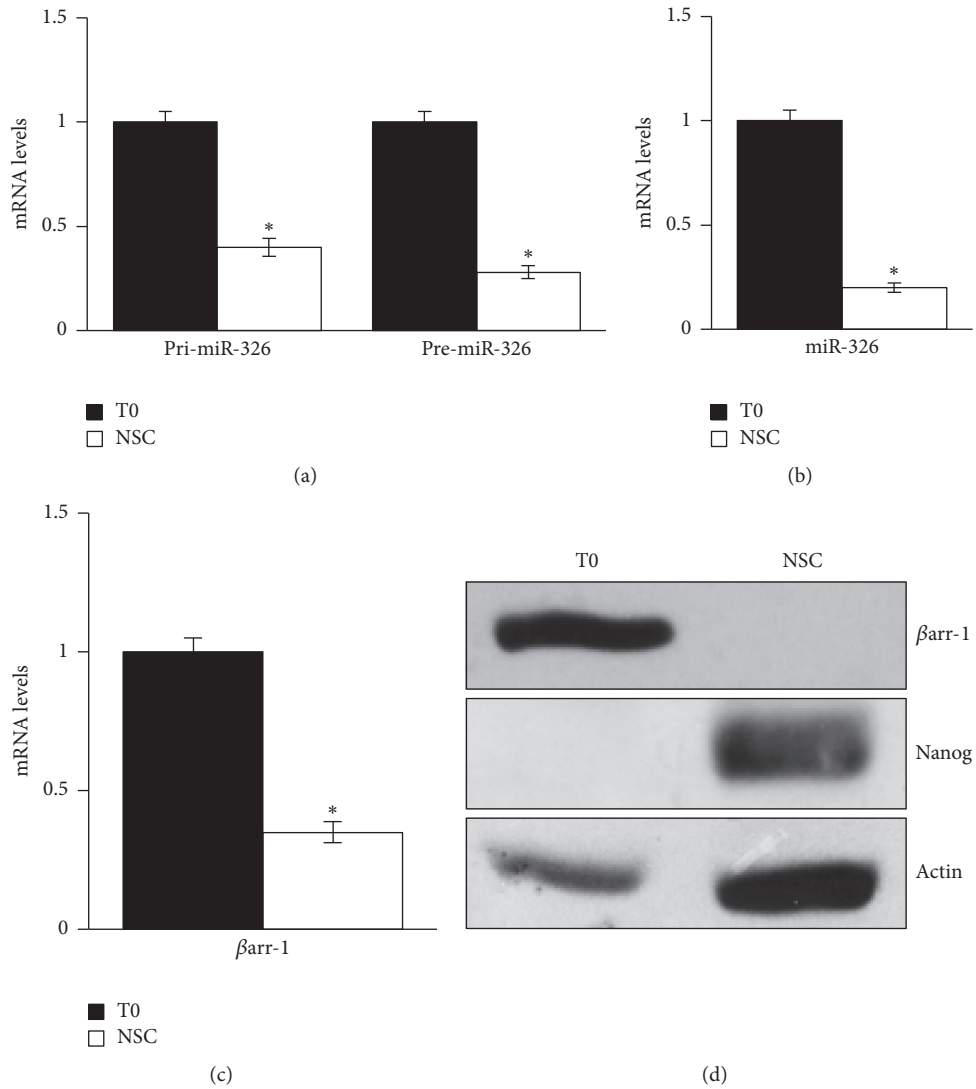

Figure 1: miR-326 and βarr-1 are downregulated in cerebellar NSCs compared to the bulk cerebellar population. (a) mRNA expression levels of the primary (pri-miR-326) and precursor (pre-miR-326) of miR-326 in NSCs compared to bulk cells (T0). (b) Mature miR-326 levels in NSCs compared to bulk cells (T0). (c) βarr-1 mRNA levels in NSCs compared to bulk cells (T0). (d) Western Blot (WB) analysis of endogenous βarr-1 and Nanog in NSCs compared to bulk cells (T0). Actin as loading control (LC). (a)–(d) data are means ± SD from 3 independent experiments. $^{*}P < 0.05$.

3.2. Epigenetic Inactivation of miR-326/βarr-1 Locus. Next we investigated the mechanism responsible for miR-326/βarr-1 locus inactivation in NSCs. There is increasing evidence that epigenetic regulation of stem cells, including "CpG island" methylation, is crucial in the preservation of their stemness by controlling the transcription switch on/off of specific developmental genes [15].

Indeed, the presence of several CpG islands in the region spanning from the first exon to part of the first intron (Figure 3(a) and Supplementary Figure 1: see Supplementary Figure 1 in the Supplementary Material available online at https://doi.org/10.1155/2017/5274171) suggested a DNA methylation-dependent control. Treatment of NSCs with the 5′-azacytidine (5-AZA) demethylating agent induced a significant increase of miR-326, its precursor transcripts, and βarr-1 levels (Figure 3(b)) and impaired stemness features while it increased cell differentiation (Figures 3(c) and 3(d)). Using methylation specific PCR we evaluated the methylation status of selected CpG islands. We found that these CpG islands were methylated in NSCs but not in the starting bulk population (T0) (Figure 3(e)). Moreover, when NCSs were treated with 5-AZA, the analyzed CpG islands lost their methylation status (Figure 3(f)), strongly suggesting that the upregulation of βarr-1 and miR-326 was due to the demethylation of their promoter region (Figure 3(b)).

These findings highlight that the methylation status of *βarr-1/miR-326* CpG islands is a mechanism to silence their expression in cerebellar NSCs.

3.3. βarr-1 Negatively Regulates NSCs Self-Renewal via Increasing p27 Nuclear Expression Levels. The above presented data suggested a role of miR-326 and βarr-1 in the establishment

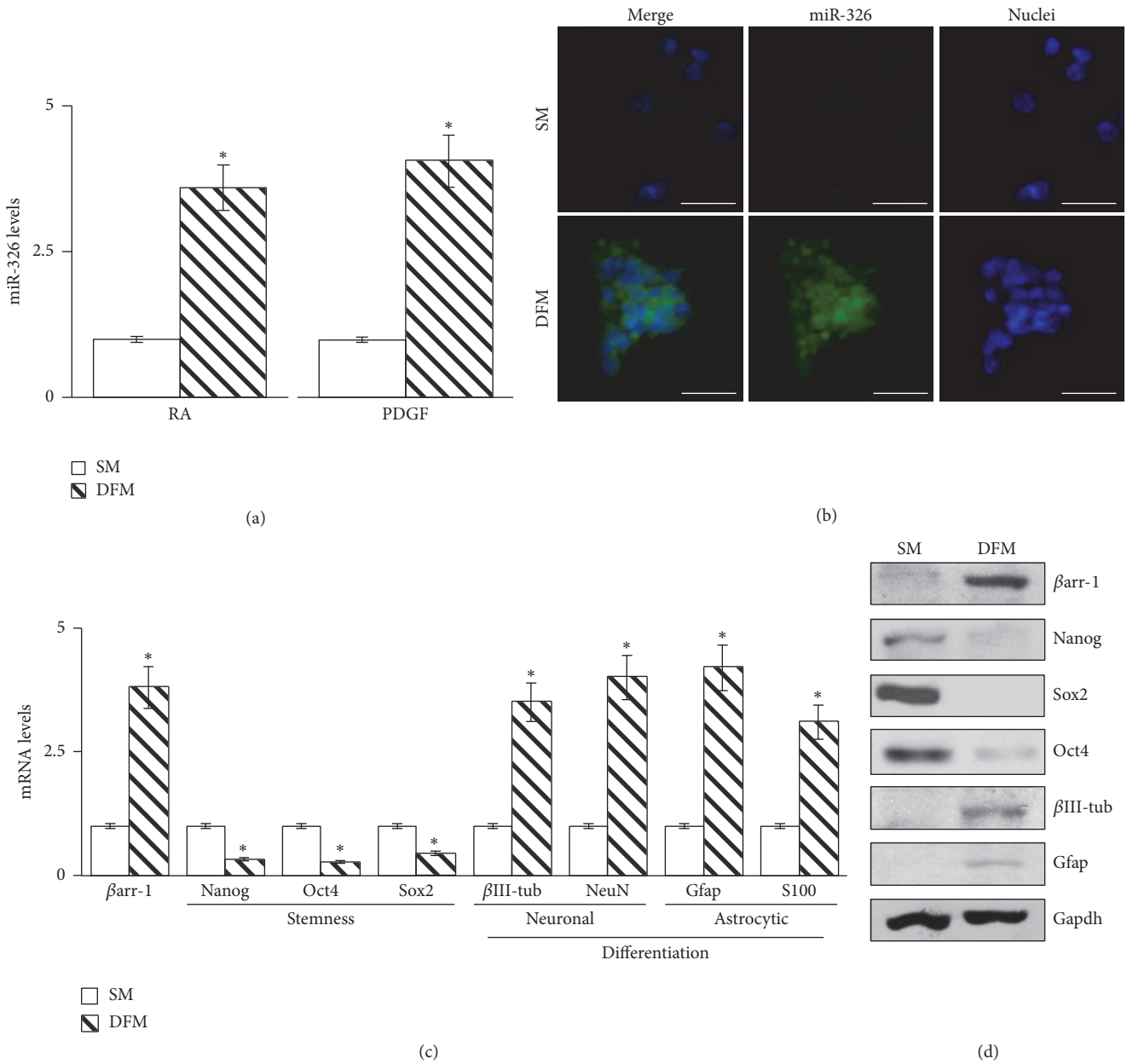

Figure 2: miR-326 and βarr-1 are upregulated in differentiated NSCs. (a) miR-326 levels evaluated in NSC exposed to differentiation stimuli (DFM) with retinoic acid (RA) or platelet-derived growth factor (PDGF) for 48 hours. Data are means ± SD from 3 independent experiments. $^{*}p < 0.05$. (b) Fluorescent in situ hybridization (ISH) staining of miR-326 of NSCs grown in stemness conditions (SM) and after RA-induced differentiation (DFM) for 48 hours. Nuclei are counterstained with Hoechst. Scale bar: 10 μm. Representative ISH images from 4 independent experiments. (c) mRNA expression levels of βarr-1 along with stemness and differentiation markers of NSC in SM and after differentiation (DFM). (d) Western blot (WB) analysis of endogenous βarr-1 and markers of stemness (Nanog, Sox2, and Oct4) and differentiation (βIII-tub and Gfap) of NSCs in SM and after differentiation (DFM). Gapdh as loading control (LC). ((c) and (d)) Data are means ± SD from 3 independent experiments. $^{*}p < 0.05$.

of a "differentiated phenotype." miR-326 is known to control several morphogenic signals that sustain stemness, such as the Hedgehog pathway and the Notch pathway [7, 16] and we found it is able to regulate Gli2 and Smo expression also in NSCs (data not shown). Indeed, miR-326 overexpression in NSCs (Figure Supplementary 2A) significantly reduced clonogenicity (Figure Supplementary 2B) and cell viability (Figure Supplementary 2C).

On the other hand, previous studies have shown that *βarr-1* functions as a cytoplasm-nucleus shuttling protein that interacts with p300/CBP to activate the transcription of the cyclin-dependent kinase (CDK) inhibitor Cdkn1b/p27kip (p27), a major determinant of cell cycle exit [16]. To gain insight into *βarr-1* functions in neuronal cells stemness and differentiation, we overexpressed *βarr-1* (Figures 4(a) and 4(b)) and found a strong impairment of the expression levels

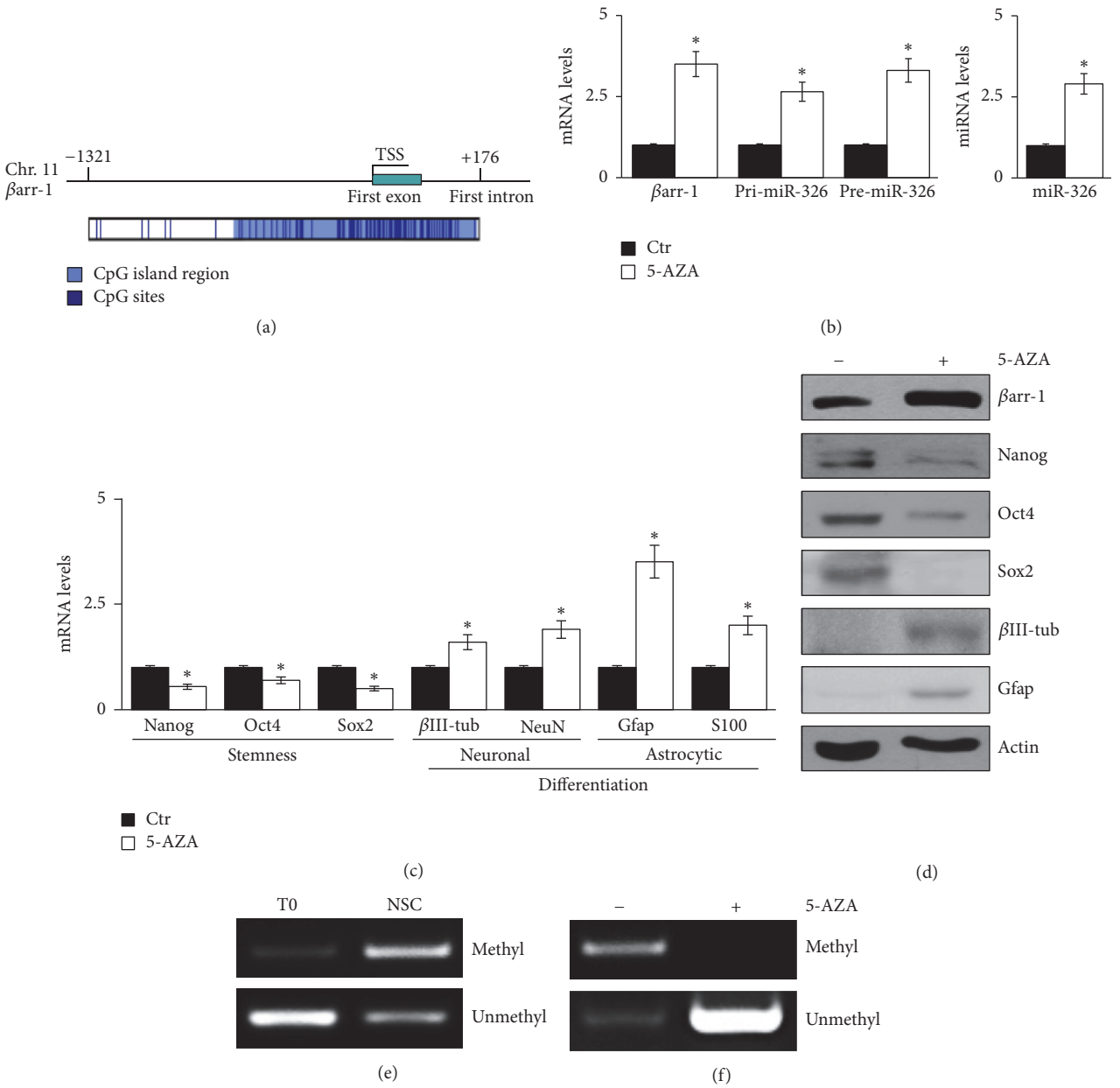

Figure 3: CpG island methylation of miR-326/βarr-1 regulatory sequence controls βarr-1 and miR-326 expression. (a) Schematic representation of mouse miR-326/βarr-1 regulatory regions, highlighting the CpG islands regions (light blue) and the CpG islands sites (blue) derived from DBCAT software. (b) mRNA expression levels of *βarr-1* and precursor forms of *miR-326* and *miR-326* expression levels in NSCs after 4 days of 5-azacytidine (5-AZA) treatment. Data are means ± SD from 3 independent experiments. $^{*}p < 0.05$. ((c)-(d)) mRNA expression levels (c) and Western blot analysis (d) of endogenous βarr-1, stemness markers (Nanog, Oct4, and Sox2), and differentiation markers (βIII-tub and Gfap) after 4 days of 5-azacytidine (5-AZA) treatment in NSCs. Actin as LC. Data are means ± SD from 3 independent experiments. $^{*}p < 0.05$. (e) Methylation specific PCR of the screened region in NSCs compared to the starting bulk population (T0). (f) Methylation specific PCR of the screened region in NSCs after 5-AZA treatment. ((e) and (f)) Data are representative images from 3 independent experiments.

of the stemness markers Nanog, Oct4, and Sox2 (Figures 4(b) and 4(c)), impaired clonogenicity together with decreased proliferation rate and cell viability (Figures 4(d) and 4(e)), and increased cell apoptosis (Figure 4(f)), suggesting that *βarr-1* may have a role both in the regulation of stemness features and in the regulation of cell cycle. Indeed, we found that, when shifted to differentiation medium, NSCs increased *βarr-1* level was paralleled by an activation of p27 transcription and the increase of p27 protein in the nucleus as observed in nucleous/cytoplasmic fractionation experiment (Figure 5). Accordingly, p27 mRNA and the nuclear protein cell fraction levels were increased by exogenously

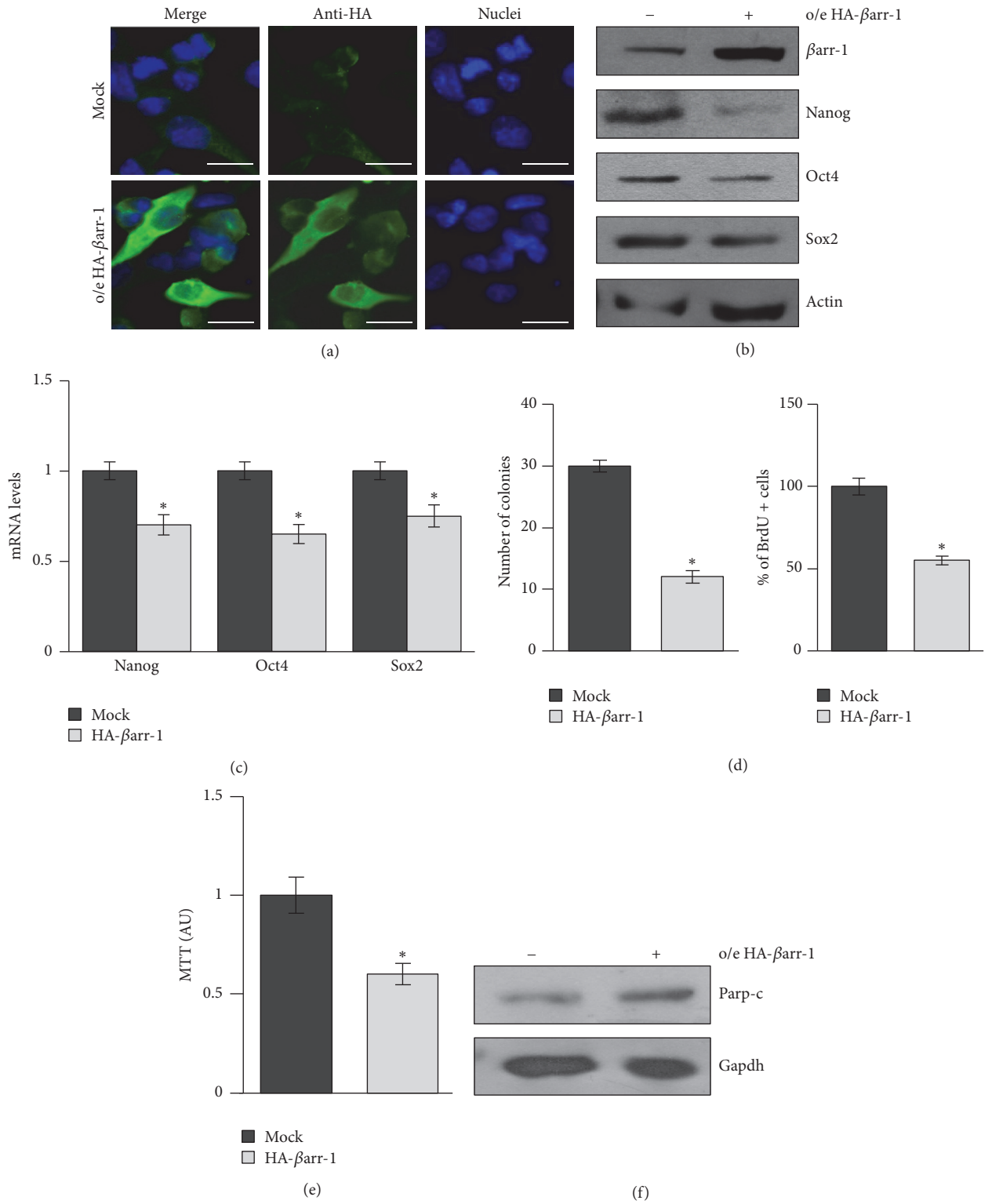

Figure 4: βarr-1 overexpression impairs NSCs clonogenicity and proliferation. (a) Representative image of immunofluorescence staining of NSCs after overexpression of HA-βarr-1. Nuclei are counterstained with Hoechst. Scale bar: 5 μm. (b) WB analysis of endogenous stemness markers in NSCs after overexpression of HA-βarr-1. Loading control: actin. Representative Western blot images from 3 independent experiments. (c) mRNA expression levels of stemness markers in NSCs after overexpression of HA-βarr-1. (d) Oncosphere forming assay (number of colonies, left panel) and bromodeoxyuridine (BrdU) uptake (right panel) in NSCs after ectopic expression of HA-βarr-1. (e) Cell viability (MTT assay) in NSC after ectopic expression of HA-βarr-1. (f) WB analysis of cleaved Parp (Parp-c) in NSCs after overexpression of HA-βarr-1. Loading control: Gapdh. Representative Western blot images from 3 independent experiments. ((c), (d), and (e)) Data are means ± SD from 3 independent experiments. $^{*}p < 0.05$.

