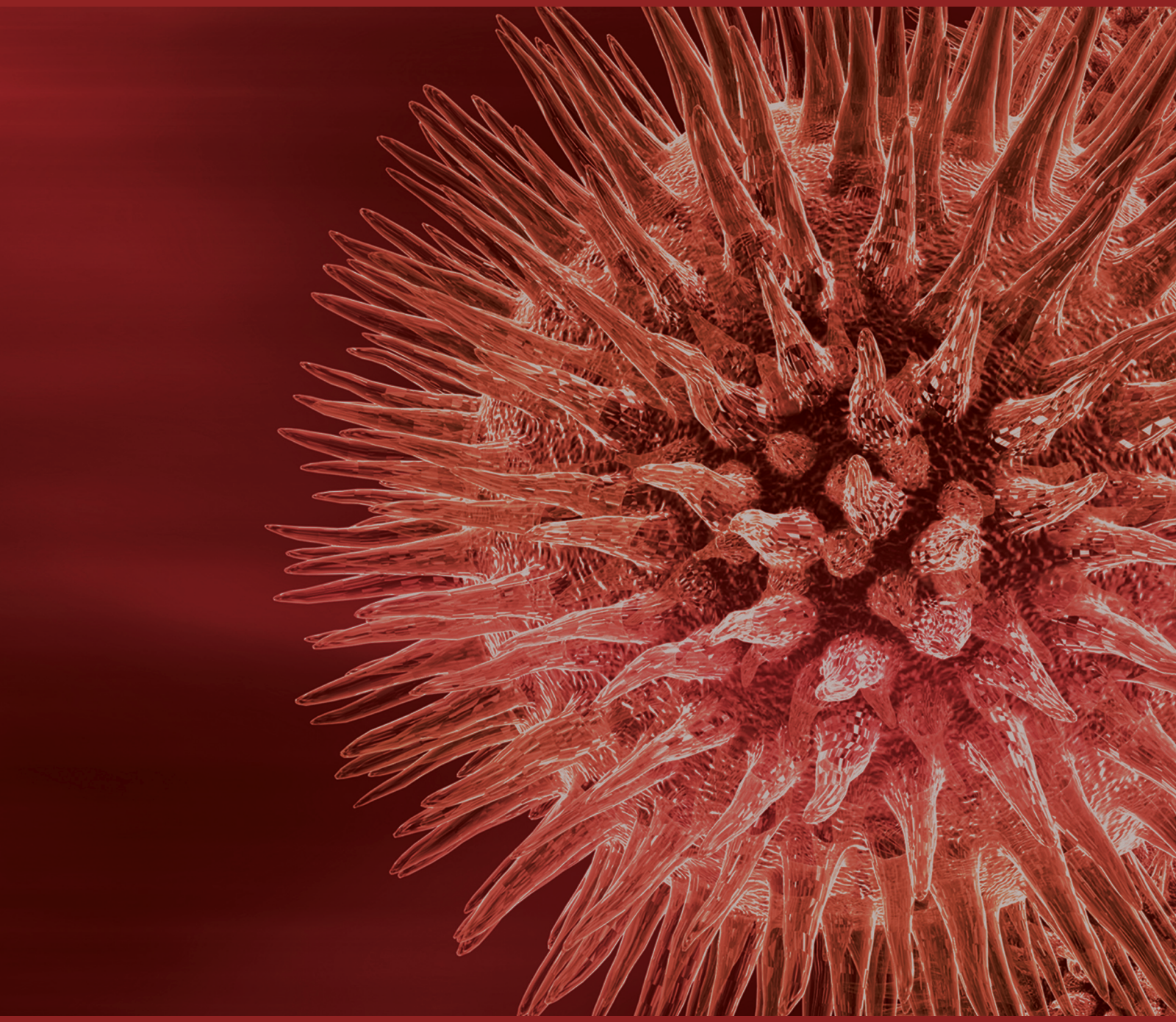
BioMed Research International

# Cellular Microenvironment in Human Pathologies

Guest Editors: Davide Vigetti, Martin Götte, Mauro S. G. Pavão, and Achilleas D. Theocharis



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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 946958, 2 pages
http://dx.doi.org/10.1155/2013/946958