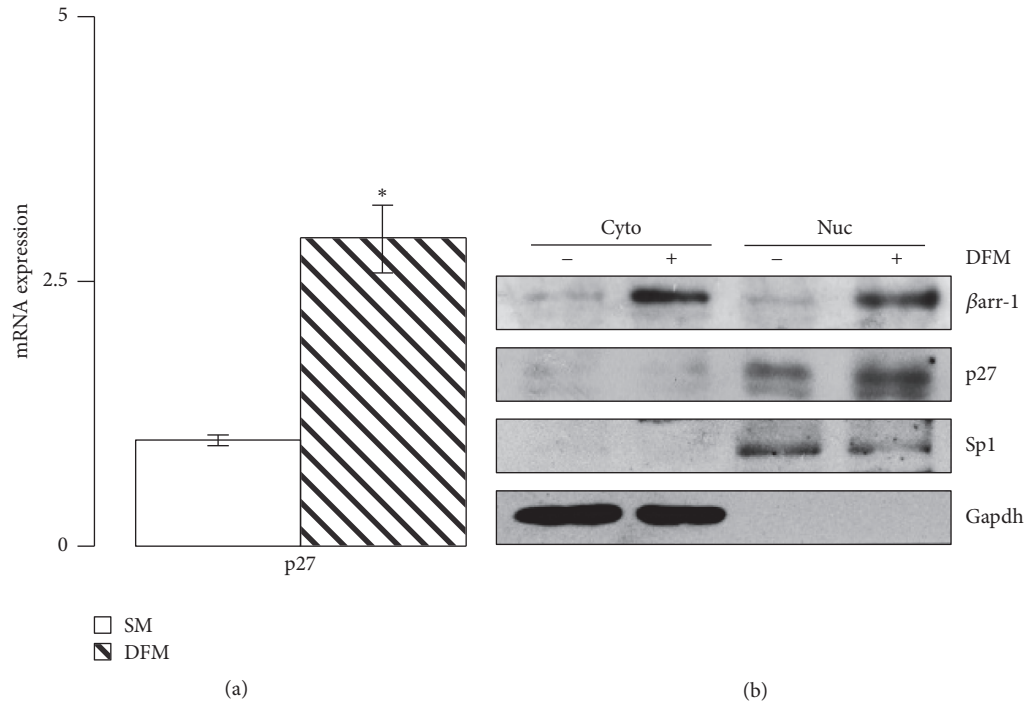

Figure 5: βarr-1 controls NSCs proliferation via p27 expression. (a) p27 mRNA expression levels evaluated by qRT-PCR in NSCs grown under stemness conditions (SM) and after differentiation (DFM) for 48 hours. Data are means ± SD from 3 independent experiments. $^{*}P < 0.05$ (DFM versus SM). (b) Western blot showing subcellular localization of endogenous βarr-1 and p27 in NSC cultured in SM or DFM for 48 hours. βarr-1 and p27 proteins levels were assessed in cytosolic (Cyto) and nuclear (Nuc) fractions. Gapdh and Sp1 protein levels were used as loading controls and markers for purity of Cyto and Nuc fractions, respectively. Representative Western blot images from 3 independent experiments.

expressed *βarr-1* (Figures 6(a) and 6(b)) and decreased by siRNA-mediated abrogation of *βarr-1* expression (Figures 6(c) and 6(d)).

Altogether these results show that *βarr-1* expression inhibits the cell cycle via activation of p27 thus blocking cell stemness features in NSCs.

4. Discussion

In this study we propose a model in which epigenetic silencing of the intragenic miR-326 and its host gene, *βarr-1*, maintains physiological neuronal stemness. While miR-326 and *βarr-1* are highly expressed in differentiated cells where they induce growth arrest, in NSCs they are kept at low levels through CpG hypermethylation. Coherently, modulation of the expression of *βarr-1* through overexpression regulated the differentiation and growth rate of cerebellar NSCs. The reactivation of the locus miR-326/*βarr-1* enhances the cell cycle inhibitor p27 while inhibiting proliferative signalling (e.g., Hh or Notch by miR-326), thus resulting in stem cell differentiation and growth arrest (Figure 7).

These findings suggest a new circuitry composed of miR-326/βArr-1, morphogenic molecules, and cell cycle modifiers which control neural stemness status.

In particular, here we identify *βarr-1* as a new player in the coordinated control of cerebellar NSCs. *βarr-1* was first identified as a gene encoding a scaffold protein that regulates G-protein-coupled receptor (GPCR), through interaction with cytoplasmic proteins linking GPCRs to intracellular signaling pathways [17, 18]. Our observations of the role of *βarr-1* in NSCs are consistent with the growth arrest observed in GCPs where *βarr-1* directly affects gene expression, translocating into the nucleus where it interacts with transcription cofactors at the promoters of the target gene p27 [19].

Consistently, previous studies investigated the role of this CDK-inhibitor in suppressing self-renewal and proliferation while driving differentiation of NSCs [17, 18, 20, 21] and cerebellar progenitors [22, 23]. A further link between p27 and stem/progenitor cell differentiation is provided by the p27-destabilizing effect induced by REST, a repressor of neuronal differentiation, resulting in maintenance of cell proliferation and blockade of neuronal differentiation [24]. In this way, loss of p27 maintains a high turnover of self-renewing cells, by coupling the ability to control both cell cycle and undifferentiated status. Of note, βarr-1 overexpression in NSCs affected also cell viability and increased cell apoptosis suggesting that such scaffold adaptor protein could have other possible functions in controlling different mechanisms besides the cell growth arrest.

Our findings underline a coordinated epigenetic mechanism that finely regulates miR-326/βarr-1 expression and cell growth in neural stem cells. Indeed, the same promoter drives the expression of βarr-1 and miR-326 through an epigenetic control in cerebellar NSC.

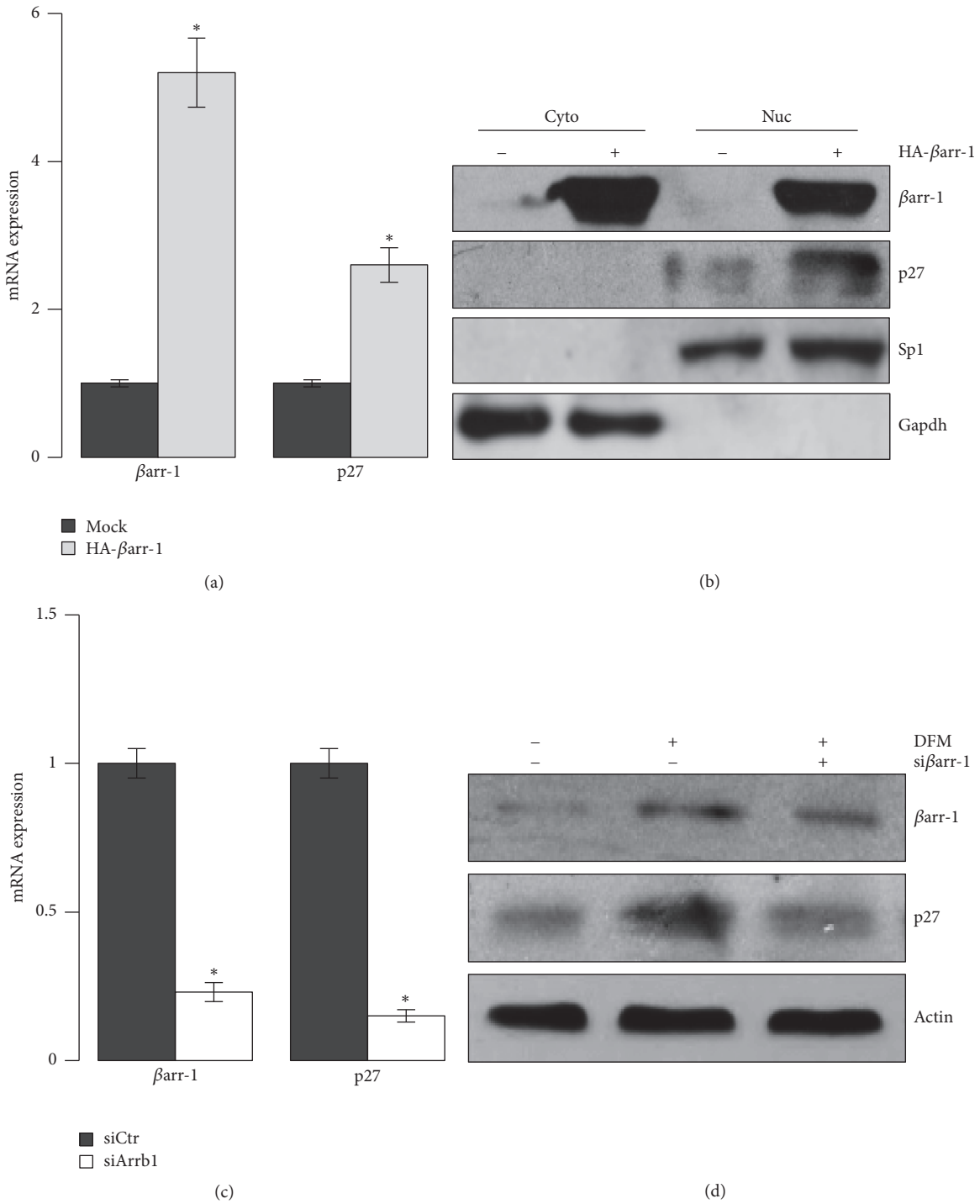

Figure 6: βarr-1 controls NSCs proliferation via p27 expression. (a) βarr-1 and p27 mRNA expression levels in NSCs transfected with the HA-βarr-1 plasmid for 48 hrs. (b) Subcellular localization of βarr-1 and p27 proteins in NSCs transfected with the HA-βarr-1 plasmid and analyzed 48 hrs after transfection. Proteins are shown in the cytosolic (Cyto) and nuclear (Nuc) fractions. Sp1: nuclear loading control; Gapdh: cytoplasmic loading control. Representative Western blot images from 3 independent experiments. (c) *βarr-1* and *p27* mRNA expression levels in NSCs transfected with control siRNA (siCtr) or βarr-1 siRNA (siβarr-1) cultured for 24 hrs in DFM. (d) Western blot showing endogenous βarr-1 and p27 protein levels in NSCs transfected with control siRNA (siCtr) or βarr-1 siRNA (siβarr-1) cultured for 24 hrs in DFM. Representative Western blot images from 3 independent experiments. ((a) and (c)) Data are means ± SD from 3 independent experiments. $^{*}p < 0.05$.

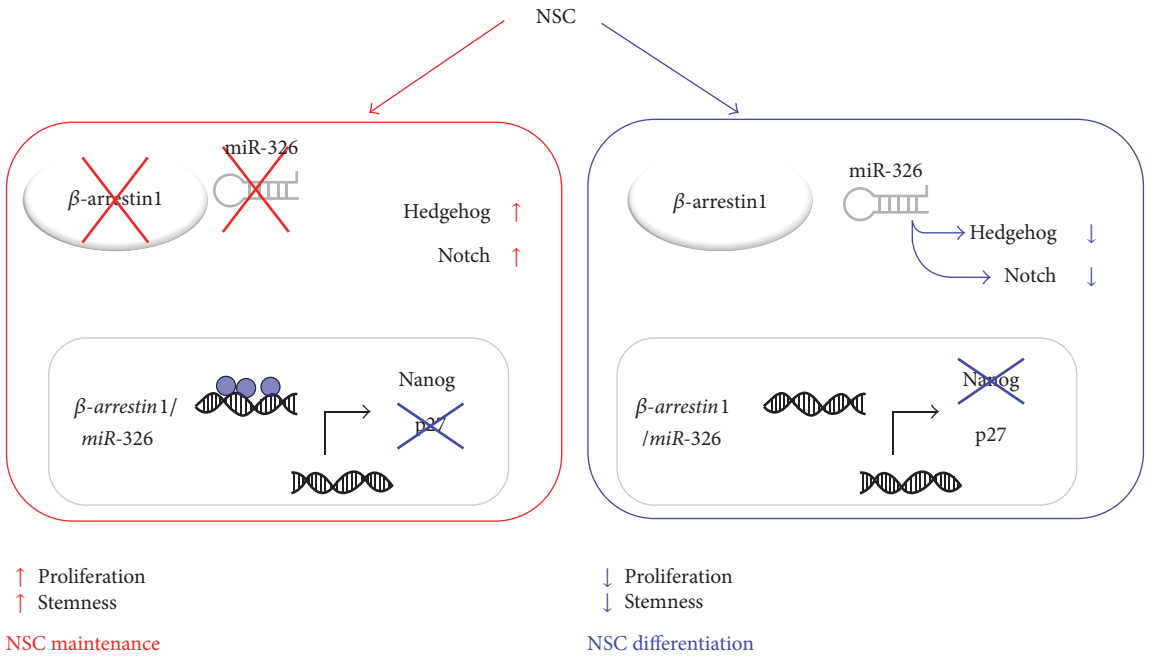

Figure 7: miR-326 and β-arrestin1 are epigenetically silenced and control stemness features in cerebellar NSCs. We propose a model in which epigenetic silencing of the intragenic miR-326 and its host gene, β-arrestin1, maintains physiological neuronal stemness. In cerebellar NSCs miR-326 and β-arrestin1 are epigenetically silenced through hypermethylation of CpG islands on their common promoter, leading to a permissive molecular environment for the expression of prostemness and proproliferative cues. Upon differentiation or demethylating treatment, the promoter of the locus miR-326/β-arrestin1 is demethylated and thus the expression levels of miR-326 and β-arrestin1 are upregulated. While miR-326 targets molecules belonging to the Hedgehog pathway and the Notch pathway, thus hampering stemness properties, β-arrestin1 acts as a cofactor for the transcriptional activation of p27, leading to cell cycle arrest.

The ability of miR-326/*βarr-1* to be epigenetically regulated also links this transcript to the processes preserving the stemness status. Indeed, increasing evidence supports the crucial role of epigenetic regulation of stem cells, including NSCs [25]. Such a regulation includes the mechanisms responsible for the maintenance of repressive chromatin states at specific loci, consisting of CpG islands methylation [26].

Accordingly, in our study miR-326 and βarr-1 are among the silenced genes in NSCs, whose regulation contributes to balance the opposing forces of cell growth and differentiation. Indeed the miR-326/βarr-1 promoter is characterized by methylated or demethylated CpG islands in NSC or differentiated cells, respectively.

Interestingly, in line with this, miR-326 and βarr-1 have been described to be key molecules in multiple sclerosis pathogenesis, being crucial for CD4+ T cell survival and differentiation. Indeed βarr-1 enhanced the expression of the protooncogene Bcl2 via the acetylation of histone H4 at the Bcl2 promoter [27] while miR-326 promoted interleukin 17- (IL-17-) producing T helper cells (TH-17) differentiation by targeting Ets-1, a negative regulator of TH-17 differentiation [28]. Of note CD4+ T cells from patients with multiple sclerosis had much higher Arrb1 expression [27] and miR-326 expression was highly correlated with disease severity in patients with multiple sclerosis [28].

Even if no specific studies of βarr-1/miR-326 coregulation have been conducted in multiple sclerosis our results together with the abovementioned observations allow speculating that the βarr-1/miR-326 transcription unit is regulated in other stem or progenitor cells during differentiation, participating in the control of cell fate, development, and disease.

5. Conclusions

We describe in the present work the role and regulation of the locus miR-326/βarr-1. miR-326 and its host gene βarr-1 represent a novel miRNA/protein network that controls cerebellar NSCs through the modulation of morphogenic signals and cell cycle modifiers, both regulating stemness and suggested to be involved in the maintenance of self-renewal feature of NSCs.

In conclusion, our findings describe a bivalent signal (miRNA and hosting protein encoding gene) converging upon the coordinated inhibition of normal stem cell functions.

Disclosure

Federica Begalli's current address is Center for Cell Signalling and Inflammation (CCSI), Department of Medicine, Imperial College London. Elisabetta Ferretti and Evelina Miele are co-last authors.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Molecular Mutations and Their Cooccurrences in Cytogenetically Normal Acute Myeloid Leukemia

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Adult acute myeloid leukemia (AML) clinically is a disparate disease that requires intensive treatments ranging from chemotherapy alone to allogeneic hematopoietic cell transplantation (allo-HCT). Historically, cytogenetic analysis has been a useful prognostic tool to classify patients into favorable, intermediate, and unfavorable prognostic risk groups. However, the intermediate-risk group, consisting predominantly of cytogenetically normal AML (CN-AML), itself exhibits diverse clinical outcomes and requires further characterization to allow for more optimal treatment decision-making. The recent advances in clinical genomics have led to the recategorization of CN-AML into favorable or unfavorable subgroups. The relapsing nature of AML is thought to be due to clonal heterogeneity that includes founder or driver mutations present in the leukemic stem cell population. In this article, we summarize the clinical outcomes of relevant molecular mutations and their cooccurrences in CN-AML, including *NPM1*, *FLT3*$^{\mathrm{ITD}}$, *DNMT3A*, *NRAS*, *TET2*, *RUNX1*, *MLL*$^{\mathrm{PTD}}$, *ASXL1*, *BCOR*, *PHF6*, *CEBPA*$^{\mathrm{biallelic}}$, *IDH1*, *IDH2*$^{\mathrm{R140}}$, and *IDH2*$^{\mathrm{R170}}$, with an emphasis on their relevance to the leukemic stem cell compartment. We have reviewed the available literature and TCGA AML databases (2013) to highlight the potential role of stem cell regulating factor mutations on outcome within newly defined AML molecular subgroups.

1. Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults. Although there have been landmark targeted therapies developed in other hematologic malignancies, such as imatinib for chronic myeloid leukemia and ibrutinib in chronic lymphocytic leukemia, induction chemotherapy for AML has not changed significantly for several decades [1, 2]. The notable exception being acute promyelocytic leukemia (APL) with the development of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) to overcome the block in myeloid differentiation due to the PML-RARα fusion protein created by the translocation 15;17. Current AML induction therapies are successful in obtaining complete remission in approximately 75% of young (age < 60 years) de novo AML patients; however, most are destined to relapse. This clinical behavior suggested the presence of an underlying leukemic cell population responsible for the relapsing nature of AML despite the attainment of a complete remission through induction chemotherapy. The existence of leukemic stem cells in AML capable of recapitulating the disease was firmly established by transplant experiments utilizing immunocompromised mouse models two decades ago [3, 4]. To date, the knowledge derived from the discovery of leukemic AML stem cells is just beginning to be used in developing new therapeutic strategies and categorizing risk groups in patients. Patient outcomes in CN-AML, in particular, are widely diverse. The clinical validation of several additional molecular markers such as *FLT3*, *NPM1,* and *CEBPA* mutations has added a great deal to the prognostic stratification of CN-AML. Therefore, it is vital to build upon these advances by continuing to elucidate the biological

characteristics and properties of leukemic stem cells and their regulating factors to assess their impact on AML treatment plans, the overarching question being what is the optimal consolidation strategy for each AML patient? Perhaps the incorporation of leukemia-stem cell mutations will add further clarity to which patients merit consolidation with allo-HCT and its attendant mortality and comorbidity and which AML patients can be safely managed with chemotherapy alone.

Historically, the French American British (FAB) classification system was used to subdivide AML into 8 subgroups (M0–M7) on a morphological basis [5]. The advent of cytogenetic studies enabled AML subtypes to be stratified into three risk groups, favorable, intermediate, and unfavorable risk. Using cytogenetics, clinicians could identify the favorable risk AML, such as the core binding factor leukemias [inv(16), t(16;16), and t(8;21)], and for this risk group, excellent long-term survival could be achieved with high dose cytarabine consolidation therapy alone. For patients with unfavorable risk, such as monosomies, 17p deletion, or complex abnormalities, there is a very low likelihood of cure with chemotherapy alone and consolidation with allo-HSCT is pursued if possible. Intermediate-risk patients include CN-AML, which comprises up to 40% to 50% of AML patients [6]. The clinical outcome of CN-AML patients varies widely and cannot be predicted based solely on cytogenetics.