*Editorial*

# Cellular Microenvironment in Human Pathologies

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Received 8 October 2013; Accepted 8 October 2013



It is well established that the cellular microenvironment dramatically influences cell behavior and is critical in many physiological functions, such as the differentiation niche of stem cells, or during development. Notably, it has pivotal roles in many pathologies. The surroundings of cells are constituted by several heterogeneous components forming a complex network of proteins and glycans. Although this extracellular matrix has been considered for decades as a mechanical scaffold where cells attach and reside, nowadays, it is known that such molecules modulate numerous cellular functions including survival, proliferation, migration, and differentiation. From a mechanistic point of view, the extracellular matrix components can interact with cells in several ways, including the modulation of soluble factor diffusion (i.e., growth factors, cytokines, morphogens, etc.), binding to their receptors, their subsequent activation, and regulation of signaling cascades.

In addition to collagenous fibrillar structures, ECM is composed of proteoglycans, a heterogeneous group of secreted or membrane bound heavily glycosylated proteins. These sugars are represented by glycosaminoglycans that consist of repetitions of disaccharides formed mainly by an uronic acid with an amine sugar. These chains can be sulfated and modified in several ways leading to the formation of heparan sulfate, chondroitin sulfate, and dermatan sulfate. The only glycosaminoglycan without chemical modification is hyaluronan. Although simple in structure, hyaluronan is an information-rich molecule, and the functions of these glycosaminoglycans depend on the polysaccharide chain lengths.

ECM turnover is fundamental in the physiological renewal of such components, and many degradative enzymes have also a role in pathology. Matrix metalloproteinases are probably the most well-known enzymes that catabolize many components of ECM including collagens and proteoglycans. More recently, other degradative enzymes specific for matrix polysaccharides have been shown to be critical in several physiological and pathological processes. Many growth factors and other mediators can bind to proteoglycans, and an altered matrix degradation can lead to an abnormal factor liberation. Further, hyaluronidases, producing low molecular weight hyaluronan, contribute to inflammation.

An increasing body of literature suggests that an altered cellular microenvironment is mechanistically involved in a variety of human pathologies. Interestingly, several extracellular components influence neoplastic diseases and cardiovascular, pulmonary, and renal pathologies that, together, represent the major causes of death in industrialized countries.

In this special issue comprising review articles and original research papers, all the above topics are deeply discussed by experts in the field.

Two works are focused on the skeletal system; M. Maldonado and J. Nam discuss the changing of ECM components in the presence of inflammation during osteoarthritis whereas S. A. Syggelos and collaborators review the effects of matrix metalloproteinases in periprosthetic loosening and osteolysis.

Different aspects of renal disorders are presented in the works of J. A. Lepedda and coauthors, who describe the use

of bikunin as a marker of Fabry's disease, and S. P. Srivastava and coresearchers, who provide an overview on the role of microRNA in fibrosis and diabetic nephrology.

The effect of biomechanical forces in the tissue microenvironment on the regulation of fibrosis is deeply reviewed by W. Carver and E. C. Goldsmith.

ECM effects on cardiac primitive cell properties and their role in normal and pathological human heart are presented by C. Castaldo and collaborators.

The effects of low-density lipoprotein receptor-related protein-1 (LRP-1) on matrix remodeling are deeply reviewed by N. Etique and coworkers.

S. Plantman reviews the role of ECM in axonal and neuronal regeneration.

Many papers are focused on cancer and metastasis and report the effect of several ECM components in different types of neoplasia.

The role of hyaluronan and its receptors CD44 and RHAMM on fibrosarcoma progression is presented by D. Nikitovic and coresearchers.

The effects of epidermal growth factor receptor on digestive tract cancers are discussed by T. Sasaki and coauthors.

Two works focused on breast cancer; A. M. Gomes and collaborators show the effects of heparanase and heparan sulfate on tumor progression, whereas D. Lymperatou and coauthors present the effects of fulvestrant and tamoxifen on cell migration.

S. Mizumoto and coresearchers present the effect of N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase in pulmonary metastasis.

The role of prostate stem cells in development of benign prostate hyperplasia or prostate cancer is reviewed by A. Prajapati and collaborators.

Syndecan-4 expression and correlation with metastatic potential in testicular germ cell tumors are presented by V. T. Labropoulou and coworkers.