The focus on improving our understanding of CN-AML prognosis and outcomes leads to the identification additional molecular markers of clinical significance. Mutations in nucleophosmin 1 *(NPM1)*, fetal liver tyrosine kinase 3 *(FLT3),* and CCAAT/enhancer binding protein α *(CEBPA)* have been shown to have clinically significant prognostic value [7]. The *FLT3* internal tandem duplication (ITD) mutation ($FLT3^{ITD}$) is present in nearly one-third of AML cases and has been associated with adverse clinical outcomes including increased relapse risk and decreased overall survival (OS) [8]. $FLT3^{ITD}$ presence in CN-AML identified a subgroup of patients with more adverse outcome, particularly patients with a high mutant allelic frequency [9]. In addition to providing prognostic information, the $FLT3^{ITD}$ is a therapeutic target as well. Sorafenib, a tyrosine kinase inhibitor targeting $FLT3^{ITD}$ mutations, has been shown to increase event-free survival (EFS) and relapse-free survival (RFS) when added to both induction and consolidation therapies, although there was no OS benefit in the three years of follow-up in the newly diagnosed AML patients aged 60 years or younger [10]. *NPM1* mutation has also been recently validated as a molecular marker of minimal residual disease (MRD) in *NPM1* mutation positive patients and the presence of MRD was shown to be the only independent prognostic factor for death in multivariate analysis [11].

Consolidative chemotherapy is utilized to eliminate residual leukemia cells and/or leukemic stem cells (LSC) after induction chemotherapy to reduce the chance of relapse. Risk of a relapse after induction chemotherapy and consolidation chemotherapy increases with the increased MRD, a condition which can be assessed by immunophenotypical detection of leukemia cells [12, 13]. Level of MRD correlates with the amount of leukemic stem cells and predicts outcome in AML [14–17]. Therefore, it is imperative to keep leukemic stem cells in mind when clinicians stratify patients for treatment purposes.

Advances in clinical genomics have identified an expanding array of recurrent molecular lesions in AML that will add layers of complexity to prognostic stratification needed to guide treatment and provide needed targets for new AML therapies. The evolving challenge is to incorporate these molecular abnormalities and their combinatorial effect on AML prognosis and in turn treatment strategies. The availability of this new AML data has created a requirement of a new classification system based on both cytogenetics and additional molecular lesions, which will be pivotal in establishing new clinical treatment guidelines. Furthermore, new classifications based on molecular abnormalities may help clinical trial design to develop targeted therapies to specific subgroups of AML patients. Recently a new AML classification system has been proposed by Papaemmanuil et al. [18]. Here the authors classified AML based on the presence of one or more driver mutations +/− other comutations into 11 different subgroups and correlated with clinical outcomes. This new classification system has provided insight regarding the effects of specific driver mutations and the additive effect seen when they are found in combination. In this paper, we summarize the significance of the most clinically relevant molecular mutations, cooccurrences of these mutations, and their functional role on leukemic stem cell population in relation to clinical outcomes based on this newly developed classification system.

2. The New AML Classification System

The proposed new AML classification system is based on a retrospective genomic analysis of 1540 AML patients in three prospective trials of the German-Austrian AML Study Group [18]. Patients received induction chemotherapy with idarubicin, cytarabine, and etoposide (ICE) with or without ATRA; high-risk patients were offered allo-HCT; intermediate-risk patients were offered a matched related donor allo-HCT, if a matched sibling was available; low risk patients received chemotherapy alone. The median follow-up period was 5.9 years.

In addition to cytogenetic analyses, 111 candidate driver genes were sequenced and 5234 somatic driver mutations were identified across 76 genes or genomic regions. Nearly all AML patients (96%) had at least one mutation and 86% patients were found to have two or more mutations. Statistical analysis of comutation patterns was utilized to define 11 mutually exclusive AML subtypes including three novel genetic subgroups that have not been described in the World Health Organization (WHO) classification in 2008 [19]. These novel subgroups are, namely, (1) AML with mutations in genes encoding chromatin, RNA-splicing regulators, or both (18% of patients); (2) AML with *TP53* mutations, chromosomal aneuploidies, or both (13%); and (3) AML with $IDH2^{R172}$ mutations (1%). Many of the mutations used to define the novel subgroups involve genes which have roles in stem cell functions. Of note, only 48% of patients were classifiable

Table 1: Molecular classification of CN-AML and clinical outcomes.

AML with *NPM1* mutation	
NPM1*	Better EFS and OS [29–32]; better CRR, high RR with EFS or OS benefits [33]; better OS [34]
NPM1/*DNMT3A*	Worse OS [41]
NPM1/*DNMT3A*/*FLT3*^{ITD}	Worse OS [18]
NPM1/*DNMT3A*/*NRAS*$^{G12/13}$	Better OS [18]
NPM1/*TET2**	Worse CRR, EFS, DFS, and OS [50]; no impact on outcomes [41]
NPM1/*IDH1** or *NPM1*/*IDH2*^{R140*}	Worse OS [55]; better OS [27, 52]
AML with mutated chromatin, RNA-splicing genes, or both	
RUNX1	Worse EFS, DFS [59, 61, 62]; worse OS [59–62]
MLL^{PTD}	Worse EFS [60, 66, 67]; worse OS [27, 41, 60, 67]
ASXL1	Worse outcomes [27, 60, 75]; worse CR, EFS, DFS, and OS [77]
BCOR	Worse EFS and OS [81]
PHF6*	Worse OS [27]
AML with *CEBPA*^{biallelic} mutation	
CEBPA^{biallelic}	Better EFS and OS [86]
CEBPA^{biallelic}/*TET2*	Worse OS [89]
CEBPA^{biallelic}/*GATA2*	Better OS [89]
AML with *IDH2*^{R172} mutation	Worse RR and OS [52, 90]; better OS [18]

EFS: event-free survival; OS: overall survival; CRR: complete remission rate; RR: relapse rate; DFS: disease-free survival.
*Without *FLT3*^{ITD}.

based on the current WHO guidelines, whereas 80% of patients could be allocated into this novel classification system. Only 8% of patients had either no detected driver mutations (4%) or ≥2 genomic subgroups (4%).

Clinical outcomes, such as OS of AML patients with genetic mutations, were found to be significantly altered by the presence or absence of other driver mutations as has been described by others [20]. For instance, *NPM1*-mutated AML, as the largest subgroup in this novel classification, had variable clinical outcomes influenced by the presence of concurrent mutations such as *FLT3*, *DNMT3A*, *NRAS*, *IDH*, *PTPN11*, or chromatin-spliceosome mutations. We focused on the clinical outcomes of the most significant single or concurrent molecular mutations based on this novel classification (Table 1) and the significance of concurrent or mutually exclusive alterations in genes of interest (Table 2). We did not include the effect of traditional cytogenetic abnormalities in this study.

3. AML with *NPM1* Mutation

Nucleophosmin is a protein encoded by the *NPM1* gene in humans. Nucleophosmin has multiple functions in various processes including histone chaperones, ribosome biogenesis and transport, genomic stability and DNA repair, control of centrosome duplications, and regulation of the ARF-p53 tumor suppressor pathway [21]. All these functions have a part in leukemic stem cell self-renewal and limited differentiation. Indeed, stem cell/progenitor cell surface marker CD34+ cells from *NPM1*-mutated AML patients are able to recapitulate leukemia in immunodeficient mice [22]. Alteration of the *NPM1* gene was found to be present at a high frequency in AML patients, ranging from 25% to 53% in all AML and 46% to 67% in CN-AML. *NPM1*-mutated AML consists of 27% of all AML and therefore forms the largest subgroup in this novel classification [18]. The identification of *NPM1* mutation in AML is important for both disease prognosis and the subsequent treatment decision-making regarding consolidation with chemotherapy alone or an allo-HCT treatment. Furthermore, a recent study demonstrated the importance of MRD analysis in *NPM1*-mutated AML [11]. Since high MRD correlates positively with high stem cell frequency in AML [16], persistence of *NPM1*-mutated transcripts in blood was associated with a greater risk of relapse after 3 years of follow-up.

AML with *NPM1* mutation is a clinically heterogeneous group likely due to the prevalence of concurrent mutations: 54% *DNMT3A*, 39% *FLT3*^{ITD}, 19% *NRAS*, 16% *TET2,* and 15% *PTPN11*. *NPM1* is usually a secondary or downstream mutation, whereas mutations in *DNMT3A*, *ASXL1*, *IDH1*/*2*, and *TET2* occur very early during clonal evolution but are typically not sufficient to cause leukemia on their own. Therefore, the analysis of comutation patterns in this group has become crucial in predicting disease prognosis.

3.1. NPM1 and FLT3^{ITD}. *FLT3*^{ITD} represents one of the most frequent genetic alterations with a 20% frequency in adult AML, 28–34% in cytogenetically normal AML [23], and 39% in *NPM1*-mutated AML [18]. *FLT3*^{ITD} activates STAT5 to maintain survival of leukemic stem cell population in AML [24]. It was not a surprise that *FLT3*^{ITD}-positive AML patients had higher relapse incidence and lower DFS [25, 26] as well as OS [27]. These observations have validated *FLT3* as a therapeutic target in AML and *FLT3* inhibitors have shown promising results when combined with standard therapy [10, 28].

With regard to *NPM1* and *FLT3*^{ITD}, several studies have shown that AML with *NPM1* mutation, but without *FLT3*^{ITD} mutation, is associated with significantly better OS and EFS

Table 2: Cooccurrent or mutually exclusive alterations and their significance in genes of interests.

Gene A	Gene B	*P* value	Odds ratio	Association	
ASXL1	*IDH2*	0.003	2.241	Tendency towards cooccurrence	Significant
IDH1	*DNMT3A*	0.005	1.266	Tendency towards cooccurrence	Significant
TET2	*DNMT3A*	0.01	1.156	Tendency towards cooccurrence	Significant
DNMT3A	*FLT3*	0.035	0.713	Tendency towards cooccurrence	Significant
KMT2A	*DNMT3A*	0.042	–1.169	Tendency towards mutual exclusivity	Significant
IDH2	*KMT2A*	0.056	1.052	Tendency towards cooccurrence	Marginal

Database used for analysis is TCGA, NEJM 2013 [94]. The database contains all 166 complete tumors of AML. Query was performed on 10 genes which include *ASXL1*, *BCOR*, *TET2*, *IDH1*, *IDH2*, *RUNX1*, *PHF6*, *KMT2A (MLL)*, *DNMT3A*, and *FLT3*. The query results contain 23 gene pairs with mutually exclusive alterations (1 significant) and 22 gene pairs with cooccurrent alterations (4 significant, 1 marginal).

[29–32]; one study demonstrated that *NPM1* mutation with or without $FLT3^{ITD}$ was only favorable in achieving complete remission but was associated with a high relapse rate with no OS and EFS benefits [33]. The German-Austrian AML Study Group conducted a study to evaluate genetic mutations and clinical outcomes in 872 adults younger than 60 years of age and again demonstrated that *NPM1* mutation without $FLT3^{ITD}$ was associated with lower risk of relapse and death [34]. The majority of these earlier studies showed that *NPM1* mutation without $FLT3^{ITD}$ is associated with better clinical outcomes, and allo-HST conferred no benefit in this patient group [34] similar to the core binding-factor leukemia patient group [35]. It is apparent that all these studies pointed to a worse clinical outcome when *NPM1* mutation and $FLT3^{ITD}$ mutation coexisted in AML. However, the most recent study argued against *NPM1* and $FLT3^{ITD}$ mutations being the sole determinants in AML prognosis, and another mutation, *DNMT3A*, must also be taken into consideration in the decision-making process of the treatment of *NPM1*-mutated AML [18].

3.2. NPM1 and DNMT3A. *DNMT3A* (DNA methyltransferase 3A) is an enzyme that catalyzes the transfer of methyl groups to specific CpG structures in DNA and hence plays an essential role in DNA methylation and gene silencing regulatory processes [36]. *DNMT3A* is important in normal hematopoietic stem cell differentiation and self-renewal [37] and its mutation produces a reservoir of preleukemic stem cells which can evolve into AML [38]. *DNMT3A* mutations were found in 22.1% of all AML and 33.7% of AML with intermediate-risk cytogenetic profile and were independently associated with a poor outcome regardless of age [39]. *DNMT3A* mutations tend to cooccur with *FLT3*, *TET2*, or *IDH1* in AML (Table 2). The combination of *DNMT3A* mutation with *FLT3*, *TET2*, or *IDH1* tends to have an adverse effect on disease-free survival in AML compared to wild-type group (Figure 1). Interestingly, hypomethylating agents, such as decitabine and 5-azacitidine, have a higher clinical remission rate in *DNMT3A*-mutated AML [40].

Patients with *DNMT3A*, *TET2*, *ASXL1*, *PHF6*, or MLL^{PTD} mutations who were in the WHO intermediate group had an adverse outcome compared to those with other genotypes [27]. *DNMT3A* mutation was found to be an adverse prognostic factor in cytogenetically normal AML with mutated *NPM1* without $FLT3^{ITD}$ in terms of OS [41]. However, this finding was not confirmed by the most recent study [18]. Instead, it was reported that patients with both *NPM1* and *DNMT3A* mutations but without $FLT3^{ITD}$ showed much better outcomes than those with $FLT3^{ITD}$. Therefore, triple-mutated AML (*NPM1*/*DNMT3A*/$FLT3^{ITD}$) yields the worst prognosis and the consolidation with allo-HCT should be considered, although prospective study is needed to confirm these results.

3.3. NPM1 and $NRAS^{G12/13}$. *NRAS* belongs to the RAS GTPase family of genes. It plays important roles in the regulation of proliferation, differentiation, and apoptosis in AML and is a fairly common mutation in AML ranging from 11% to 30% [42]. *NRAS* mutation consists of 19% of *NPM1*-mutated AML [18]. The prognostic impact of *NRAS* mutation has been reported to be insignificant for OS, EFS, and disease-free survival (DFS) [42]. However more recently mutations in *NPM1* and *DNMT3A* in the presence of $NRAS^{G12/13}$ in AML patients were associated with a more favorable outcome [18].

3.4. NPM1 and TET2. *TET2* (ten-eleven translocation) protein is an epigenetic modifier that converts methylcytosine to 5-hydroxymethylcytosine and plays a role in DNA methylation and myelopoiesis. Normal expression and function of *TET2* are essential in maintaining the hematopoietic stem cell (HSC) pool and in controlling HSC differentiation [43]. Studies using conditional knockout mouse models have revealed that complete loss of *TET2* ($TET2^{-/-}$) or *TET2* haplodeficiency ($TET2^{+/-}$) impaired hematopoietic stem cell differentiation, held cells in a more immature state, and initiated aberrant hematopoiesis both in vitro and in vivo [44–46]. *TET2* expression is tightly regulated by the master stem cell transcription factors *Oct4* and *Sox2* [44]. *TET2* mutations are present in 5–25% of adult AML cases, with the highest frequency in the elderly [47, 48]. *TET2* mutations are significantly correlated with *NPM1* (16%) in this most recent study [18] and were found to be mutually exclusive with MLL^{PTD} [48] and *IDH1*/*2* mutations [48, 49]. *TET2* mutation resulted in a lower complete remission rate, shorter EFS and DFS in patients with mutated *NPM1* without $FLT3^{ITD}$ [47], and shorter OS in patients with mutated *NPM1* without $FLT3^{ITD}$ [47, 50] and with mutated *NPM1* [50]. However, *TET2* mutations were also reported to have no impact on the

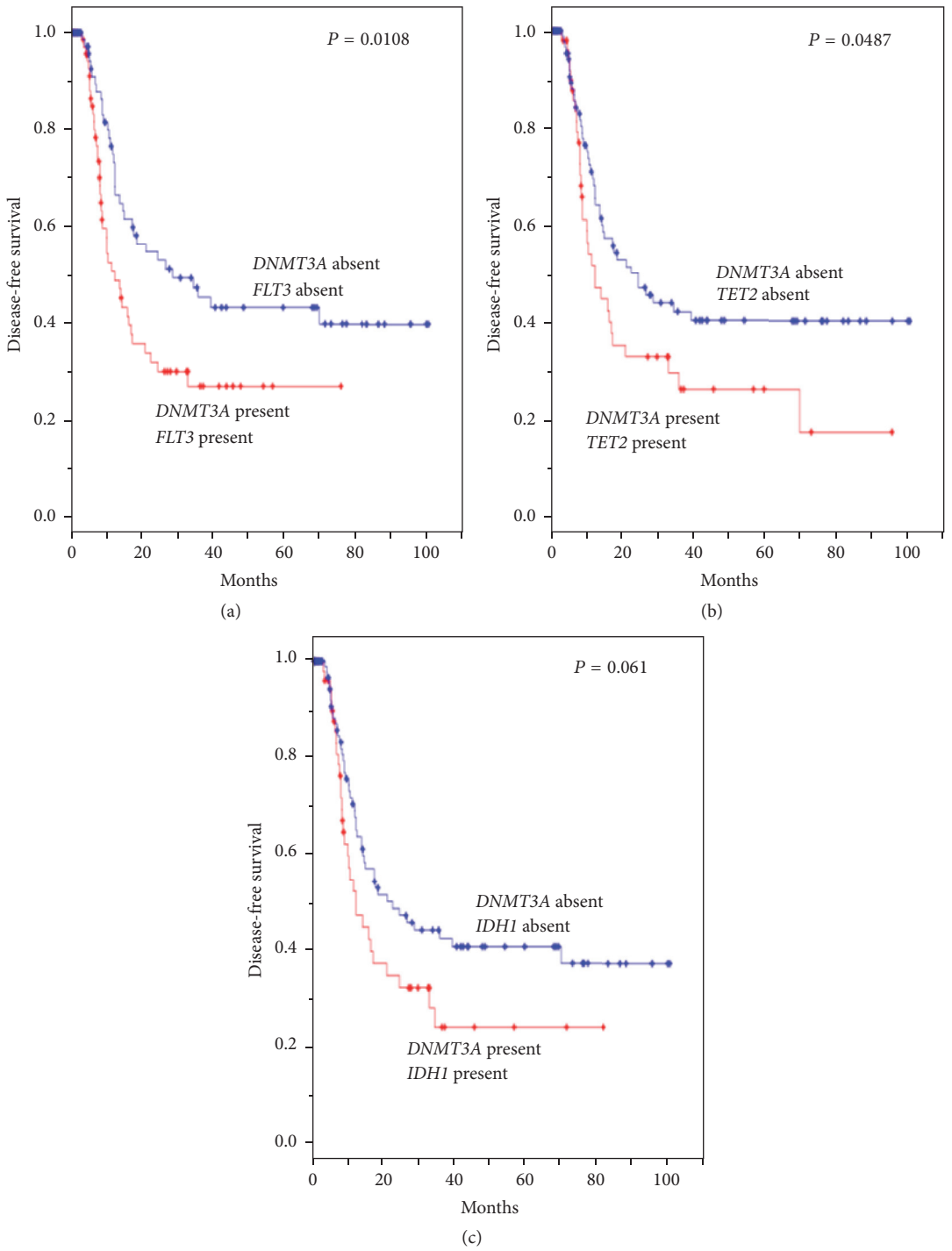

Figure 1: Kaplan-Meier curves for disease-free survival according to the presence or absence of the specific gene alterations. Gene alterations include mutations, deletions, fusions, and gene amplifications. All the alterations for *IDH1* are mutations. Over 95% of the alterations are mutations for *DNMT3A*, *TET2,* and *FLT3*. The rest of the alterations are multiple alterations for *DNMT3A* and *TET2* and deletions for *FLT3*. Database used for analysis is TCGA, NEJM 2013 [94]. The cBio Cancer Genomics Portal was used for the analysis [95] (http://cbioportal.org).

clinical outcomes of de novo AML [48, 49], CN-AML [49, 50], and CN-AML with mutated *NPM1* or *CEBPA* without $FLT3^{ITD}$ [41]. The full clinical impact of *TET2* mutations has yet to be fully understood.