Overall, the papers presented in this special issue not only underscore the importance of the cellular microenvironment for a variety of physiological and pathophysiological processes with considerable relevance for human disease, but also provide insight into the molecular diversity of regulatory interactions exerted by the microenvironment on a wide range of cell types. A deeper understanding of the functional properties of the cellular microenvironment thus emerges as an important prerequisite for the development of more efficient therapeutic approaches in tissue repair and regeneration and diseases ranging from chronic and acute inflammation to cancer.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 981695, 11 pages
http://dx.doi.org/10.1155/2013/981695

*Review Article*

# Proregenerative Properties of ECM Molecules

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Received 30 April 2013; Revised 4 July 2013; Accepted 7 August 2013

Academic Editor: Mauro S. G. Pavão



After traumatic injuries to the nervous system, regrowing axons encounter a complex microenvironment where mechanisms that promote regeneration compete with inhibitory processes. Sprouting and axonal regrowth are key components of functional recovery but are often counteracted by inhibitory molecules. This review covers extracellular matrix molecules that support neuron axonal outgrowth.

## 1. Introduction

The extracellular matrix (ECM) has a profound influences on individual cells and influences and/or controls several basic cellular processes such as adhesion, differentiation, survival, growth, and migration. In order to mount an effective regenerative response after axonal injury, the injured neuron must be able to initiate a number of changes at the cellular level including an increase in expression of relevant genes and protein transport with the aim of forming an extruding process known as a growth cone [1–3]. For functional recovery to occur, the growth cone must then successfully navigate back to its target by reacting to inhibitory and permissive cues in its surrounding and finally reestablishing proper connection with its original target. The ECM heavily influences all these processes.

When a peripheral nerve is injured, a series of cellular events collectively referred to as Wallerian degeneration occur. Macrophages invade the site of injury and in a coordinated effort together with Schwann cells start to clear the injured area of debris. A new neuronal growth cone is subsequently formed and starts to advance to its original target. The denervated Schwann cells proliferate and differentiate to a phenotype that aids in regeneration, by producing neurotrophic molecules, basement membrane components, and cell adhesion receptors. Schwann cells subsequently align along tubes of remaining endoneural basal lamina forming that so-called bands of Büngner. The growth cone advances in close contact with Schwann cells and the basal lamina. Although peripheral nervous system (PNS) lesions generally heal better than central nervous system (CNS) lesions, PNS lesions still cause significant physical impairment. It has been argued that current microsurgical techniques have reached a plateau where further advancements are unlikely to occur [4]. The situation is particularly complicated when a large portion of the nerve is lesioned and a gap occurs that requires nerve grafts harvested from another site (usually the sural nerve is used) that requires multiple surgeries and loss of function at another site. With this in mind, PNS regeneration is still a great clinical challenge, and current knowledge of factors that contribute to axonal regeneration could be of importance for tissue engineering strategies to develop artificial nerve grafts [5].

After a CNS lesion, retrograde neuronal cell death is generally more pronounced, and Wallerian degeneration is slower and less complete, although the inflammatory response is pronounced. In the CNS there are also other factors that contribute to the regenerative failure: (I) formation of fluid filled cysts, (II) lack of an organized basal lamina like the one seen in the PNS, and (III) the glial scar that is formed after a lesion inhibits growth cone advancement [6]. This scar is usually filled with inhibitory ECM molecules such as chondroitin sulphate proteoglycans. The current review has its focus on proregenerative matrix molecules, and for readers interesting in inhibitory ECM molecules, several excellent reviews are available [6–8].

## 2. Experimental Techniques to Study Neuronal Regeneration

*2.1. Cell Cultures.* The majority of the physiological findings on neuronal regeneration/outgrowth cited in the current study have been described using either cell-culture techniques or *in vivo* models of injury. Given the inherent vulnerability of adult CNS neurons to hypoxia, physical trauma, and dependence on cell-cell interaction, the majority of cell-culture studies cited in this review have relied on the culture of embryonic or early postnatal neurons from rodents, chick, or human. Since regenerative capacity of neurons diminishes with age [9, 10], caution must be taken when extrapolating these studies to the adult situation. The biggest exception to this being adult sensory neurons that are routinely cultured from adult animals in serum—and growth factor—free medium [11]. However, a recently developed protocol [12] has made it possible to successfully culture large numbers of neurons from the adult brain. This was achieved through a strategy whereby the dissociation and purification processes as well as the composition and pH of the culture media were optimized to improve survival and reach a high degree of purity. At least one study using adult CNS neurons to examine neuron-ECM interaction has been published [13], but the author of this review expects to see this technique used more routinely in the future.

*2.2. Lesion Models.* A number of different lesion models are currently in use. The lesion models mentioned in the text are summarized in Figure 1.

A peripheral nerve injury (compression, crush, or transection) is an injury in the PNS, followed by a regenerative response [2]. Commonly used experimental models are injuries to the sciatic, facial, or laryngeal nerve. After a dorsal root injury, regrowing axons are halted at the border of the spinal cord (the dorsal root entry zone), and functional recovery is not seen [14]. A ventral funiculus lesion is a lesion of the white matter in the ventral part of the spinal cord [15]. This lesion creates an injury of the motoneuron axon in a CNS environment.

Spinal cord injury models can be either partial, such as dorsal column injury, hemisection (depicted), or complete. In addition, compression and weight-drop models are commonly used [16]. Optic nerve injury (cut or compression) is a lesion of a nerve with CNS biology (oligodendrocytes, etc.) [17]. This lesion axotomizes the retinal ganglion cells which covey visual information from the retina in the eyeball to the lateral geniculate nucleus in the thalamus. Stab wounds create a small, well-defined lesion to study the reaction of neighbouring cells in the CNS [18]. Experimental stroke models are created by occlusion of one of the arteries supplying the brain with oxygenated blood [19]. This lesion is complex both in terms of the graded response observed postinjury (a severely injured lesion core, surrounding penumbra, and uninjured brain tissue) and the different secondary complications that follow, such as inflammation and oedema [19]. Finally, injection of neurotoxins is a useful tool in neurotrauma research. A variety of toxins are used for different purposes. For example, kainic acid is commonly used to mimic seizures or glutamate toxicity that occurs after traumatic brain [20], and injection of LPS (lipopolysaccarides) is used to mimic inflammatory conditions [21]. There are also toxins with a high degree of specificity for certain groups of neurons: 6-hydroxydopamine which kills dopaminergic (and noradrenergic) neurons can be injected into the substantia nigra and is thus used as an animal model for Parkinson's disease [22].

## 3. Neuronal ECM Receptors

*3.1. Integrins.* Integrins are expressed on all cell types and phylogenetically conserved. Plants, fungi, and prokaryotes do not express integrin homologues [23]. In mammals, 18 $\alpha$ and 8 $\beta$ genes have been identified, and to date 24 different receptors have been identified, see Figure 2. The phenotypes of knockout animals range from very severe to apparently normal, and ten published knockouts are lethal, namely, $\alpha$3, $\alpha$4, $\alpha$5, $\alpha$6, $\alpha$8, $\alpha$9, $\alpha$V, $\beta$1, $\beta$4, and $\beta$8 [24]. The phenotypes of knockout animals have been thoroughly reviewed elsewhere [24–26] and will not be further addressed in this review. Integrins bind a variety of proteins, such as ECM molecules, cell-surface receptors, or blood proteins. Whereas some $\alpha$-$\beta$ combinations display a high degree of specificity in their ligand, others are much more promiscuous. Integrins containing the $\beta$1 or $\alpha$V subunits bind ECM proteins such as collagen, laminin, vitronectin, osteopontin, and fibronectin, whereas $\beta$2 integrins, which are found on immune cells, bind cell surface receptors such as ICAMs (intercellular adhesion molecules). Integrins containing $\alpha$4, $\alpha$5, $\alpha$8, $\alpha$IIb, and $\alpha$V subunit bind proteins and peptides carrying the RGD (Arg-Gly-Asp) sequence such as fibronectin and vitronectin [27]. Integrins containing $\alpha$1, $\alpha$2, $\alpha$3, $\alpha$6, and $\alpha$7 have long been known to bind members of the ECM proteins known as laminins. This picture is, however, constantly changing. For example, $\alpha$9$\beta$1 has been shown to bind laminin [28].