3.5. NPM1 and IDH1/IDH2^{R140}. *IDH1* and *IDH2* (isocitrate dehydrogenases 1 and 2) are enzymes that catalyze the interconversion of isocitrate and alpha-ketoglutarate and appear to play an epigenetic role in histone and possibly DNA

methylation. *IDH1* or *IDH2* mutations confer a hypermethylation phenotype in leukemia and inhibit hematopoietic stem cell differentiation [51]. These phenotypic characteristics are shared by *TET2* loss-of-function mutations [46]. The most common *IDH1* mutation is in the arginine residue at position 132 (*IDH1*^{R132}), occurring in 6–9% of adult AML, while *IDH2* mutations occur in 9–19%, predominantly *IDH2*^{R140} in 8–12% [18, 27, 52–54]. *IDH1* and *IDH2* mutations are mutually exclusive in AML. Furthermore, *IDH1* and *IDH2*^{R140} are strongly associated with *NPM1* mutations [18]. *IDH1* [53, 54] and *IDH2* [53] mutations have been reported to carry an unfavorable prognosis with regard to survival in normal karyotype AML lacking *NPM1* and *FLT3*^{ITD} mutations. In patients with cooccurring *DNMT3A* and *IDH2*^{R140} mutations, the OS was significantly poorer than those with wild-type or a single mutation [18].

An earlier study demonstrated that the *IDH1* and *IDH2* mutations constitute poor prognostic factors in cytogenetically normal AML with *NPM1* mutation without *FLT3*^{ITD} [55]. In some other studies, however, patients with mutated *IDH1* or *IDH2*^{R140} had good prognoses for OS in AML patients with the *NPM1* mutation without *FLT3*^{ITD} [27, 52], and it was further concluded that the favorable effect of *NPM1* mutations was restricted to patients with cooccurring *NPM1* and *IDH1* or *IDH2*^{R140} mutations [27].

4. AML with Mutated Chromatin, RNA-Splicing Genes, or Both

This chromatin-spliceosome group is the second largest subgroup in this new classification [18]. This is also an extremely heterogeneous group, consisting of genes regulating RNA splicing (*SRSF2*, *SF3B1*, *U2AF1*, and *ZRSR2*), chromatin (*ASXL1*, *STAG2*, *BCOR*, *MLL*^{PTD}, *EZH2*, and *PHF6*), and transcription *(RUNX1)*. Functional proteins encoded by these genes have functions in hematopoietic stem cell self-renewal and differentiation. Using the European LeukemiaNet (ELN) guidelines, the majority (84%) of the patients in this new chromatin-spliceosome group would be classified as having intermediate prognostic risk. However, this new subgroup demonstrated resistance to induction chemotherapy and inferior long-term outcomes [18] suggesting a reclassification of AML patients with chromatin-spliceosome mutations as an adverse prognostic group. Nearly all of the genetic mutations in this subgroup have been previously reported to be adverse prognostic markers.

4.1. RUNX1. The *RUNX1* (runt-related transcription factor 1, formerly known as AML1) gene encodes the alpha subunit of the core binding factor involved in transcription and is required for definitive hematopoiesis [56]. *RUNX1* protein also plays an essential role in mesenchymal stem cell proliferation and promotes cell survival in AML [57, 58]. *RUNX1* mutations are present in 5% to 18% of AML [59–62]. They are associated with *ASXL1* [59], *MLL*^{PTD} [62], and *IDH1*/*IDH2* mutations [62] and are essentially mutually exclusive of *NPM1* mutations [59, 62]. *RUNX1* mutations were found to be associated with resistance to chemotherapy, inferior DFS, EFS [59, 61, 62], and OS [59–62]. More importantly, *RUNX1* mutations were deemed to be an independent prognostic marker for shorter EFS in multivariable analysis [62]. An explorative subgroup analysis demonstrated that *RUNX1*-mutated AML patients benefited from allo-HSC in terms of RFS [62].

4.2. MLL^{PTD}. The *MLL* (Mixed Lineage Leukemia) gene, located on chromosome 11q23, is frequently involved in translocations that recur in AML and have been classified into an individual subgroup, AML with *MLL* fusion genes, t(x;11)(x;23) [18]. MLL fusion proteins are capable of transforming normal hematopoietic stem cells into malignant leukemic stem cells [63]. A *MLL* partial tandem duplication (*MLL*^{PTD}), the result of a tandem duplication of an internal portion of the *MLL* gene that spans either exons 2 to 6 or exons 2 to 8, is present in approximately 10% of CN-AML [64, 65]. *MLL*^{PTD} mutation has been identified as a poor prognostic factor for EFS [60, 66, 67] and OS [27, 41, 60, 67]. Furthermore, it is clear that the OS was shortened when the intermediate-risk group patients had mutated *MLL*^{PTD} regardless of the presence of *FLT3*^{ITD} [27].

4.3. ASXL1. The *ASXL1* (additional sex combs like-1) gene is a human homologue of the *Drosophila* additional sex combs *(Asx)* gene, which is highly conserved across multiple species. The *ASXL1* protein functions in both epigenetic activation and repression of gene transcription [68–70]. Its regulation of histone modification affects hematopoietic stem cell pool maintenance and its loss causes severe defects in HSC development [71, 72]. *ASXL1* mutations are more common in the aberrant karyotypes, the elderly, and MDS-associated and secondary AML [73–77], while 9–12% of *ASXL1* mutations are detected in cytogenetically normal AML [75–77]. *NPM1* and *ASXL1* mutations appear to be mutually exclusive [76–78]. Several studies have shown that AML patients with *ASXL1* mutations had worse outcomes when compared to those without these mutations [27, 60, 75]. Specifically in CN-AML, *ASXL1* mutations were associated with inferior complete remission, DFS, OS, and EFS [77].

4.4. BCOR. The *BCOR* (*BCL6* corepressor) gene is located on chromosome X and encodes a nuclear protein that is a key transcriptional regulator of hematopoiesis [79]. Studies demonstrated that normal *BCOR* retains hematopoietic stem cells in a quiescent, undifferentiated state and loss-of-function *BCOR* mutations result in enhanced HSC cell proliferation and differentiation [80]. *BCOR* mutations occur in 3.8% of CN-AML, and *DNMT3A* mutations are detected in 43.5% of these patients. *BCOR* mutations tend to be associated with an inferior EFS and OS [81].

4.5. PHF6. The *PHF6* (plant homeodomain finger 6) gene, also located on chromosome X, plays a key role in chromatin remodeling [82]. *PHF6* mutations are found in approximately 3% of adult AML and confer worse OS in intermediate-risk patients that are *FLT3*^{ITD} negative [27].

5. AML with *CEBPA*$^{\text{biallelic}}$ Mutation

The *CEBPA* gene is located on chromosome 19 band q13.11 and encodes a 42 kDa size protein that is a member of the basic region leucine zipper transcription factor family [83]. Normal function of *CEBPA* is crucial in maintaining adult hematopoietic stem cell in a quiescent state and *CEBPA* gene knockout in mice results in impaired HSC differentiation [84]. The CEBPA protein is expressed in myelomonocytic cells and is critical for neutrophil development [83]. Mutated *CEBPA* regulates Sox4 expression which affects self-renewal of leukemic stem cells [85]. The frequency of *CEBPA* mutations is reported to range from 7% to 22% in patients with AML [86] and 15% to 18% in CN-AML [87, 88]. About two-thirds of *CEBPA*-mutated patients are biallelic-mutated (*CEBPA*$^{\text{biallelic}}$), and the remaining one-third carry a single mutation (*CEBPA*$^{\text{monoallelic}}$). In a meta-analysis of 10 clinical studies covering 6219 patients, *CEBPA*$^{\text{biallelic}}$ mutation was found to be associated with favorable clinical outcomes with regard to EFS and OS in patients with AML or CN-AML; conversely, no significant difference was found between *CEBPA*$^{\text{monoallelic}}$ mutation and wild-type *CEBPA* in patients with AML or CN-AML [86]. In a long-term follow-up study (median follow-up time of 9.8 years), patients with *CEBPA*$^{\text{biallelic}}$ mutations showed longer OS, longer relapse-free survival, and a lower cumulative incidence of relapse compared to those with *CEBPA*$^{\text{monoallelic}}$ mutation. The ten-year OS rate for patients ≤60 years and with *CEBPA*$^{\text{biallelic}}$ mutation was 81%. *CEBPA*$^{\text{biallelic}}$-mutated AML was associated with *TET2* mutation in 34% of the patients, and the combination resulted in significantly worse OS, whereas it was associated with *GATA2* (GATA binding protein 2, a transcription factor) mutation, found in 21% of *CEBPA*$^{\text{biallelic}}$-mutated AML, resulting in improved OS [89].

6. AML with *IDH2*$^{\text{R172}}$ Mutation

IDH2$^{\text{R172}}$ is a distinct *IDH2* mutation that occurs in AML with a frequency of 1–3% [18, 52, 90]. Unlike *IDH2*$^{\text{R140}}$ that significantly correlates with *NPM1* mutation, *IDH2*$^{\text{R172}}$ is generally not associated with other molecular mutations. The gene expression and DNA methylation profiles of *IDH2*$^{\text{R172}}$-mutated AML differ from those of other IDH mutations and lead to more severe aberrations in metabolic activity [91, 92]. Thus, *IDH2*$^{\text{R172}}$ mutation has been defined as a unique subgroup in the new classification scheme [18]. In previous studies, *IDH2*$^{\text{R172}}$ mutation was associated with a higher relapse rate and lower OS that were comparable with those of the adverse-risk cytogenetics patients [52, 90]. In this most recent study, however, the presence of *IDH2*$^{\text{R172}}$ mutation was associated with a favorable prognosis with regard to OS, similar to patients with *NPM1*-mutated AML [18].

7. Conclusions and Future Directions

The progress in AML risk stratification using next-generation sequencing technologies over the past decade has been truly remarkable. As an example, the identification of *NPM1*- or *CEBPA*$^{\text{biallelic}}$-mutated CN-AML to have favorable risk has significantly impacted the clinical management of these patient groups. The novel AML classification system proposed by Papaemmanuil et al. has been especially valuable in organizing the growing array of AML mutations in terms of additive effects on prognosis. As an example, the subgroup "AML with *NPM1* mutation" is the largest subgroup and has a large number of comutations. The different comutation combinations do not have a strictly additive effect on clinical outcomes indicating further risk stratification in this group is necessary. Conversely, the subgroup "AML with mutated chromatin, RNA-splicing genes, or both" is more consistent as an adverse prognostic group, at least among the five genetic mutations we reviewed here: *RUNX1*, *MLL*$^{\text{PTD}}$, *ASXL1, BCOR, and PHF6*. A central theme of this adverse-risk group is that the majority of these genes have roles in maintaining normal HSC quiescence by their epigenetic regulation and their mutations result in transformation of HSC into leukemic stem cells. These malignant stem cells, in turn, are thought to be the wellspring of leukemic cell expansion, likely directly responsible for the relapsing nature of AML. Of note, mutations in epigenetic modifiers or regulators such as *DNMT3A*, *TET2*, and *IDH1/2* alter normal HSC quiescent state and prime HSC to a preleukemic state [93]. These epigenetic factors function as stem cell regulators and impact DFS (Figure 1). This observation may be true beyond AML and therapies targeting epigenetic modifiers or stem cell regulating factors may hold promise in improving disease-free survival of patients with other hematologic malignancies. These observations of recurrent AML mutations and comutation patterns await validation in larger prospective clinical trials. Regardless, this new classification strategy is an important step forward in understanding the molecular complexity of AML and has the potential to yield many new therapeutic targets to be exploited to someday eradicate this aggressive disease.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Regulation of Stem Cell Properties of Müller Glia by JAK/STAT and MAPK Signaling in the Mammalian Retina

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In humans and other mammals, the neural retina does not spontaneously regenerate, and damage to the retina that kills retinal neurons results in permanent blindness. In contrast to embryonic stem cells, induced pluripotent stem cells, and embryonic/fetal retinal stem cells, Müller glia offer an intrinsic cellular source for regenerative strategies in the retina. Müller glia are radial glial cells within the retina that maintain retinal homeostasis, buffer ion flux associated with phototransduction, and form the blood/retinal barrier within the retina proper. In injured or degenerating retinas, Müller glia contribute to gliotic responses and scar formation but also show regenerative capabilities that vary across species. In the mammalian retina, regenerative responses achieved to date remain insufficient for potential clinical applications. Activation of JAK/STAT and MAPK signaling by CNTF, EGF, and FGFs can promote proliferation and modulate the glial/neurogenic switch. However, to achieve clinical relevance, additional intrinsic and extrinsic factors that restrict or promote regenerative responses of Müller glia in the mammalian retina must be identified. This review focuses on Müller glia and Müller glial-derived stem cells in the retina and phylogenetic differences among model vertebrate species and highlights some of the current progress towards understanding the cellular mechanisms regulating their regenerative response.

1. Introduction

In humans and other mammals, the retina, like most other regions of the central nervous system (CNS), does not spontaneously regenerate; and damage to the retina or neurodegenerative disease that kills retinal neurons results in permanent blindness. Worldwide, more than 12% of people over the age of 40 have visual impairment or blindness caused by age related macular degeneration and glaucoma, two of the neurodegenerative diseases affecting the retina [1, 2]. As life expectancy continues to increase, the increasing prevalence of blinding neurodegenerative disease is reducing productivity and quality of life and imposing significant economic as well as social burdens to individuals, their families, and society. Current therapies can slow progression and delay vision loss but cannot restore lost vision. Consequently, there is increasing interest in identifying approaches for therapeutic retinal regeneration.

A variety of stem cells, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells, and fetal-derived neural and retinal stem cells, are currently under investigation for regeneration and subsequent transplantation of retinal neurons (see reviews in [3–10]). With advancements in gene editing using CRISPR/Cas9 technologies and the ability to expand cells in culture prior to differentiation, extrinsic sources such as ESCs and iPSCs are promising for developing strategies to correct preexisting genetic defects in vitro [11]. However, there are potential ethical concerns with the use of ESCs or progenitors from embryonic or fetal tissues, making them less attractive for therapeutic regeneration. Further, extrinsic stem cells will require surgical transplantation and integration of new neurons into existing circuitry. Although the retina is normally an immune privileged tissue, retinal damage and degenerative disease compromise the blood/retinal barrier, allowing ingress of immune cells [12–15]. Therefore, transplantation therapies may also require immunosuppression for long-term viability of the engrafted cells. An intrinsic retinal stem cell would alleviate concerns of integration and immune response and

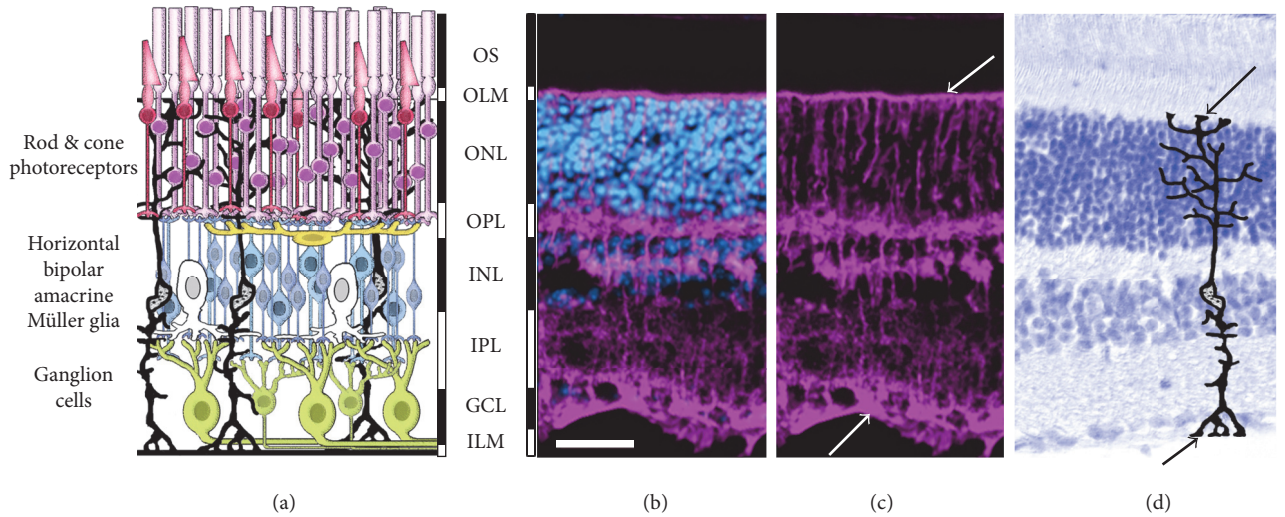

Figure 1: Retinal structure and cellular organization. (a) Diagram shows organization of retinal neurons and Müller glia. The cell bodies of rod (purple) and cone (red) photoreceptors are in the outer nuclear layer (ONL) and the photoreceptor outer segments (OS) contain the photopigments that absorb light. Rod and cone bipolar (blue), horizontal (yellow), and amacrine (white) cells are in the inner nuclear layer (INL), with retinal ganglion cells (green) located in the ganglion cell layer (GCL). Between the nuclear layers are the outer and inner plexiform layers (OPL, IPL) containing the synaptic terminals. Müller glia (black) have cell bodies located in the INL and extend processes throughout the retina. (ILM, OLM). (b, c, d) Photomicrographs of adult mouse retina. (b, c) Müller glia are immunostained for glutamine synthetase (magenta) revealing their radial processes that extend the full thickness of the retina. The Müller glial endfeet form the inner limiting membrane (ILM) and outer limiting membrane (OLM) (arrows). (d) Photomicrograph showing histology of adult mouse retina stained with toluidine blue, showing retinal lamina and overlaid with a diagram of a Müller glial cell. Arrows indicate glial endfeet at ILM and OLM. Scale bar = 50 microns in (b), (c), (d).

would provide an alternative strategy to complement the use of extrinsic stem cells.

Müller glia are intriguing candidates for intrinsic retinal stem cells. Müller glia are radial glial cells within the retina and are generated from the same lineage as retinal neurons. In the mature retina, Müller glia maintain retinal homeostasis, buffer ion flux associated with phototransduction, and form the blood/retinal barrier within the retina proper. Although they contribute to gliotic responses and scar formation following retinal injury, Müller glia also show regenerative capabilities that vary across species. This review focuses on Müller glia and Müller glial-derived stem cells in the retina and the phylogenetic differences among model vertebrate species and highlights current progress towards understanding and harnessing their regenerative response.

2. Retinal Structure and the Origin of Müller Glia

The retina is a thin layer of neural tissue located at the posterior pole of the eye. It consists of (a) photoreceptors (rods and cones) that convert light stimuli into neurochemical signals, (b) three major classes of interneurons (horizontal, amacrine, and bipolar cells) that perform initial information processing, (c) Müller glia that perform a multitude of support functions, and (d) projection neurons (retinal ganglion cells) that extend axons through the optic nerve and optic tract to convey the visual image information to higher processing centers within the brain [16–18]. The retinal cells are organized in a highly ordered laminar structure (Figure 1), which allows identification of cell types by position, morphology, and gene expression. The retina is developmentally part of the CNS. Lineage analysis has shown that the multipotent retinal progenitors that make up the embryonic retinal neuroepithelium generate all types of retinal neurons, as well as the Müller glia [16, 19, 20]. Apart from its importance in vision, the neural retina serves as a model system for studying the CNS, as it is the only portion of the central nervous system located outside of the cranium and can be noninvasively imaged and functionally tested in vivo. The process of retinal regeneration recapitulates many aspects of retinal development, with similar patterns of gene expression, cell fate specification, and the order of neurogenesis.

3. Müller Glia: Stem Cells for Retinal Regeneration in Fish

The initial evidence for the stem cell characteristics of Müller glia came from research to discover the cellular source of ongoing neurogenesis and regenerative responses in the retinas of fish. The capacity for neurogenesis in the mature retinas of fish appears to be teleologically related to their overall pattern of indeterminate growth and the associated continuous growth of their eyes. Eye growth in fish results in large part

from a general expansion/stretching of the retina, leading to decreasing retinal density for most types of retinal neurons within the central retina [21]. There is also ongoing neurogenesis, which occurs at two sites: (1) the circumferential germinal zone (CGZ), where a population of retinal progenitors persists and continually adds concentric rings of new neurons to the retinal margin, and (2) the central retina, where new rods are added continually to the existing photoreceptor mosaic [22]. Neurogenesis of rod photoreceptors in the fish retina begins after hatching and continues throughout life [23–28], with new rod photoreceptors generated from a population of slowly proliferating, PAX6-expressing progenitors within the inner nuclear layer (INL) [29]. These INL progenitors arise from the Müller glia and continue to divide as they migrate to the outer retina to become rod precursors, which subsequently differentiate into rod photoreceptors [26, 28, 29]. Ongoing neurogenesis in the fish retina appears to keep the Müller glia poised to respond to retinal damage by initiating intrinsic neurogenic programs.