Historically, the ability of integrins to mediate adhesion and cell spreading led to the idea that their function was to serve as a link between the ECM and the cytoskeleton via linker proteins such as talin, paxillin, and $\alpha$-actin, reviewed by [29]. Integrins also play a role in initiating intracellular signalling. Integrins lack catalytic activity, but upon ligand binding, they initiate intracellular signalling cascades by activating kinases such as integrin linked kinase (ILK), focal adhesion kinase (FAK), PI3-kinase, and protein kinase C (reviewed by [23, 29, 30]).

*3.2. Integrins in the Nervous System.* Although expression of integrins has been detected in the developing nervous system [31], information on the expression of these molecules in the adult brain was largely lacking until a very thorough mapping study was performed by Pinkstaff and colleagues [32]. They used *in situ* hybridization to examine the expression of 14 different integrin mRNAs in the adult rat brain and brainstem. Notably, they were unable to detect any expression of integrins $\alpha$2, $\beta$2, and $\beta$3 and very restricted expression of $\beta$4 and $\alpha$4. In contrast, they described a widespread expression of integrins $\alpha$1, $\alpha$3, $\alpha$6, $\alpha$7, $\alpha$V, and $\beta$1. Motoneurons in the facial nucleus and in the sciatic motor pool express integrins $\alpha$3 and $\alpha$6 and particularly high

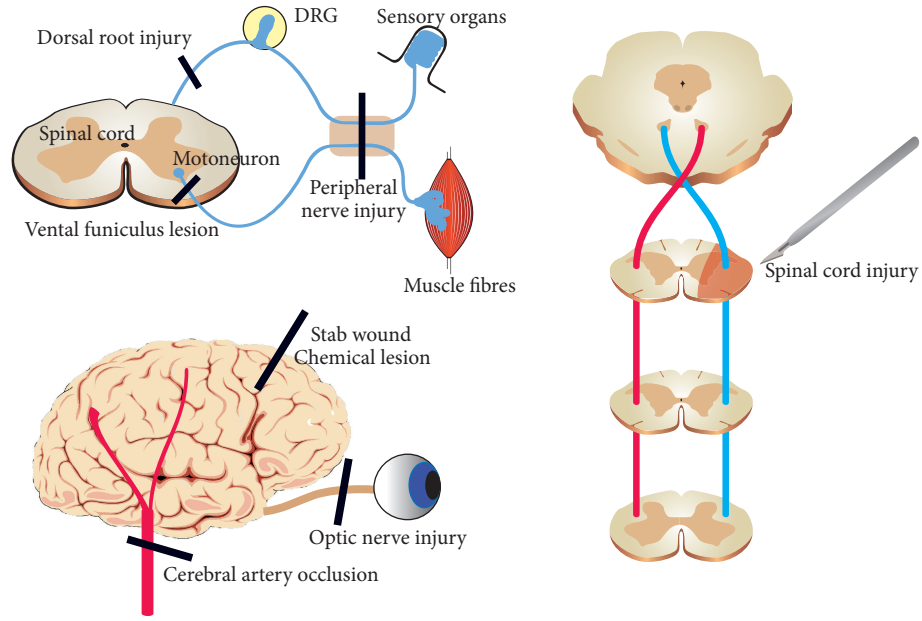


Figure 1: Lesion models discussed in this review.

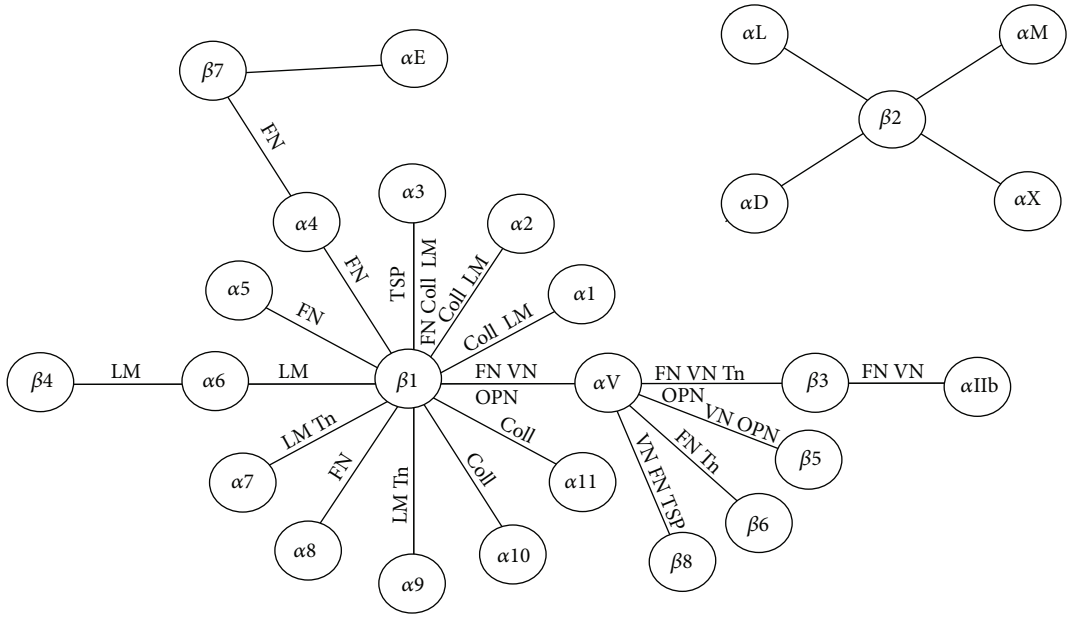


Figure 2: Integrins and ligands discussed in this review. LM: Laminin, Tn: Tenascin, OPN: Osteopontin, FN: Fibronectin, VN: Vitronectin, and TSP: Thrombospondin.

amounts of $\alpha 7$ and $\beta 1$ [32–34]. Dorsal root ganglion (DRG) neurons have been found to express a number of integrins: $\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, and $\beta 1$ [34–39]. Readers interested in thorough descriptions of integrin-mediated signalling, and surface to cytoskeletal interactions in neural regeneration are encouraged to examine high quality in-depth reviews of these matters such as [40, 41].

*3.3. Other ECM Receptors.* In addition to integrins, a number of neuronal ECM receptors with functions in neurite outgrowth exist. Dystroglycan binds laminin [42], CD44 binds osteopontin [43] and certain collagen isoforms, and CD47 interacts with thrombospondin.

## 4. ECM Molecules and Neuronal Regeneration

*4.1. Laminin.* Laminins are heterotrimeric proteins composed of one $\alpha$, one $\beta$, and one $\gamma$ chain. Currently 5 $\alpha$, 3 $\beta$, and 3 $\gamma$, chains are known, and 16 $\alpha\beta\gamma$ combinations have been described [44]. Laminins range in size from approximately 400 to 900 kDa. All laminins have a coiled-coil structure where all three subchains intertwine and a series of five

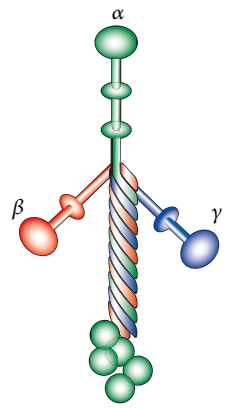


Figure 3: The molecular anatomy of laminins.

globular domains in the $\alpha$ chain C-terminal, see Figure 3. Over the years, the laminin nomenclature has changed several times, with the latest (and probably most convenient) being published in 2005 [44]. Table 1 lists laminin isoforms and chain composition. In detail descriptions of laminin subchains and specific domains can be found in [44, 45].

Laminin immunoreactivity has been detected in areas of axonal growth in the developing CNS and PNS [46, 47]. It is also seen in those CNS areas where adult regeneration is observed, such as the olfactory system [48] in the ventral funiculus after injury, where lesioned axons regenerate over spinal cord scar tissue [49]. Given that this family of proteins has received extensive attention, various isoforms will be addressed individually.

*4.1.1. Laminin-111.* In 1979, Timpl and coworkers isolated laminin-111 from mouse Engelbreth-Holm-Swarm sarcoma cells [50]; hence the previous name is EHS-laminin. The fact that this was the first isoform to be described and the ease with which it can be purified has led to being regarded as the "prototype" laminin and used extensively. Although available preparations of this laminin isoform are generally of good quality, it can be in complex with nidogen, which may influence physiological properties [51].