Retinal regeneration in fish has been studied for over 50 years [30–34]. A variety of traumatic, surgical, neurotoxic, or phototoxic injuries have been used to induce regenerative neurogenesis, and the outcome is a fully laminated retina, albeit with some relatively minor organizational differences, and a restoration of circuitry and function [34–39]. Although multiple studies had reported clusters of mitotically active cells in the INL following retinal injury [40, 41], the source of the new retinal cells was not identified as Müller glia until 2007 [40–42]. Regardless of the injury paradigm, there appear to be several stages to the retinal response [43]. There is an initial, nonproliferative stage that occurs during the first 2 days after injury, during which Müller glia transiently upregulate expression of the intermediate filament, glial fibrillary acidic protein (GFAP), a key marker of gliosis [44, 45]. The gliotic response of Müller glia is not prominent, and the subsequent, regenerative response in zebrafish begins with an initial, limited proliferation of the Müller glia and generation of a pool of Müller glial-derived progenitors in the INL [46–49]. The Müller glial-derived progenitors continue to proliferate, forming columns of dividing cells that span the retinal layers and, around 7 days after injury, differentiate into retinal neurons [46, 50–52]. Interestingly, the switch from gliosis to proliferation is critical for the regenerative response in zebrafish, as blocking injury-induced proliferation of Müller glia, using 5-fluorouracil or morpholinos against PCNA, enhances the gliotic response, resulting in robust and persistent upregulation of GFAP, and prevents injury-induced neurogenesis [45]. Müller glial-derived progenitors are capable of regenerating all neuronal cell types in an injured retina and restoring visually guided behaviors [34–36, 38, 39, 53–58]. Although early studies suggested that only those subpopulations of neurons that were lost as a result of the initial injury were regenerated [49, 59, 60], recent evidence shows that, even in cases of localized injury to one cell type [e.g., phototoxic injury to photoreceptors or N-methyl-D-aspartate (NMDA) injury to retinal ganglion cells], additional cell types can be produced [48].

4. Müller Glia: Gliosis and Injury Response in Mammals and Birds

In warm blooded species, spontaneous regeneration by Müller glia does not occur to any appreciable extent in vivo, leading to questions about the designation of Müller glia as retinal stem cells in these species, particularly in mammals [64]. In the mammalian retina, the primary glial response to retinal injury is gliosis, which is characterized by robust upregulation of GFAP, limited (if any) proliferation, cellular hypertrophy, and formation of glial scars (reviewed in [65, 66]; see also [67–72]). In posthatch birds, there is a slightly more robust response to retinal injury, with gliosis accompanied by a limited neurogenic response that declines with age [64, 73–75].

Evidence for neurogenesis by Müller glia in vivo following acute retinal injury has been demonstrated in posthatch chick, rat, and mouse [62, 64, 71, 73–77] but requires manipulation of various exogenous factors (see below) or overexpression of neurogenic or proneural genes such as *Ascl1a* [78, 79] and *Atoh7* [80, 81]. Intraocular injection of selective neurotoxins that kill either photoreceptors or ganglion/amacrine cells in the adult rodent eye generates large numbers of reactive Müller glia but little proliferation [62, 82]. The proliferative response is enhanced by intraocular injection of a variety of extrinsic growth factors, including CNTF, EGF, FGF1, FGF2, and insulin in posthatch chickens [73–75] and in rodents [62, 71, 83, 84]. However, the overall extent of the neurogenic capacity of mammalian or avian Müller glia is low, even with growth factor stimulation, and, in the mammalian retina, low numbers of neuronal cells are generated and the majority of proliferating progenitors fail to survive in the long term [62]. A better understanding of the mechanisms that promote the proliferative and neurogenic responses is needed before clinically relevant levels of regeneration are achieved in vivo.

5. Mechanisms Regulating Gliosis versus Neurogenesis: JAK/STAT versus MAPK Signaling

Promoting clinically relevant levels of regeneration from Müller glia in the mammalian or human retina will require suppression of gliosis and enhancement of their proliferative and neurogenic responses. Multiple signaling molecules and their downstream signal transduction cascades have been implicated in regulating the injury and regenerative responses in the retina, including notch [85–88], tumor necrosis factor alpha (TNF-α) [89], transforming growth factor beta (TGF-β) [84, 90–92], insulin [70, 73], midkine (MDKN) [93–95], ciliary neurotrophic factor (CNTF) [75, 92, 96–99], epidermal derived growth factor (EGF) [62, 74, 84, 100], and fibroblast growth factors (FGFs) [62, 75, 101, 102]. Many of these signaling pathways converge on JAK/STAT (Janus kinase/signal transducer and activator of transcription) and MAPK (mitogen-activated protein kinase) signal transduction cascades (Figure 2). Retinal injury stimulates release of a variety of cytokines and mitogens, including EGF, FGF1, FGF2, and

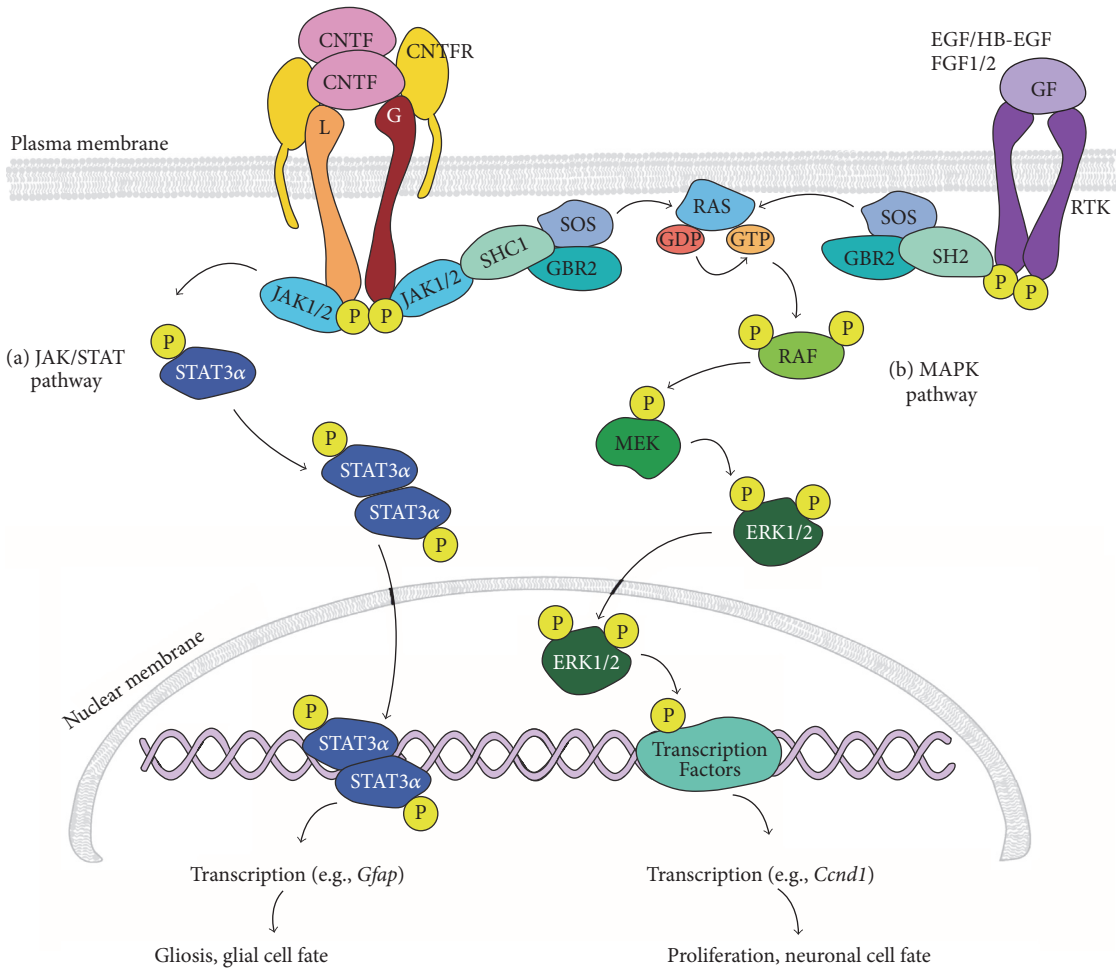

Figure 2: Summary diagram of JAK/STAT and MAPK signal transduction. (a) To activate JAK/STAT signaling, CNTF binding to the GPI linked CNTFRα initiates recruitment of LIFRβ (L) and GP130 (G) to form the hexameric CNTF receptor complex. Recruited LIFRβ and GP130 are phosphorylated on their cytoplasmic domain by JAK1/2. Activated JAK1/2 phosphorylate STAT3α, which forms homodimers that translocate to the nucleus. pSTAT3 homodimers bind to DNA and activate transcription of target genes, such as *Gfap*, to initiate gliosis. (b) The MAPK signaling pathway can be activated downstream of ligand binding to receptor tyrosine-kinases (RTKs) by growth factors including HB-EGF, EGF, FGF1, and FGF2 or by activation of CNTFR by CNTF. In both pathways, adaptor proteins such as GBR2 recruit SOS to the activated receptor, and subsequent activation of SOS leads to phosphorylation of RAS, MEK, and finally ERK1/2. Activated ERK1/2 translocate into the nucleus and phosphorylate several transcription factors involved in cell proliferation, cell survival, and cell differentiation.

CNTF, which have been implicated in various aspects of glial activation and proliferation [103–106]. However, endogenous expression levels of growth factors and cytokines following injury are insufficient to promote significant proliferation of mammalian Müller glia. Injury-induced proliferation can be enhanced by intraocular injection of EGF, FGF1, FGF2, and CNTF, either alone or in various combinations with each other or with insulin [62, 70, 73–75, 92, 98, 101, 107]. All of these factors can activate intracellular signal transduction cascades via JAK/STAT and/or MAPK signaling pathways. Therefore, examination of these signaling pathways and how their activation relates to gliosis and retinal regeneration in fish, birds, and mammals is important to begin to understand the mechanisms contributing to the differential injury responses.

Intracellular signaling through the JAK/STAT pathway is activated by receptor binding of a variety of ligands, including cytokines (e.g., interleukins [108], interferons [109], and CNTF [110]), growth factors (e.g., EGF [111], FGF [112]), and hormones (growth hormone [113], thyrotrophin stimulating hormone [114]). In JAK/STAT signaling, ligand binding to cognate receptors results in phosphorylation of receptor-associated JAK1/2, which causes rapid (within minutes) phosphorylation of STAT3 and a delayed (within hours) phosphorylation of STAT1 [115, 116]. Phosphorylated STAT3 (pSTAT3) and pSTAT1 form homodimers or heterodimers via phosphotyrosyl peptide interaction of their SH2 (Src homology 2) domains, resulting in translocation to the nucleus, binding to DNA at the consensus binding sequence TTCC[C/G]GGAA, and transcription of target genes [116].

CNTF can activate MAPK signaling downstream of its receptor; however activation of MAPK signaling more typically occurs downstream of receptor tyrosine-kinase activation by a variety of ligands, including EGF, FGFs, and insulin. In the MAPK signaling cascade, binding of a signaling ligand to its receptor causes a series of sequential phosphorylation reactions. Each step in the cascade can be performed by multiple proteins, making the cascade both diverse and complex (see reviews in [117, 118]). Briefly, phosphorylation of the cytoplasmic domains of the cytokine receptors causes adaptor molecules to recruit proteins that activate RAS (rat sarcoma oncogene), which phosphorylates RAF (rapidly accelerated fibrosarcoma), which phosphorylates MAPK kinases (including MAPK/ERK kinases 1 and 2; a.k.a. MEK1/MEK2), which phosphorylate MAPKs, including extracellular signal-regulated protein kinase (ERK), c-Jun-N-terminal kinase (JNK), and protein 38 (p38). Phosphorylated MAPK translocates to the nucleus and phosphorylates a variety of transcription factors that activate target gene transcription.

6. CNTF Activation of JAK/STAT and Gliosis

Among the factors that can activate JAK/STAT and MAPK signaling, CNTF plays multiple roles in the retinal injury response and particularly in activating the gliotic responses of Müller glia. CNTF expression is upregulated in Müller glia following a variety of retinal injuries in the zebrafish [98], posthatch chicken [75], and mammals [103, 104, 106, 119]. Müller glia of all species express little, if any, GFAP in the absence of injury, neurodegenerative disease, or other insults [67, 120, 121]. However, intraocular injection of CNTF into otherwise uninjured eyes of zebrafish [75], posthatch chickens [92], mice [99], and rats [115] increases GFAP in Müller glia, mimicking a gliotic response [98]. Upregulation of GFAP by CNTF is mediated by STAT3 signaling via direct binding of phosphorylated STAT3 (pSTAT3) dimers to the GFAP promoter [99, 122, 123]. Consistent with a role in gliosis, STAT3 expression also increases in Müller glia following ouabain- or light-induced injury in zebrafish [47, 98], and STAT3 phosphorylation is similarly increased in Müller glia following injury to the avian [75] and mouse retina [124, 125].

7. JAK/STAT and MAPK Regulation of Müller Glial Proliferation

In addition to activating JAK/STAT signaling, injury activates MAPK signaling in Müller glia in zebrafish and mice [98, 126]. Various growth factors, including EGF, FGFs, and insulin, activate MAPK signal transduction directly downstream of tyrosine-kinase receptors, whereas CNTF activates MAPK via JAK activation of SHP2/RAS (Figure 2) [98, 127, 128]. Following a penetrating injury, combinatorial treatments of insulin combined with either heparin binding EGF-like growth factor (HB-EGF; an EGF-related but more potent mitogen [129]) or FGF2 increase proliferation of Müller glia in the zebrafish retina, an effect that is reduced by inhibition of MAPK or JAK/STAT [74, 100, 101]. Similarly, in both avian and mouse retinas, NMDA injury increases pERK and pSTAT3 in Müller glia [74, 75, 115]. Proliferation of Müller glia is increased by exogenous HB-EGF, FGF2, insulin, or combinations of CNTF and FGF2, in NMDA injured, but not in uninjured, retinas [75]. Although phosphorylation of ERK1/2 and STAT is increased by these same factors, the ability of CNTF/FGF2 to increase proliferation of Müller glial-derived progenitors in NMDA injured chicken retinas requires activation of JAK/STAT [74, 130]. Similarly, the proliferative effects of CNTF can be blocked at the receptor level by inhibition of the gp-130 coreceptor or postreceptorally by inhibition of either JAK2 phosphorylation or STAT3 dimerization/nuclear translocation [75, 99]. If JAK/STAT signaling mediates both gliosis and proliferation of Müller glia in response of CNTF or injury, a key question is how to promote proliferation of Müller glia-derived stem cells to repopulate areas of retinal degeneration, without also inducing gliosis.

8. Müller Glia In Vitro

Despite similarities between fish, birds, and mammals in the Müller glial response to exogenous growth factors, modulation of JAK/STAT and MAPK signaling in the mammalian retina remains insufficient to stimulate clinically relevant regenerative responses. There is evidence that both intrinsic and extrinsic mechanisms contribute to inhibition of the neurogenic/regenerative response of Müller glia in the retinas of higher vertebrates. Although regeneration in the fish retina can occur throughout life, there is an age-associated decline in the proliferative response of Müller glia to retinal injury and exogenous mitogens in rodents [83, 131]. The genetic background in different mouse strains also has significant influence on the ability of Müller glia to proliferate in response to injury and exogenous growth factors, although specific genetic factors have yet to be identified [132]. Since intraocular injection of exogenous factors can promote Müller glial proliferation, it is likely that there are insufficient endogenous levels of known growth factors. In addition, there are likely additional, unidentified factors that are required to activate key signaling pathways or that actively inhibit the proliferative and regenerative capacity in vivo. Consistent with this, isolated mammalian Müller glia show increased proliferation and neurogenic potential in vitro. This may reflect the greater ability to manipulate exogenous factors in vitro, as well as the removal of the Müller glia from any local inhibitory signals. Thus, even though the ultimate goal for using Müller glial-derived progenitors is to stimulate neural regeneration in situ, the use of primary Müller glia and permanent cell lines offers an opportunity to examine mechanisms underlying their proliferative and regenerative responses under more controlled conditions in vitro.

Several Müller glial cell lines have been described: ImM10, conditionally immortalized Müller glia from P10 mouse retinas [61, 133]; MIO-M1, spontaneously immortalized Müller glia from adult human retinas [134]; rMC-1, SV-40 immortalized Müller glia from light-injured rat retinas [135]; TR-MUL5, conditionally immortalized Müller glia from rat [136]; and MU-PH1, conditionally immortalized Müller glia from 2-month-old mice. Direct comparisons are complicated by differences in the age and species of origin, variable culture conditions, and the panels of genes that

have been analyzed in different studies. However, in standard culture conditions, all express at least some genes typical of Müller glia in vivo (e.g., vimentin, *Sox2*), as well as nestin, an intermediate filament typically associated with neural stem cells. Only rMC-1 has robust GFAP expression, consistent with its origin from light-injured retinas [135], although one study also reported low levels of GFAP immunoreactivity in MIO-M1 cells [137]. There are concerns that overexpression of oncogenes will fundamentally change the identity and cellular responses of Müller glial cell lines. The conditionally immortalized cell lines, ImM10 (mouse) and TR-MUL5 (rat), contain an inducible, temperature sensitive SV40T-antigen, thereby allowing elimination of oncogene expression under appropriate conditions.

There is a temporal change in cell cycle kinetics of primary Müller glia, which initially proliferate slowly, even in the presence of serum containing medium [133]. After continued culture, primary Müller glia become more highly proliferative, consistent with spontaneous immortalization [133, 134]. Within the first two weeks in culture, Müller glia change morphology and downregulate key genes associated with glial function [e.g., glutamine synthetase (GS), cellular retinaldehyde-binding protein (CRALBP/RLBP1)] [138]. We observed reduced proliferation of ImM10 cells when grown in serum-free medium with nonimmortalizing conditions, although rates of proliferation increased and the differences between immortalizing and nonimmortalizing conditions diminished at higher passage numbers [133]. Thus, careful comparative studies of cultured Müller glia with their in vivo counterparts will be needed for final validation of any identified mechanisms regulating proliferation and neurogenic competence.

Immortalized Müller glia can be induced to generate cells expressing neuronal genes [61, 134, 139–142]. ImM10, MIO-M1, and MU-PH1 generate neurospheres in response to specific growth factors, typically a combination of epidermal derived growth factor (EGF), fibroblast growth factor 2 (FGF2), and/or insulin (Figure 3(b)), and upregulate a variety of genes typical of retinal progenitors, including *Pax6* and nestin [61, 134, 137, 139]. Using a variety of in vitro differentiation protocols, Müller glial-derived progenitors from neurospheres will alter their morphology to resemble cultured neurons, showing condensed nuclei and long, branching neurite-like processes (Figure 3(d)). Redifferentiated human Müller glial-derived progenitors express markers of most retinal cell types and have been shown to respond to light [137]. Transplantation of in vitro differentiated photoreceptors from Müller glial-derived progenitors partially restored light response in a rat model of rapid photoreceptor degeneration, as measured by increases in the a-wave of the electroretinogram (a measure of photoreceptor function) [140]. Transplantation of in vitro differentiated Müller glia into rat retinas, following pharmacological depletion of retinal ganglion cells, partially restored the negative scotopic threshold response of the electroretinogram (an indicator of retinal ganglion cell function) [139]. However, despite the presence of the newly generated cells in their appropriate lamina and evidence of some synapse formation with upstream neurons, the new cells failed to extend axons into the optic nerve or connect to visual centers in the brain. In all differentiation paradigms reported, relatively large numbers of cells continue to express glial genes and retain a glial morphology. Additionally, the number of neurons generated is relatively low, their morphology is inconsistent, and gene expression profiles have yet to demonstrate expression of all genes necessary for specification and functional maturity of individual retinal cell types.

9. JAK/STAT and MAPK in Müller Glia In Vitro

There have been few systematic studies of the activity of specific signal transduction cascades in Müller glia in vitro. Since activation of JAK/STAT or MAPK signaling in Müller glia can regulate gliosis and proliferation, we analyzed activation of STAT3 and MAPK pathways in ImM10 cells by western blot. There were no changes in total STAT3 or pSTAT3 in neurosphere or differentiation cultures; and, unexpectedly, despite the presence of both EGF and FGF2 in sphere forming media, there was no change in pERK1/2 in neurospheres [143]. In contrast, pERK1/2 increased in ImM10 cells in differentiation conditions despite the absence of exogenous EGF or FGF2 [143]. However, EGF and FGF2 mRNA levels are upregulated in differentiation cultures of ImM10 cells [61], suggesting that endogenously produced factors contribute to activation of MAPK signaling in these cells.

During neonatal retinal development, CNTF has been proposed to modulate a "molecular switch" that promotes either a glial or neuronal cell fate via concentration dependent activation of STAT3 versus MAPK signaling, respectively [144, 145]. In rat P1 retinal explants and dissociation cultures, low concentrations of CNTF (<50 ng/ml) increase the number of cells expressing neuronal genes via MAPK signaling, whereas high concentrations (100 ng/ml) increase the number of cells expressing glial genes via STAT3 signaling [144]. Inhibition of STAT3 signaling abolishes CNTF's repression of photoreceptor markers in mouse retinal progenitor cell cultures [146, 147], whereas inhibition of MAPK signaling abolishes the increase in the number of cells expressing neuronal markers in dissociation cultures of neonatal rat retina treated with low concentrations of CNTF [144].