Expression of laminin $\alpha$1 (possibly forming laminin-111, $\alpha 1\beta 1\gamma 1$) has been detected in trigeminal nerve bundles [52], indicating that Schwann cells in some locations express this subunit. Also, in peripheral nerves of laminin $\alpha$2 deficient mice, laminin $\alpha$1 can be detected [53, 54], but the functional effects of this compensatory upregulation are not yet clear. Expression of this subunit has also been described in Pacinian corpuscles in human skin [55], suggesting that LM-111 could serve as an instructive role in axonal guidance.

*4.1.2. Laminin-211.* Expression of laminins $\alpha$2, $\beta$1, and $\gamma$1 has been detected in both developing and adult peripheral nerves of rodents or human origin [56–58]. These subunits are also upregulated in the proximal stump of injured rat sciatic nerve [58], possibly as part of a regenerative response in providing a substrate for axonal outgrowth. Loss of laminin $\alpha$2 leads to peripheral neuropathy as seen in several animal models such as the dy/dy mice, reviewed by [59], and in the human condition known as merosin-deficient congenital muscular dystrophy (MDCMD) [60]. In the peripheral nerve, the lack of laminin $\alpha$2 is manifested as reduced myelination, discontinuous basal lamina, atypical Schwann cell ensheathment, and abnormal impulse propagation, reviewed in [61]. In addition, LM-211 subunits are also expressed in Meissner's corpuscles in human skin [55]. Several lines of evidence suggest that laminin-211 supports neurite growth under some conditions: cultured embryonic spinal motoneurons from rat extend neurites on LM-211 to a greater extent than on LM-811 or collagen [62], adult DRGs extend neurites on laminin-2 in the presence of nerve growth factor (NGF) [55], Schwann cells from $\alpha$2-deficient mice are less supportive for embryonic DRG growth [61], and addition of $\alpha$2 blocking antibodies reduces growth of DRGs on nerve sections [63]. However, studies of adult regeneration *in vivo*, using any of the laminin $\alpha$2 deficient mice or rat strains, have thus far not been performed. Laminin-211 (merosin) has been available commercially (and used extensively) as a preparation from human placenta, but these preparations have been of suboptimal quality regarding purity and molecular integrity [64]. Protocols for production of recombinant LM-211 have since been developed [65].

Table 1: Laminin chain composition and nomenclature.

| Laminin | Chain composition | New nomenclature [44] |
|---|---|---|
| Laminin-1 | $\alpha 1\beta 1\gamma 1$ | 111 |
| Laminin-2 | $\alpha 2\beta 1\gamma 1$ | 211 |
| Laminin-3 | $\alpha 1\beta 2\gamma 1$ | 121 |
| Laminin-4 | $\alpha 2\beta 2\gamma 1$ | 221 |
| Laminin-5 or 5A | $\alpha 3A\beta 3\gamma 2$ | 332 or 3A32 |
| Laminin-5B | $\alpha 3B\beta 3\gamma 2$ | 3B32 |
| Laminin-6 or 6A | $\alpha 3A\beta 1\gamma 1$ | 311 or 3A11 |
| Laminin-7 or 7A | $\alpha 3A\beta 2\gamma 1$ | 321 or 3A21 |
| Laminin-8 | $\alpha 4\beta 1\gamma 1$ | 411 |
| Laminin-9 | $\alpha 4\beta 2\gamma 1$ | 421 |
| Laminin-10 | $\alpha 5\beta 1\gamma 1$ | 511 |
| Laminin-11 | $\alpha 5\beta 2\gamma 1$ | 521 |
| Laminin-12 | $\alpha 2\beta 1\gamma 3$ | 213 |
| Laminin-14 | $\alpha 4\beta 2\gamma 3$ | 423 |
| — | $\alpha 5\beta 2\gamma 2$ | 522 |
| Laminin-15 | $\alpha 5\beta 1\gamma 3$ | 523 |