To test if low levels of CNTF could promote neurogenesis in differentiation cultures of ImM10 cells, we analyzed gene expression following addition of low concentrations of CNTF (20 ng/ml) [61]. Consistent with our previous study, genes associated with multiple neuronal types were detected in differentiation cultures, including photoreceptors *(Rhodopsin, Opn1sw,* and *Nr2e3)*, retinal ganglion cells *(Sncg, L1Cam)*, and bipolar cells *(Prkca)*. However, addition of CNTF did not alter the overall patterns or levels of gene expression [143]. In addition, GFAP was not detected in differentiation cultures, either with or without CNTF, suggesting that there was no enhancement of gliosis. Surprisingly, CNTF did not change phosphorylation of STAT3 or ERK in ImM10 cells, reflecting the failure of CNTF to activate either JAK/STAT or MAPK signaling. These findings suggest that the ImM10 cells in vitro respond to CNTF differently than Müller glia in vivo. One potential explanation is that the effects of CNTF may require additional

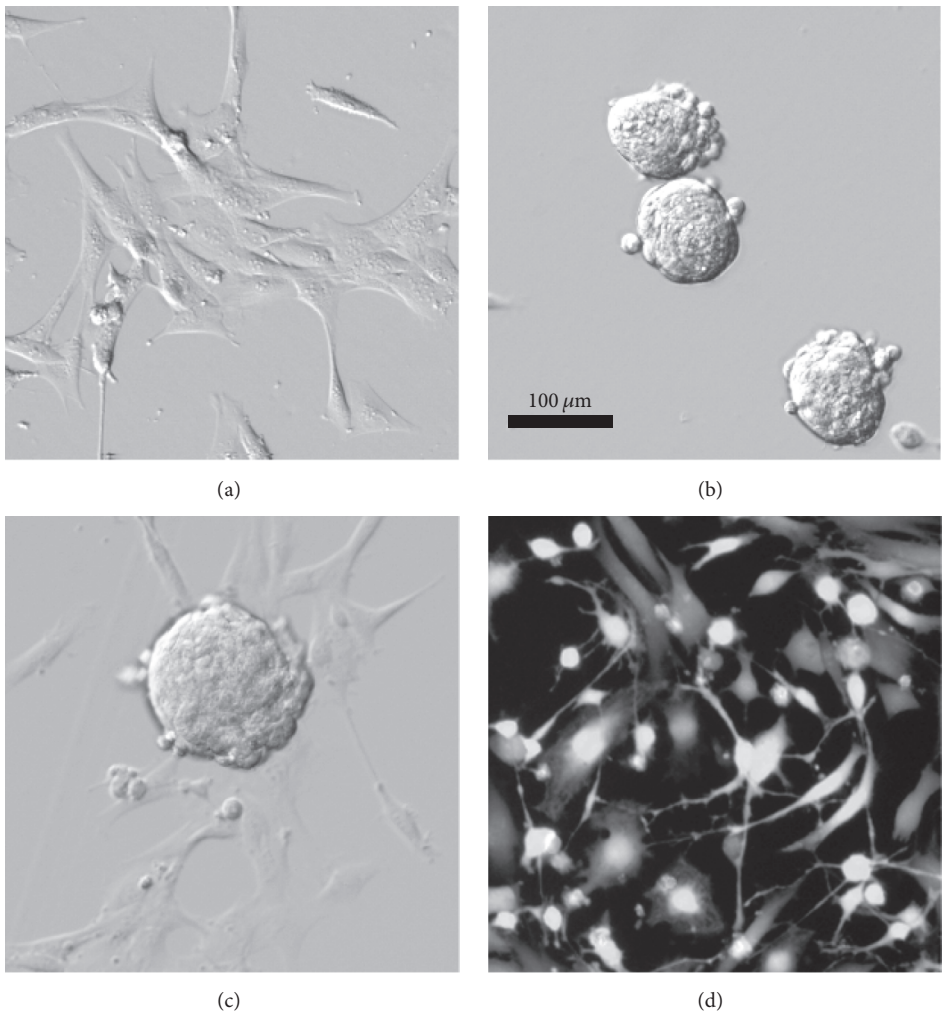

Figure 3: Morphology of mouse Müller glia in growth, neurosphere, and differentiation cultures in vitro. Conditionally immortalized mouse Müller glia (ImM10 cell line) at different stages of in vitro differentiation as previously described [61]. (a) ImM10 cells in growth media (Neurobasal, 2% fetal bovine serum, B27 supplement, and penicillin/streptomycin) under immortalizing conditions (33°C, 50 U/ml interferon gamma) show typical morphology of cultured Müller glia. (b) ImM10 cells following 4 days in sphere forming medium (Neurobasal, B27 supplement, and modified G5 supplement with 20 ng/ml EGF, 20 ng/ml FGF2, and penicillin/streptomycin) in nonimmortalizing conditions to prevent T-antigen expression (39°C, without interferon gamma), showing typical nonadherent neurospheres. (c) Spheres at 1 day following transfer to priming medium (Neurobasal, G5 supplement modified to contain EGF (20 ng/ml) but without FGF2, and penicillin/streptomycin; nonimmortalizing conditions), neurospheres adhere to plate, and cells begin to migrate onto dish. (d) Following priming, ImM10 cells in differentiation medium (Neurobasal, B27, and pen/strep; nonimmortalizing conditions) for 2 days and stained with CalceinAM show variable morphologies and include cells with distinct neuronal morphology (small cell body, multiple thin processes).

cells or factors not present in the cell line. Consistent with this idea, stem cells from dental pulp treated with conditioned media from injured rat retinas upregulated rhodopsin expression in vitro, whereas those treated with conditioned media from purified Müller glia did not [148].

The JAK/STAT and MAPK signaling pathways are implicated in retinal cell fate choice, proliferation, and gliotic hypertrophy after retinal injury and can be activated by a variety of growth factors including EGF, HB-EGF, FGF2, insulin, and CNTF. The JAK/STAT pathway appears to mediate both beneficial (proliferation) and detrimental (hypertrophy/gliotic) aspects of the injury response in vivo (Figure 4). Our findings that MAPK activation increased in ImM10 cells cultured under conditions that increased neuronal gene expression are consistent with a role for MAPK signaling in mediating a neuronal cell fate over glial fate choice. In mammalian retinal injury, the gliotic response predominates over the regenerative response, although the regenerative response can be enhanced with the addition of exogenous growth factors and cytokines that activate JAK/STAT and MAPK signaling. Unfortunately, this enhancement is still insufficient to make regeneration predominate over gliosis.

10. Other Mechanisms

Although activation of JAK/STAT and MAPK signaling by CNTF, EGF, and FGFs can promote proliferation and the

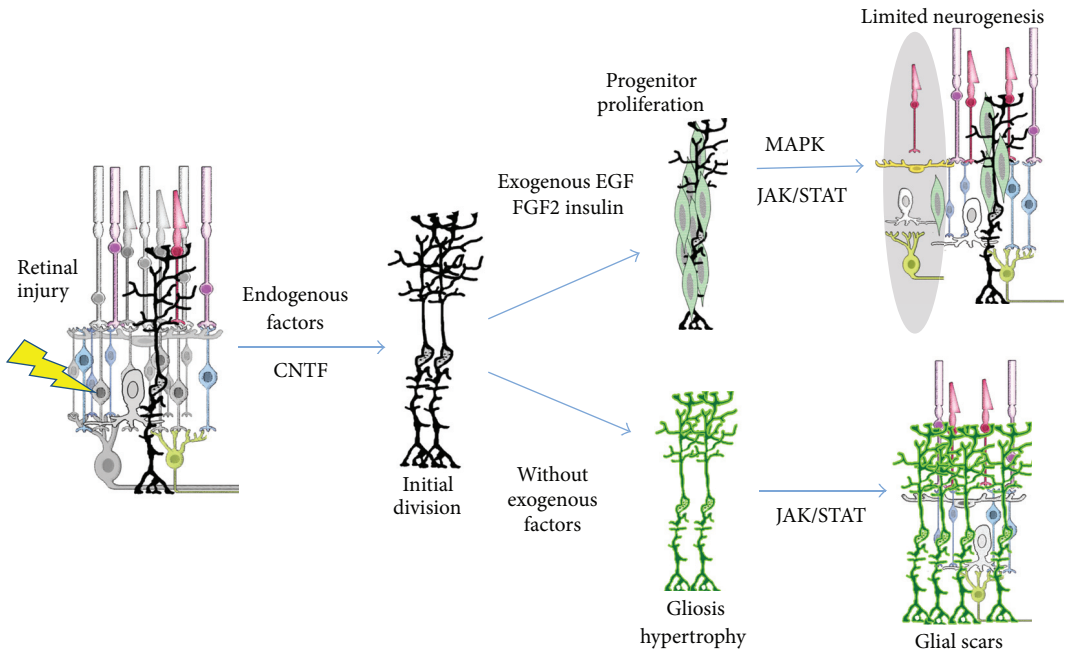

Figure 4: The proposed involvement of CNTF, JAK/STAT, and MAPK signaling in gliosis and neural regeneration by Müller glia in mammalian retina. Retinal injury (lightning bolt) kills retinal cells (gray cells) and stimulates release of growth factors, including CNTF, resulting in limited cell division of Müller glia (MG). In the absence of exogenous growth factors, increased JAK/STAT signaling (lower arrows) in activated MG promotes gliosis (bright green), resulting in glial scars, but neurons are not regenerated. Activation of MAPK and JAK/STAT signaling by exogenous factors, including EGF, FGF2, and insulin (upper arrows), produces proliferative progenitors (light green), which can regenerate some retinal neurons (in gray shaded oval), such as amacrine cells [62] and photoreceptors [63]. Some undifferentiated progenitor cells (light green) persist following resolution of the regenerative response. Even with exogenous factor stimulation, mammalian retinas fail to restore all lost cells.

glial/neurogenic switch, these signal transduction pathways do not act in isolation. Rather, they function within the context of a wide variety of other cellular mechanisms that contribute to the retinal injury response and regeneration in various contexts. Extensive discussion of all the cellular mechanisms regulating the injury and regenerative responses of Müller glia is beyond the scope of this article; several recent reviews can provide more information [22, 64, 149–154]. In addition to its roles in regulating gliosis and cell fate specification, CNTF is also implicated in neuroprotection and axonal outgrowth through activation of the MAPK, JAK/STAT, and/or PI3K (phosphatidylinositol-3 kinase) pathway [155–159]. Other pathways that are important in regulating cell cycle reentry and exit, gliosis, neurogenesis, and differentiation of Müller glia following retinal injury include the following: notch [85, 87, 160] and WNT signaling [161, 162]; activation of a variety of signal transduction cascades by TNF-α [87, 89], TGF-β [163], and BMP/SMADs [84, 91, 164]; regulation of cell cycle by *ccnd1* and *p27*(*kip1*) [83, 85, 165]; microRNAs [78, 166–168]; and the effects of transcriptional regulators that regulate retinal development, such as the proneural gene *Ascl1a* [79, 142, 162] and the neurogenic gene *Atoh7* [80, 81, 141]. Thus, expanding our understanding of how multiple pathways integrate to regulate the injury and regenerative responses of Müller glial will be important for continued progress in the field.

Analysis of retinal regeneration in fish has identified a variety of signaling molecules and their downstream signal transduction cascades that have shown promise for enhancing regenerative responses of Müller glia and warrant continued study. One intriguing, yet understudied, molecule that plays a role in retinal neurogenesis and regeneration in zebrafish is midkine (MDKN) [169]. MDKN is a heparin binding protein that interacts, either directly or indirectly, with a number of receptors, including ALK, LRP, notch, and protein tyrosine phosphatase-zeta (PTP-ξ), to modulate downstream signal transduction cascades [94, 169–174]. Binding of MDKN, or the structurally related pleiotrophin, to PTP-ξ blocks its phosphatase activity, resulting in increased phosphorylation of a variety of tyrosine-kinase receptors and their substrates and potentiating downstream signal transduction cascades [94]. In zebrafish, MDKNa/MDKNb are expressed in both retinal progenitors and Müller glia and are upregulated following retinal injury [93, 95]. Morpholino inhibition of MDKNa reduces proliferation of Müller glial-derived progenitors and limits regeneration of rod photoreceptors following light damage in zebrafish [93]. Much less is known about MDKN in the mammalian retina, although it is neuroprotective for rod photoreceptors following light damage in mice [175] and rats [176]. We found that *Mdkn* mRNA is upregulated in ImM10 cells under differentiation condition in vitro [61]. Given the pleiotropic effects of MDKN and its

potential to modulate multiple signal transduction cascades that impact the proliferative and neurogenic responses of Müller glia, it would be interesting to assess combinatorial effects of MDKN and growth factor stimulation of JAK/STAT, MAPK, or other signal transduction cascades on Müller glial proliferation and retinal regeneration.

11. Conclusions

Müller glia are particularly appealing as a cellular source for retinal regeneration because they are intrinsic to the retina and offer the potential to regenerate neurons in situ, without transplantation. Despite a growing body of research showing the neurogenic potential of Müller glia in the mammalian retina, a level of regenerative response sufficient for potential clinical applications has yet to be achieved. Given the overall modest outcomes to date, cultured Müller glia seem unlikely to provide a clinically relevant path for generating sufficiently large numbers of retinal progenitors for transplantation. Successful regenerative strategies using transplantation are more likely to build on the ongoing progress in generating retinal progenitors and neurons from other sources, such as induced pluripotent stem cells [3–6, 15, 177–184]. Therefore, the importance of studies to promote in vitro differentiation of Müller glia lies in the ability to manipulate the cellular environment and to dissect cellular mechanisms that regulate their regenerative responses. Nevertheless, there is clear evidence that Müller glia change patterns of proliferation and gene expression as they adapt to culture conditions and that the ability of Müller glia to respond to some ligands and activate key signaling pathways is different in vitro and in vivo. This raises questions about whether in vitro assays will recapitulate all aspects of the in vivo glial injury response and how in vitro findings will translate into promoting regeneration in vivo. Thus, caution is warranted in interpreting results obtained using Müller glial cell lines. Research to promote clinically relevant levels of retinal regeneration from mammalian Müller glia will benefit from the use of more complex model systems and such as ex vivo retinal explants and three-dimensional substrates and the inclusion of multiple retinal cell types to better model the retinal environment.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Transcriptional and Genomic Targets of Neural Stem Cells for Functional Recovery after Hemorrhagic Stroke

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Hemorrhagic stroke is a life-threatening disease characterized by a sudden rupture of cerebral blood vessels, and it is widely believed that neural cell death occurs after exposure to blood metabolites or subsequently damaged cells. Neural stem cells (NSCs), which maintain neurogenesis and are found in subgranular zone and subventricular zone, are thought to be an endogenous neuroprotective mechanism for these brain injuries. However, due to the complexity of NSCs and their microenvironment, current strategies cannot satisfactorily enhance functional recovery after hemorrhagic stroke. It is well known that transcriptional and genomic pathways play important roles in ensuring the normal functions of NSCs, including proliferation, migration, differentiation, and neural reconnection. Recently, emerging evidence from the use of new technologies such as next-generation sequencing and transcriptome profiling has provided insight into our understanding of genomic function and regulation of NSCs. In the present article, we summarize and present the current data on the control of NSCs at both the transcriptional and genomic levels. Using bioinformatics methods, we sought to predict novel therapeutic targets of endogenous neurogenesis and exogenous NSC transplantation for functional recovery after hemorrhagic stroke, which could also advance our understanding of its pathophysiology.

1. Introduction

Hemorrhagic stroke, including intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH), is linked to high mortality and morbidity [1, 2]. Despite long-standing and worldwide efforts, the incidence of hemorrhagic stroke has not declined, according to a meta-analysis [3]. Currently, no effective medical treatment is available to improve the neurological outcomes in patients with hemorrhagic stroke. Although surgical decompression for cerebral hemorrhage benefits the survival of patients, defined pathogenesis and targets of prevention and treatment of hemorrhagic stroke have yet to be elucidated [4, 5]. Therefore, potential therapeutic strategies targeting secondary brain injury are attracting a lot of attention in translational studies of hemorrhagic stroke.

Neurogenesis is traditionally considered as an endogenous neuroprotective mechanism after acute central nervous system injuries, and it has been found to mainly occur in the subventricular zone (SVZ) along the lateral wall of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus [6]. Emerging evidence demonstrates that neurogenesis occurs after hemorrhagic stroke onset to repair the lesions of secondary brain injury and restore brain connections [7–9]. In addition, researchers have made great efforts to transplant exogenous neural stem cells (NSCs) to the brain lesions from different sources, including but not limited to embryonic stem cells, mesenchymal stem cells, and tissue-derived stem cells, with/without a variety of preinterventions. However, due to the complexity of the NSC microenvironment or niche, these strategies have either been proved unsatisfactory or resulted in serious side effects during clinical translation [10–12].

Recently, emerging evidence from the use of new technologies, such as next-generation sequencing and transcriptome profiling, has provided new insight into our understanding of genomic function and the regulation of NSCs. In this article, we will present current available data on controlling NSCs from both transcriptional and genomic

levels. Using bioinformatics methods, we sought to summarize novel therapeutic strategies involving endogenous neurogenesis and exogenous NSC transplantation for functional recovery after hemorrhagic stroke, which could also advance understanding of the pathophysiology of hemorrhagic stroke.

2. Pathophysiology of Hemorrhagic Stroke

Primary brain injury after ICH happens in a few hours after the rupture of arteries resulting in bleeding and is mainly a result of hematoma formation with mechanical damage to adjacent tissues [1, 13]. For SAH, bleeding into the subarachnoid space due to aneurysm rupture leads to vasospasm and brain ischemia [14]. The hemorrhagic location and volume are highly associated with neurofunctional outcomes. However, the Surgical Trial in Intracerebral Hemorrhage (STICH trials I and II) has failed to provide convincing evidence to support the use of early surgical hematoma removal versus initial conservative therapy [15, 16]. In addition, recombinant activated factor VII significantly reduces hematoma growth without improving survival or functional outcomes in ICH patients (Clinical Trial: NCT00127283) [17]. Meanwhile, the treatment of SAH has not improved; the calcium channel blocker, nimodipine, is still the only proven drug to show beneficial outcomes for those patients with/without angiographic vasospasm. Additionally, treating vasospasm does not always lead to improvement in functional outcomes. This was recorded in randomized, placebo-controlled clinical trials (CONSCIOUS-2 and CONSCIOUS-3) using the endothelin receptor antagonist, Clazosentan, which reduced vasospasm in patients after SAH but failed to reduce mortality or attenuate neurological deficits.

Based on these disappointing results, researchers have turned their focus to mechanisms of secondary brain injury after hemorrhagic stroke, which play a critical role in the neurological deterioration in these patients [18–20]. Secondary damage is triggered from the blood components present that subsequently activate cytotoxic, excitotoxic, reactive oxygen species-related, and inflammatory-mediated pathways, and so forth. Nevertheless, neuroprotective agents, which have improved outcomes in animal studies, have failed to exhibit clinical benefits [21, 22]. Thus, strategies targeting NSCs and endogenous neurogenesis may be a potential and promising way to improve neurological outcomes after hemorrhagic stroke.

3. Current Understanding of the Neuroprotective Effects of NSCs for Hemorrhagic Stroke

3.1. NSCs for Hemorrhagic Stroke. The role of NSCs has been well defined in rodents, but neurogenesis in humans is more complicated. Histopathological examination of hippocampus tissue from cancer patients postmortem revealed the presence of nascent neurons [23, 24], providing the first evidence for human neurogenesis [23]. More recently, Spalding et al. retrospectively marked the hippocampal cells, by using the ratio of ^{14}C to ^{12}C in DNA of postmortem patients exposed to nuclear testing before death. Amazingly, they found that the turnover rate of new neurons in the dentate gyrus could be as high as 700 per day [25]. Meanwhile, by using two-photon laser scanning confocal microscopy, Shen et al. obtained specimens from patients with primary ICH and found that NSC specific proteins and cell proliferation markers were localized in cells in the perihematomal areas of basal ganglia and the parietal lobe after ICH [7]. These data suggest that ICH could induce de novo neurogenesis in the adult human brain. In addition, cerebral samples from SAH patients with aneurysm demonstrated the existence of many NSC markers, such as Nestin, vimentin, SOX-2, Musashi-1, and Musashi-2, which possibly contribute to the neural regeneration and functional recovery after aneurysm rupture [8]. However, elucidating the role of NSCs after hemorrhagic stroke in human still needs a large sample size of patients who vary in medical histories, cognitive ability, sportsmanship and lifestyles, and so forth, because all these factors can influence neurogenesis in experimental animals.

3.2. Neuroprotective Effects of NSCs after Hemorrhagic Stroke. Since the protective effects of neurogenesis are well reported in other acute central nervous system injuries, numerous researchers also support the beneficial role of NSCs after hemorrhagic stroke including proliferation, migration, and differentiation. Back in 2004, Tang et al. found that Nestin-stained or BrdU-labeled cells were mainly located in the basal ganglion and nearby SVZ around hematoma and ependyma after ICH in rats. Additionally, no cells positive for these markers were found in control or sham groups or in non-lesioned parenchyma [26]. Masuda et al. injected BrdU for two weeks after ICH in rats and found that BrdU-labeled cells significantly increased in both the contralateral and ipsilateral SVZs. Meanwhile, doublecortin-positive, immature, and migratory neurons were also seen in the dorsal striatum and perihematoma area two weeks after ICH. In addition, they also noticed clusters of doublecortin-stained cells in the striatum surrounding the hemorrhagic lesion four weeks after ICH. These findings implicate that experimental ICH induces the proliferation and migration of endogenous NSCs to repair the hemorrhagic lesion [9].

In addition to endogenous NSCs, exogenous NSC transplantation also exhibits the potential to attenuate neurological deficits after hemorrhagic stroke. In 2003, Jeong et al. intravenously transplanted human NSCs into experimental ICH rats. Their results indicated that NSCs can cross blood brain barrier and enter the rat brain with ICH. Interestingly, those surviving NSCs in the rat brain helped with the functional recovery [27]. Another investigation transplanted all-trans retinoic acid-induced NSCs into the contralateral ventricle up to 7 days after ICH and found new neurons and astrocytes surrounding the hematoma lesions of the brain four weeks later in all rats receiving the transplantations [28]. Moreover, these results were confirmed by superparamagnetic iron oxide- (SPIO-) labeled human NSCs detected by 3 T Magnetic Resonance Imaging, which indicated the presence of prominent NSCs in the periventricular region at four

and six weeks after transplantation [29]. Most importantly, compared with the control group, the NSC-transplanted rats exhibited excellent functional performance on neurofunctional tests after two to eight weeks, which indicates that the exogenously supplied NSCs may be used for the functional recovery after hemorrhagic stroke [30].

3.3. Complexity of NSCs in Hemorrhagic Stroke Treatment. Despite the potential neuroprotective effects of NSCs, a lot of factors could influence the efficacy of NSC therapy for the hemorrhagic stroke treatment, such as intervention timepoint, administration routes, microenvironment of NSC, the source and status of NSCs, and possible immune responses.

According to a meta-analysis review, stem cell transplantation, particularly mesenchymal stem cell transplantation, significantly induces stem cell migration to lesion sites, decreases associated neural apoptosis and inflammation, improves ultrastructural integrity of cerebral tissue, and aids in improving neurologic function after SAH [31]. Additionally, intracerebral transplantation was the most effective route of administration for functional and structural recovery after ICH [32]. However, the effectiveness of the therapy in clinical practice remains to be determined [32].

Many factors such as metabolism regulators, epigenetic modifiers, vascular constrictors or dilators, modulators of immune response, and activators or inhibitors of signal transduction pathways can influence adult neurogenesis. Moreover, proliferation, differentiation, maintenance, and self-renewal of NSCs in the stem cell niche are controlled by a network of intrinsic and extrinsic regulators, such as neurotrophins, cyclins and cyclin-dependent kinases, and transcription factors. These factors act in concert within their biological network during the establishment and maintenance of neural connections. Epigenetic modulations during hippocampal development can also have impacts on one's learning and memorizing abilities. Genetic polymorphism in genes involving neurogenesis may have essential roles in variations of NSC differentiation between individuals in adult neural regeneration [33]. Elucidation of favorable genetic variations in neurogenesis may have therapeutic implications [33].

In mammals, new neurons are constantly generated in the SVZ and SGZ throughout developmental stage and adult life. This continuous neurogenesis after birth may be important in processing information, daily learning, memorization, and so forth. During hippocampal neurogenesis, doublecortin-positive immature neurons and neuronal precursor cells mature into neurons. In the immature stage, cells are sensitive and susceptible to extrinsic stimuli. However, knowledge on the dynamics which lead to neuron maturation is limited. Moreover, to date, purification of NSCs in vitro proves to be a challenging task to allow for investigation of their biology and application in clinical medicine.

By examining gene expression at single-cell level using RNA-seq technology, Gao et al. found that two subgroups existed among immature neurons with distinct gene expression profiles and different molecular markers. Comparisons of the two subgroups indicated that Notch and Sonic hedgehog (Shh) and the Hippo pathways are all important in neuron maturation and NSC activity [34, 35].

A complex network of elements, consisting of macromolecules of the extracellular matrix (ECM), support cells (glial cells/astrocytes/oligodendrocytes), adhesion molecules for cell-cell and cell-ECM connections, blood vessels, neurotrophins, and so forth, has an impact on tissue homeostasis and maintenance of a homing microenvironment for NSCs. Among these components, ECM derived from NSCs provides a unique and indispensable microenvironment that helps with stem cell differentiation and neural regeneration. Analysis of protein expression by two-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometry (LC-MS/MS) provided proteomic profiles that corresponded to unique niche properties for each group tested. Proteomic results demonstrated that NSC-derived ECM can impact the decision-making process of stem cell fate by offering microenvironment for specialized stem cell niches in the process of tissue development and regeneration [36].

4. New Insight into Genomic Function and Regulation of NSCs

Due to the development of omics (referring to the field of study in biology ending in -omics, such as genomics, proteomics, or metabolomics) technology, emerging evidence has demonstrated that both transcriptional and genomic pathways play important roles in ensuring the normal function of stem cells. At the transcriptional level, sequence-specific transcription factors and coregulators work together to orchestrate the transcriptional landscape of stem cells, which determines the on/off state of target genes, thereby controlling the cell fate of stem cells. At the genomic level, the replication and repair machineries maintain the genomic stability of stem cells.

The zebrafish is an excellent animal model because it can repair several organs, such as the damaged retina, severed spinal cord, injured brain and heart, and amputated fins. Recent technological developments of exquisite molecular tools for research in zebrafish, including cell ablation, lineage analysis, and novel and substantial microarrays, together with advancements in stem cell biology, have allowed scientists to investigate how progenitor cells contribute to the generation of appropriate structures and various underlying mechanisms, including reprogramming [37], and the appearance of various types of proliferating progenitor cell populations, such as $SOX2^+$, $A2B5^+$, and $NG2^+$, of neural, glial/astrocyte, and oligodendrocyte progenitor cells, respectively. Among several essential factors for pluripotency, SOX2 and POU5F1 are significantly increased in neuron regeneration, which is linked to the pathway activation of progenitor cells. Elucidation of the fundamental mechanism for the endogenous neurogenesis and neuron network remodeling in adult zebrafish spinal cord has provided investigators with important ideas for future therapeutic strategies in acute brain injury repair

and functional recovery in mammals [38]. Upon brain injury, neuronal progenitors of various types are recruited to the lesion site by different molecules. These progenitors are produced by the pool of NSCs to perform the task of regeneration. An imbalance of stem cell asymmetric division and self-renewal results in abnormal divisions and leads to the depletion of NSCs over time, which has been demonstrated in the alterations of the behavior of NSCs responsible for producing additional neurons in the process of neurogenesis [39].

Factors which form a regulatory network to support NSC self-renewal have not been fully elucidated up to now. Understanding of the key transcription factors (TF), the promoter region and other noncoding regions that they bind, and the target genes that they regulate, will be essential in unleashing the full potential of these cells for therapeutic use. At the center of this regulatory network are SOX family and FOX family TFs, nuclear factor I (NFI), and basic helix-loop-helix (bHLH) transcription factor family. Coordinated action of these factors to promote proliferation and at the same time prevent untimely differentiation and quiescence is crucial to NSC self-renewal [40]. By analyzing the region-specific regulatory networks based on available published databases on SVZ and SGZ, Ertaylan et al. discovered the potential microenvironment associated differences based on membrane and nuclear receptors via HIF-1α, Ar, and NR3C1. They also performed cell fate determinant test for NSCs from SVZ to the interneurons of olfactory bulb and NSC populations from SGZ to the granule cells of the granular cell layer. The existence of membrane and nuclear receptors in this region-specific regulatory network shows the importance of niche-derived extracellular molecules and region-specific factors for the neurogenesis in SGZ and SVZ [41].

Genomic approaches in modern time have facilitated unprecedented advances in our understanding of the development, function, and evolution of central nervous system. By contrast, little is recorded or published about the possible interplay between different genetic factors, epigenetic modulators, noncoding RNAs, and environmental factors in causing or modulating neurological disorders in populations from underdeveloped countries [42]. Both pharmacological intervention and genetic manipulation of epigenetic modulators can trigger profound changes in molecular expression, neuron identity, and complex behavioral and cognitive phenotypes. Apparently, epigenetics plays a nontrivial role in the pathogenesis of neurological disorders. Emerging paradigms in possible connections between epigenetics and hemorrhagic stroke include the following: how gene mutations of epigenetic factors induce hemorrhagic stroke; how genetic polymorphism of epigenetic factors is linked to disease risk of hemorrhagic stroke; how changes in the expression, localization, or function of epigenetic factors affect hemorrhagic stroke; how epigenetic factors modulate disease-linked genomic loci, protein expression, and cellular pathways; and how differential epigenetic profiles from patient-derived tissue samples affect disease outcome [43].

5. Bioinformatics Methods for Analyzing the Novel Therapeutic Targets of NSCs

Transcriptomic analysis, proteomic discovery, epigenetic status, and metabolic states during endogenous neurogenesis have the potential to lead to important discoveries and improve care of hemorrhagic stroke. Recent advances in analytic techniques present a new opportunity to discover potential targets that are of therapeutic values and provide new concepts which could change our perspectives of physiology, pathology, and biology in the near future.

Many research groups have studied the transcriptomics of NSCs and the process of NSC differentiation and cell fate determination to identify key regulators of NSC proliferation. Traditionally, Oct4 was found to be sufficient to reprogram human NSCs to pluripotency, with capacities for following proliferation and differentiation [44]. By doing transcriptome analysis at the single-cell level and weighted gene coexpression network analysis, Luo et al. were able to delineate the molecular characteristics of $CD133^+/GFAP^-$ ependymal cells from the forebrain neurogenic zone of adult mouse [45]. Single-cell sequencing has indicated that NSCs in many different activation states cooccur in the SVZ of adult brain [46] and that the network from adult NSCs forms a continuous linear trajectory [47]. Developmental genes such as Bcan, Fbln2, Itih3, Ncam1, Tnr, and Vcan modulate NSC differentiation via Wnt/β-catenin pathway at early stage of differentiation and TGF-β signaling pathway at later (7 day) stage. Of note, TGF-β pathway regulates epithelial to mesenchymal transition during development [48]. Transcriptome changes during the differentiation of human embryonic stem cells into neural lineage were identified to investigate the underlying mechanisms of neural differentiation [49]. TGIF1 and MARK1 have been found to be important during the development of cerebral cortex based on studies using human embryonic stem cells [50]. Moreover, Selective Reaction Monitoring-based proteomic profiling has allowed the creation of human pluripotent stem cell-derived neuronal model with reproducibility and physiological relevance. Combined with the quantification of proteins related to central nervous system diseases, this model provides the platform for potential drug discoveries [51].

Protein modifications posttranslationally are also a central part of NSC characterization which offer enormous information on such processes as cellular signaling, proliferation, differentiation, and maintenance. Studies based on expression profiles suggest that miRNAs are critical regulators in NSC biology [52]. Recently, neural stem cell maintenance was found to be regulated by an E2F1–miRNA feedback loop [53, 54]. A total of 10 miRNAs were identified to be differentially up- or downregulated in stem cells of glioblastoma versus normal NSCs, which may provide clues to develop miRNA-based therapies that target cancer stem cells specifically [55]. Recent studies have indicated key roles of miRNAs in reprogramming of somatic cells into NSCs or neurons [56–60]. In addition to miRNAs, transcripts over 200 nucleotides long which may not code for proteins and lncRNAs can have important biological functions in neuronal differentiation

[61, 62]. Except for noncoding RNAs, other epigenetic mechanisms, such as DNA methylation and histone modifications, also play major roles in regulating and orchestrating gene expression during the course of neurogenesis as well as in neurological and psychiatric disorders [63–65]. The balanced DNA methylation status is essential for the maintenance and cell fate determination of neural stem cells during early development and in preventing malignant transformation [66, 67]. By using acetylated histone H3 ChIP-sequencing, the histone H3 acetylation level was found to increase overtime on the neural gene loci in the course of mouse embryonic stem cell differentiation to neurons, which revealed how the epigenetic modulation of histone acetylation/deacetylation coordinates with signals outside of the cells to determine the fate of NSCs [68]. However, our knowledge on the active roles of histone modifications in neurogenesis is only at the start line waiting to be developed [69].

The power of integrating different platform-based proteomics with the monitoring of multiple reactions was demonstrated [70], because small number of differentially expressed proteins did not show statistically significant differences in the outcomes of experimental group versus the control. A comprehensive review of NSC biology and epigenetics along with proteomics is beyond the scope of this manuscript [71]; we instead briefly summarize some basic information to show how proteomic technology has been widely used to indicate potential cellular targets mediating the differentiation of NSCs with regard to different aspects of multiple neurological diseases. Comparative proteomic analysis revealed HDGF as a novel angiogenic secreted factor during endogenous neurogenesis [72]. Membrane proteins expressed by the undifferentiated NSC line were identified [73].

A lot of work is now being devoted to developing innovative tools to ascertain the relationship between "omics" and analyzing the novel therapeutic targets of endogenous neurogenesis and exogenous NSC transplantation. For example, identification of cell fate determinants for directing stem cell differentiation remains a challenge. The gene-regulatory networks-based model of stem cell differentiation and computational method can guide differentiation experiments in stem cell biology and regenerative medicine [74]. However, the continuous development of computational and statistical methodologies will for sure provide greater precision and relevance of all "omics" research, without exceptions.

Additionally, identifying biomarkers of central nervous system disorders is one of the urgent goals of medicine in modern times. Most neurological disorders, including hemorrhagic stroke, are diagnosed too late due to the unavailability of biomarkers that can recognize early signs of pathological processes in the living brain. Like other omics fields, metabolomics may offer enormous information on the status of the brain at a given time point. By using proton magnetic resonance spectroscopy, Maletić-Savatić et al. discovered a metabolic biomarker of NSCs for the analysis in the live human brain, which connected systems with cellular neurobiology through the uses of certain specific metabolites. Thus, they give a functional observation into the living human brain, which may pave the way to the eventual discoveries of useful biomarkers of the diseases in clinic [75].

Reprogramming technology enables the production of NSCs from somatic cells by direct transdifferentiation. However, not much is recorded regarding how neuron processes in these NSCs or induced neural stem cells (iNSCs) behave differently from those of other stem cell populations both in vitro and in vivo. Hallmann et al. did transcriptome analyses on mouse iNSCs, which demonstrated unique, global, neural, metabolic, and cell cycle-related markers in these populations [76]. Xi et al. employed a mix of cytokines and small molecules to maintain the primitive and quiescent NSCs derived from mouse embryo stem cells and induced NSCs from rat fibroblasts by ectopic expression of three different transcriptional factors, including Oct4, Sox2, and c-Myc [77]. Clarification of the behavior of NSCs, in both clinical use and preclinical research, could predict well for the future brain tissue repair by transplantation of a patient's own-isolated stem cells [78]. However, poor reprogramming efficiency and the lack of proliferation of some somatic cell types make it hard to produce large numbers of neurons with this method and thus difficult to translate the technology into clinical use [79].

6. Perspective and Conclusion

In the past twenty years or so, multiple technologies have been developed to utilize the regenerative potential of NSCs and the plasticity of neural cells in central nervous system to help preserve brain tissue after injury or improve structural and functional recovery upon acute brain injury, including hemorrhagic stroke [80]. Based on the pathophysiology of secondary brain injury after hemorrhagic stroke, targets regarding prediction, diagnosis, treatment strategies, and neurofunctional recovery need to be further identified and verified in large cohorts of patients, especially those controlling NSCs at both the transcription and genomic levels (Figure 1). Novel bioinformatics methods may provide much more information about therapeutic strategies for endogenous neurogenesis and exogenous NSC transplantation in hemorrhagic stroke management.

Competing Interests

The authors declared no potential conflict of interests.

Authors' Contributions

Le Zhang and Wenjing Tao contributed equally to this work.

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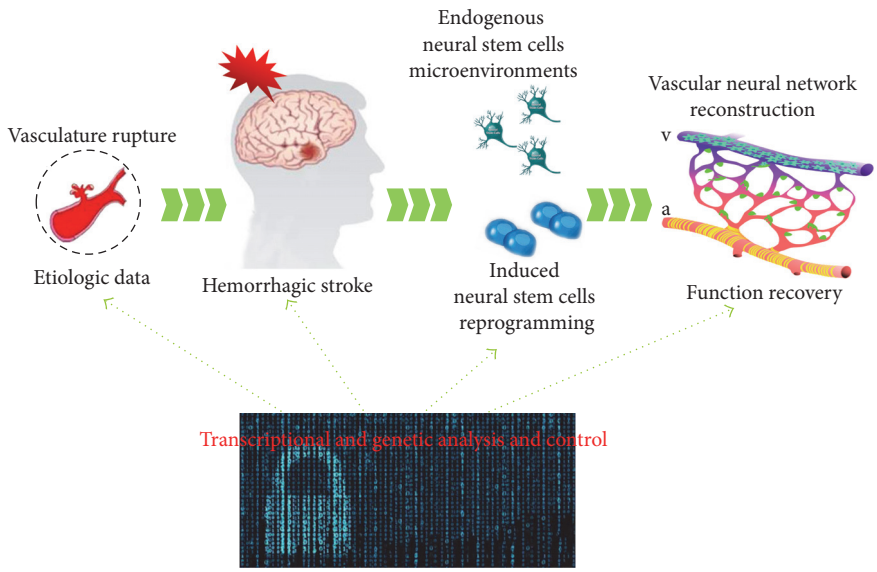

Figure 1: Diagram of transcriptional and genetic analysis and control for the function recovery in hemorrhagic stroke.

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Review Article

Tumor Budding, Micropapillary Pattern, and Polyploidy Giant Cancer Cells in Colorectal Cancer: Current Status and Future Prospects

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We previously reported that polyploid giant cancer cells (PGCGs) induced by $CoCl_2$ could form through endoreduplication or cell fusion. A single PGCC formed tumors in immunodeficient mice. PGCCs are also the key contributors to the cellular atypia and associate with the malignant grade of tumors. PGCCs have the properties of cancer stem cells and produce daughter cells via asymmetric cell division. Compared with diploid cancer cells, these daughter cells express less epithelial markers and acquire mesenchymal phenotype with importance in cancer development and progression. Tumor budding is generally recognized to correlate with a high recurrence rate, lymph node metastasis, chemoresistance, and poor prognosis of colorectal cancers (CRCs) and is a good indicator to predict the metastasis and aggressiveness in CRCs. Micropapillary pattern is a special morphologic pattern and also associates with tumor metastasis and poor prognosis. There are similar morphologic features and molecular phenotypes among tumor budding, micropapillary carcinoma pattern, and PGCCs with their budding daughter cells and all of them show strong ability of tumor invasion and migration. In this review, we discuss the cancer stem cell properties of PGCCs, the molecular mechanisms of their regulation, and the relationships with tumor budding and micropapillary pattern in CRCs.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors and its incidence ranks the third of malignant tumors [1]. The metastasis and relapse of CRC are the main reasons of tumor recurrence and patient death [2]. Twenty percent of CRCs have lymph node and/or distant metastasis at diagnosis [3]. The overall 5-year survival rate for CRCs patients is 64% and this rate drops to 12% in metastatic CRC patients [1]. The high death rate of metastatic CRCs is well known. If we can identify characteristic features in the primary lesion which are highly correlated to recurrence and metastasis of CRCs, then these characteristics can be used as prognostic markers to predict the recurrence or metastasis [4, 5]. The essential step in tumor invasion and metastasis is the tumor dedifferentiation and dissociation at the invasion front [6]. However, the degree of tumor differentiation in CRCs is hard to evaluate and is not exactly in accordance with the metastasis. Recently, increasing evidences confirmed the important role of polyploid giant cancer cells (PGCCs) and tumor budding in predicting the metastasis and patient's prognosis of CRCs [7, 8].

Normal human cells contain 46 chromosomes, but tumors cells contain abnormal numbers (usually between 60 and 90) of chromosomes, with cell-to-cell variability. Structural abnormalities of chromosomes such as inversions, deletions, duplications, and translocations are commonly observed in cancer cells but are rare in normal cells [9, 10]. The cells with abnormal number of chromosomes are named polyploid cells. PGCCs contribute to solid tumor heterogeneity and are the main histological feature of malignant tumor in pathologic diagnosis. Commonly, the number of PGCCs is higher in high-grade malignant tumor than in low-grade malignant tumor, in recurrent tumor after chemotherapy

than in tumor before chemotherapy, and in the metastatic foci than in the primary tumor [11, 12]. PGCCs are previously considered to be at the stage of mitotic catastrophe and believed to be nondividing senescent cells. Polyploid giant cells appear in skeletal muscles, osteoclasts, and senescent cells [13] and can be formed via cell fusion or abortive cell cycles [14]. We previously found that PGCCs isolated from the ovarian cancer and breast cancer cell lines can revert to regular cancer cells through budding [12, 15]. PGCCs can express cancer stem cell markers including CD44 and CD133. The daughter cells budded from PGCCs expressed EMT-related proteins and show strong ability of tumor invasion and migration.

Tumor budding is similar to the morphological features of micropapillary pattern [16–20]. Based on the morphologic features, protein expression, and biologic behaviors, we speculate that these daughter cells budded from PGCCs fall into the broad term of tumor budding and micropapillary cancer pattern [12, 15, 21]. This review will discuss the recent development of PGCCs and its association with tumor budding and micropapillary pattern in CRCs.

2. PGCCs and Cancer Stem Cells

Cancer stem cells, often referred to as tumor-initiating or tumor-propagating cells [22, 23], are capable of generating entire tumor mass. These cells are considered as the seed cells to fuel the development, chemoresistance, and recurrence of human cancer. The history of the cancer stem cell can be traced to Coneheim who proposed the embryonic nest theory of cancer stem cells 150 years ago [24, 25]. The early definitive evidence of cancer stem cells was found in leukemias [26]. Later, Al-Hajj et al. and other groups showed that cancer stem cells were present in solid cancers, including breast carcinoma and glioblastoma [27, 28]. Intensive efforts have been devoted to identifying and characterizing cancer stem cells. To date, stem cell-like populations have been characterized and isolated by flow cytometry using so-called cancer stem cell markers [27–29]. However, these markers were neither pure nor specific for cancer stem cells [30–32]. Furthermore, the phenotypes of these marker-enriched cancer cells were not stable and could change from marker-positive to marker-negative [33]. Thus, characterizations of markers that unequivocally identify a population of cancer stem cells remain challenging. American Association for Cancer Research (AACR) consensus conference workshop described cancer stem cell as "a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor" [34].

PGCCs are often considered as the senescent cells. PGCCs in tumors have not attracted major attention due to lack of extensive study and their poorly understood biology [12]. Actually, PGCCs are the key contributors to cancer heterogeneity and form the basis for differential diagnosis of benign and malignant tumors. They associate with the malignant grade and lymph node metastasis. The relationship between PGCCs and cancer differentiation has long been known, but it is not clear if PGCCs contribute to tumorigenesis or they are only the consequence of malignant transformation [35–37]. Clinical evidence is accumulating in support of the view that the number of PGCCs positively correlates with the malignant degree of cancer. In cancer, multiple stresses including antimitotic chemotherapy drugs, radiotherapy, hypoxia, or poor microenvironment can increase the number of PGCCs.

We recently reported that PGCCs can be purified from human ovarian and breast cancer cells lines and primary human ovarian tumors with the use of chemical hypoxic mimetic, cobalt chloride ($CoCl_2$) [12, 15, 21, 36–38], and confirmed that these cells were formed through cell fusion and produced daughter cells via asymmetric cell division. On the other hand, PGCCs are slow-cycling in nature and express stem cell markers. These cells are prone to differentiation into other benign tissues including adipose, cartilage, and bone, which has been confirmed both in vitro and in vivo [12]. A single PGCC can form spheroids in medium and Matrigel in vitro through time-lapse observation. A single PGCC can also form tumor when it was subcutaneously injected into the SCID mice. PGCCs express a distinct signature of proteins involved in hypoxia, invasion, chromatin remodeling, and cell cycle regulation [39–41]. These features of PGCCs suggest that PGCCs may represent a novel type of cancer stem cells which can be defined by size and morphology without using cell surface markers.

PGCCs generate daughter cells via asymmetric cell division which is a hallmark of stem cells. In multicellular eukaryotes, mitosis is the recognized process for somatic cell division, ensuring the accurate separation of genetic material [42, 43]. Asymmetric cell division is important in producing cell diversity during normal tissue development. In contrast to symmetric cell divisions, asymmetric cell division produces two daughter cells including stem cell and non-stem cell which have different cellular fates [44]. Asymmetric cell division is a fundamental process involving many physiological and pathological processes. In a typical outcome, the stem cell generates a copy of itself, and a second daughter cell programmed to differentiate into a non-stem cell type [45, 46]. Asymmetric division is a key mechanism ensuring tissue homeostasis, maintaining the stem and progenitor cell population, and allowing the development of diverse functional cells.

3. Tumor Budding and Its Clinical and Pathologic Significances

Prognosis of CRC patients has been associated with several morphological features including the infiltration depth, tumor cell differentiation, and lymph node or distant metastasis. These morphological features are the primary parameters for prognostic evaluation and tumor staging of CRCs in clinics [47]. However, other pathological indicators including tumor budding should be noted in the pathologic records to help the clinicians to judge the prognosis of patients with CRC [48]. Tumor budding is an increasingly recognizable feature to indicate the lymph node metastasis in CRCs [49–53]. "Tumor budding" was first described by Imai who noticed that cells sprouting from the edge of tumor entity are indicative of a tumor with high growth rate [54]. It is generally thought that tumor budding is a histological feature that is

observed by pathologists from microscopy. The term "tumor budding" is referred to as a cluster of cancer cells which are located in the invasive front microscopically [55, 56]. Tumor budding is defined as an isolated single cancer cell or a cluster composed of fewer than five cancer cells and has been reported to be highly related to the recurrence rate and poor prognosis in CRCs [48, 49, 57–61]. Tumor cells with minimally differentiated CRCs show strong ability of tumor invasion and migration and lymph node metastasis. The number of tumor buddings has negative correlations with the degree of tumor differentiation. There are more tumor buddings in minimally differentiated CRCs than in the highly differentiated and moderately differentiated CRCs [57, 62–65]. In clinical practice, Dukes staging system has been widely used for CRC classification for many years because it can effectively predict the disease prognosis. However, some patients with the same Dukes stage have different prognosis and different response to chemotherapy. Comprehensive evaluation system including the degree and type of differentiation, tumor budding, lymph node metastasis, and infiltrative depth should be used to guide the clinical treatment and predict prognosis. The comprehensive evaluation system should be reproducible and have substantial predictive value for the patients with CRC.

Several large studies tried to establish the criteria of clinical significances of tumor budding in CRCs and confirmed that tumor budding correlates with the lymph node or distant metastasis and associates with patient prognosis. Hase et al. studied 663 patients with CRCs; they divided patients into two groups according to the number of buddings including none or mild group and moderate or severe group [55]. They found that the presence of moderate-to-severe tumor budding indicated a bad biological behavior of CRCs; they also proposed that meticulous follow-up and adjuvant chemotherapy may be beneficial to patients with moderate-to-severe tumor budding regardless of their Dukes staging. In a multivariate analysis, tumor budding but not diffuse infiltration was identified as an independent prognostic factor. Other studies showed that tumor budding is an independent factor to predict the prognosis of CRCs. Ueno et al. reported that the number of tumor buddings is associated with tumor metastasis in CRCs [66–69]. A count of 0 to 9 tumor buddings per field (magnification of ×250) was marked as low grade, and a count of 10 or more was considered as high grade [68]. Like the Gleason scores in cancers of the prostate, tumor budding may be one of the important scoring factors to predict the prognosis in CRC patients.

The morphologic characteristics of tumor budding reflect the loss of adhesive epithelial phenotype in cancer cells and are accompanied by the metastasis of cancer [70–73]. Results from Pyke et al. confirmed that laminin-5 is a marker of invading cancer cells because of similar distributions between laminin-5-positive budding cancer cells at the invasive front in CRCs and the receptor for urokinase-type plasminogen activator [74]. It was suggested that laminin-5 might represent a valuable marker for tumor budding. Furthermore, laminin-5 and urokinase-type plasminogen activator receptor colocalized in CRCs could be important in the invasion and metastasis of cancer cells [74].

4. The Role of EMT in the Process of Tumor Budding and PGCCs with Daughter Cells

EMT is involved in many physiological processes including mesoderm formation and neural tube formation and pathological processes including wound healing and organ fibrosis [75, 76]. It should be pointed out that EMT plays an essential role in cancer metastasis and progression as epithelial cells lose their cell polarity and cell-cell adhesion and become mesenchymal cells with migratory and invasive properties [77]. During EMT, cells lose their epithelial morphology because of the cytoskeleton reorganization. Low cell-cell adhesion resulted from E-cadherin dysfunction and different expression of tight and adherent junction proteins increased invasive properties of cancer cells [78].

EMT has also been demonstrated to play an essential role during the formation of cancer stem cells [79]. Previously, we confirmed that PGCCs can be induced by $CoCl_2$ and paclitaxel. When cancer cell lines recovered from the treatment of $CoCl_2$ and paclitaxel, PGCCs could produce daughter cells via asymmetric cell division. Western blot analysis was performed to confirm that cytokeratin (AE1/AE3) expression was lower in the daughter cells than in cancer cells without treatment. The increased expression of mesenchymal markers such as vimentin was evident in daughter cells [12, 15, 21]. Particularly after paclitaxel treatment, the daughter cells developed an elongated, spindle cell, fibroblastic morphology, which was consistent with mesenchymal cells [15]. A high-throughput iTRAQ-based proteomic methodology was used to determine the differentially expressed proteins between PGCCs treated with $CoCl_2$ and the control cells. Results showed that a panel of stem cell-regulating factors and EMT-related proteins were upregulated in PGCCs [21]. PGCCs with budding daughter cells had higher Snail, TWIST, and Slug expression than the diploid control tumor cells [21].

5. Wnt/β-Catenin Signal Pathway in Tumor Budding

Jass et al. reported that APC (adenomatous polyposis coli) mutation was much less frequent in sporadic microsatellite instability-high (MSI-H) cancers than in MSI-low or microsatellite stable cancers [7]. APC can regulate the expression of β-catenin and is an important component of β-catenin degradation complex. Tumor budding was characterized by increased expression for both β-catenin and p16 (cyclin-dependent kinase inhibitor 2A) [68]. β-Catenin is important for linkage of E-cadherins to the cytoskeleton [80, 81]. The downregulation of E-cadherin in carcinoma cells is associated with increased invasive ability of cancer cells [82, 83]. Furthermore, epithelial cell adhesion molecule (Ep-CAM) has been confirmed to be involved in tumor budding [84]. Loss of membranous Ep-CAM regulates the β-catenin subcellular localization. When β-catenin translocates from the cytoplasm to the nucleus, epithelial adhesion was reduced and migratory potential increased. Loss of Ep-CAM of CRC cell membrane is highly correlated with tumor budding, cancer grade, and local recurrence [84].

6. Tumor Budding, Micropapillary Pattern, and PGCCs in CRCs

Micropapillary pattern was first reported in breast cancer, in which a small cluster of tumor cells appear in tumor tissue and there are no vessels and stromal cells in the middle of tumor cluster [16]. Micropapillary pattern has been detected in about 20% of CRCs, a phenomenon analogous to the more familiar one seen in some carcinomas of breast, bladder, lung, pancreas, ovary, urothelial tract, and stomach [85–88]. Micropapillary pattern in carcinomas is associated with a greater frequency of lymphovascular invasion and lymph node metastases and poor prognosis [89]. Tang et al. reported that the average number of metastatic lymph nodes, the proportion of CRC with distant metastasis, and the number of cases with lymphovascular tumor emboli were significantly higher in CRCs with micropapillary pattern compared to those in CRCs without micropapillary pattern. Furthermore, micropapillary pattern often appears in tumor with minimal differentiation and the expression of E-cadherin in tumor cells of micropapillary pattern is lower than that in regular tumor cells [89]. The difference between tumor budding and micropapillary pattern is mainly based on the location and cell number. Micropapillary pattern can appear in both the edge and center of tumor mass, and the cell number is often more than five. Tumor budding is located in the invasion front and the cell number is less than five. Verdú et al. reported that there is an "inside-out" MUC1 immunohistochemical staining feature in micropapillary pattern [90]. MUC1 plays an important role in the detachment of cells from the stroma and determines the characteristic morphological features of the invasive potential and aggressive behavior via regulating the intercellular adherence [91]. Micropapillary pattern has similar morphological features to tumor budding in the invasion front of CRCs [90].

PGCCs appear in most of micropapillary carcinoma patterns and tumor budding. In some micropapillary patterns, the absence of PGCCs may be due to the five-micrometer thickness for slide processing. According to the morphological observations in CRCs with large sample, we speculate that tumor budding and micropapillary pattern may have the same origin, which derive from PGCCs with their budding daughter cells. We previously reported that single PGCCs which appeared after paclitaxel treatment from an invasive breast cancer cell line MCF-7 formed cancer organotypic structure (COS) including glandular, vessel-like, and papilla-like structures in vitro [15]. The papilla-like structures derived from single PGCCs were made of PGCCs and their daughter cells. The morphology of the papilla-like structure resembled that of micropapillary pattern in human CRCs [15]. As described above, daughter cells budded from PGCCs in tumors have strong ability to invade and migrate. Both tumor budding and micropapillary pattern originated from PGCCs with budding daughter cells show strong invasive ability.

There is a close association among tumor differentiation, tumor budding, PGCCs, and micropapillary carcinoma pattern. Lv et al. also reported that single stromal PGCCs with their budding daughter cells were often associated with tumor metastasis in OSCs [92]. The number of single stromal PGCCs between low-grade and high-grade OSCs was different and statistically significant. High-grade OSCs have more single stromal PGCCs number [41, 92]. Single PGCCs generating daughter cells via budding can be observed in paraffin-embedded CRCs slides. Tumor tissue with single stromal PGCCs is more aggressive than tumor tissue without single stromal PGCCs. In another unpublished paper by us, we have confirmed that there are more tumor buddings and PGCCs in minimally differentiated CRCs than in highly differentiated and moderately differentiated CRCs. PGCCs can generate daughter cells via budding (Figures 1(a) and 1(b)). Because of the fact that the cell number in micropapillary pattern is more than that in tumor budding, there is a space separation between the tumor tissue and mesenchymal tissue. However, PGCCs always appear in micropapillary pattern (Figures 1(c) and 1(d)) and tumor budding (Figures 1(e) and 1(f)). Thus, the criterion of using single stoma PGCCs with their budding daughter cells may be better representative than tumor budding or micropapillary pattern. The association among tumor differentiation, tumor budding, PGCCs, and micropapillary pattern can help us further understand tumor metastasis and patient's prognosis.

7. Future Prospects of Tumor Budding and Single Stromal PGCCs

CRCs with tumor budding were identified to respond to chemoradiotherapy poorly and have adverse prognosis. Tumor budding is mostly unreported in daily diagnostic practice due to the lack of a standardized evaluating system. Karamitopoulou et al. suggested a method using 10 high-power fields to assess tumor budding at the invasive front. Their opinion showed that using 10 high-power fields to evaluate tumor budding had independent prognostic value and could show good interobserver consistency [93]. Jass et al. described "discrete clusters" of cells as buds [5]; Hase et al. gave definition of buds as clusters "appearing to bud from a larger gland" [55]. However, the identification of tumor budding is often confused with the fibroblasts, histiocytes masquerading, and fragmentation of a larger gland.

Since tumor budding is related to the prognosis of CRC patients, the standardized evaluating index including tumor budding and PGCCs could improve the grading of CRCs. Thus, it is worth paying more attention to the relationships between PGCCs with their newly budding daughter cells and tumor budding. The molecular mechanisms of tumor budding are involved in the expression of EMT-related proteins and Wnt/β-catenin signaling pathway which may provide clinicians with new hints to treat tumor with high-grade malignancy through targeting these proteins involved in tumor budding and PGCCs formation.

Competing Interests

The authors have no competing interests.

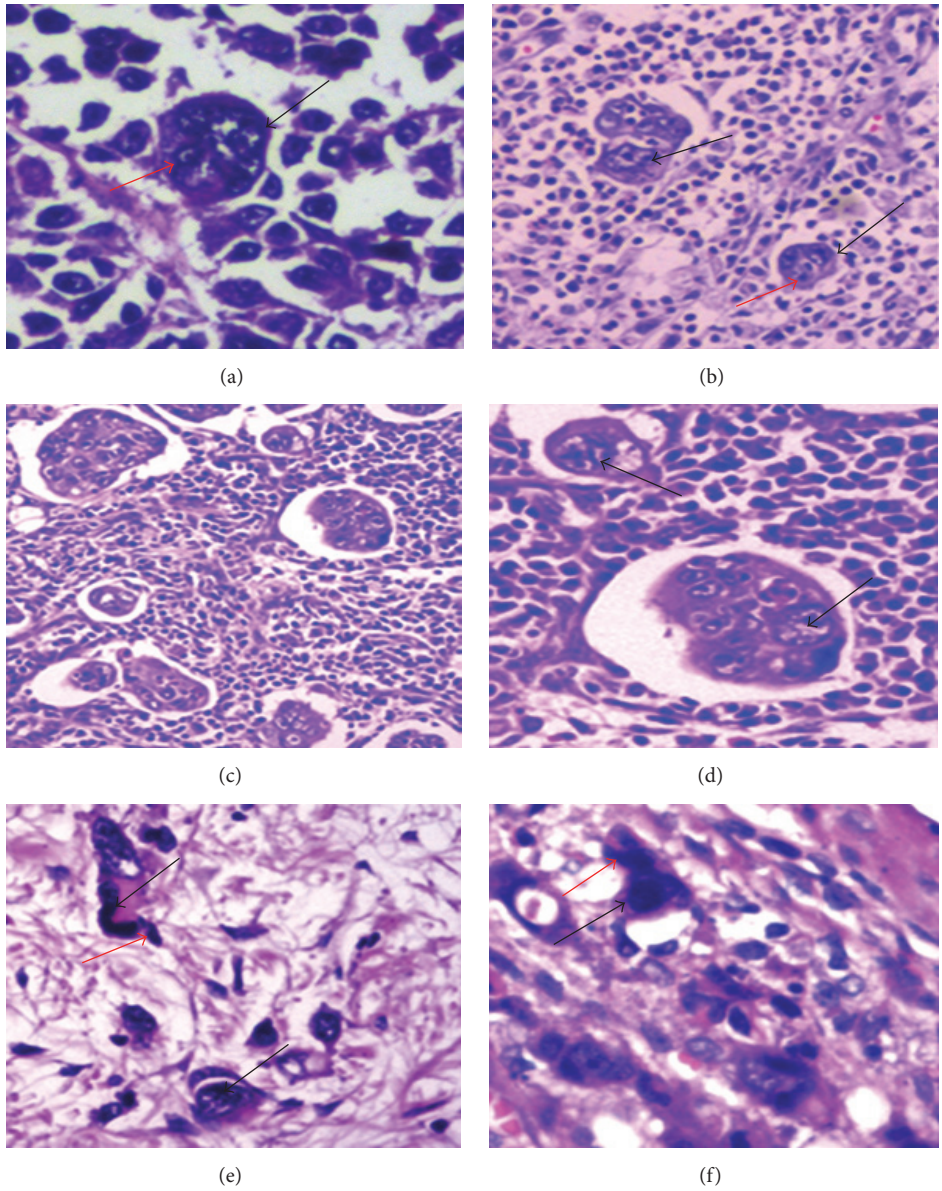

Figure 1: Tumor budding, PGCCs, and micropapillary carcinoma pattern in CRCs. ((a) and (b)) PGCCs with budding appear in minimally differentiated CRCs. Black arrows indicate PGCCs and red arrows indicate daughter cells generated by PGCCs (HE, ×200). (c) Micropapillary patterns appear in minimally differentiated CRC (HE, ×100). (d) PGCCs appear in micropapillary patterns (HE, ×100, black arrows). ((e) and (f)) Single PGCCs with their budding daughter cells in CRCs; and the structure is similar to tumor budding. Black arrows indicate PGCCs and red arrows indicate daughter cells budded by PGCCs (HE, ×200).

Authors' Contributions

Dan Zhang and Zhengduo Yang contributed to collection of literatures. Shiwu Zhang and Xipeng Zhang contributed to conception and design, financial support, and manuscript writing. All authors read and approved the final manuscript.

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