*4.1.3. Laminin-411.* Similar to LM-211, laminin-411 subunits have been detected in peripheral nerves [52, 56–58] and upregulated after injury [58]. LM-411 was purified from a human glioblastoma cell line in 2001 [66], and a recombinant version was created in 2006 [67]. Neonatal trigeminal sensory neurons extend neurites when cultured on LM-411 and LM-111, but not on LM-211, in contrast to the results of spinal motoneurons [62]. Adult DRG neurons grow neurites only slightly better on LM-411 compared to LM-211 [55]. These results indicate possible cell-type specific preference of different neural types for outgrowth. The LM-alpha4

chain is subject to chondroitin sulphate modification [68]. However, according to unpublished results, we did not see any difference in neurite growth when neurons were grown on LM-411 that had been pretreated with chondroitinase. At current, the *in vivo* situation is not clear since studies using laminin $\alpha$4 deficient animals did not show hampered regeneration [62]. However, deletion of the laminin $\gamma$1 subunit in the peripheral nerve (thus ablating all functional laminin trimers) causes a profound reduction of the regeneration of spinal motoneurons after sciatic nerve crush [69].

*4.1.4. Laminin-511.* In addition to laminins-211 and -411, expression of laminin $\alpha$5 (thus possibly forming laminin-10, $\alpha5\beta1\gamma1$) has also been described in peripheral nerves [53, 70], but much less abundant. The full function of this isoform in peripheral nerves is not known, but it has been suggested that it may participate in the positioning of sodium channels in the nodes of Ranvier [53]. Similar to $\alpha$2 and $\alpha$4, the $\alpha$5 subunit was also detected in Meissner's corpuscles in human skin [55], and a preparation containing LM-511 enhanced sensory recovery in grafted skin [71]. The function of laminin-511 was initially examined *in vitro* using preparations from human placenta [72], but in order to improve quality recombinant human LM-511 was produced by Doi and colleagues [73]. Of four laminins tested (LM-111, -211, -411, and -511), LM-511 induced the most extensive outgrowth from adult DRG neurons *in vitro* [55], mediated via integrin $\alpha6\beta1$. Spinal motoneurons also grow well on LM-511, but less extensive than those on LM-211 [62]. Recent studies suggest that the neurite growth-promoting properties of laminin-511 are located to the L4a domain [74]. In the CNS, laminin is quite absent from the neuropil, with one exception, the hippocampal formation. The laminin matrix here has a neuroprotective function (since destruction of the laminin matrix is essential for excitotoxicity) [75, 76]. By immunohistochemistry, work from the same lab suggested that laminin 511 is likely the major isoform in the hippocampus [77]. This finding was subsequently expanded on by Fusaoka-Nishioka and colleagues [78], who cultured embryonic hippocampal neurons on laminins-111, -211, -411, and -511 and found 511 to have a growth-promoting capacity greater than the other isoforms.

*4.1.5. Laminins-121 and -221.* Sasaki and coworkers [79] recently produced recombinant laminins-121 and -221. In the same study, both isoforms were shown to stimulate growth from adult mouse DRG neurons, and growth on LM-121 was more extensive than EHS-laminin, recombinant laminins-111, and -211. Future studies will likely answer if this isoform also supports growth from other neuronal types.

*4.1.6. Exogenous Administration of Laminins after CNS Lesions.* So far, exogenous administration of laminins into lesion sites in the CNS has not been able to improve functional regeneration. However, a recent report by Menezes and coworkers showed that a polymerized form of laminin (produced by treating commercially available laminin preparations with a low-pH buffer) supports axonal regeneration and functional recovery after spinal cord injury in rats [80]. In addition, functional recovery has also been seen in spinal cord injury after injection of self-assembling nanofibres that present the laminin-derived IKVAV epitope at high density [81]. Injection of another laminin-derived peptide (KDI) protects dopaminergic neurons from 6-hydroxydopamine-induced injury [82] and improves regeneration and functional recovery after spinal cord injury in rats [83].

*4.2. Fibronectin.* Fibronectin (FN) is a large glycosylated protein, composed as a dimer and exists in both a soluble form (e.g., in plasma) and as an ECM constituent in the developing nervous system [47]. Although adult levels are relatively low in the PNS, it undergoes a profound upregulation after injury [84]. FN was first found to support growth from embryonic retinal ganglion cells by Akers and coworkers, [85] and has since then been shown to increase outgrowth from several types of neurons such as embryonic dorsal root ganglion neurons, sympathetic ganglions, and spinal cord neurons [37, 86, 87]. The glycosylation of FN has been shown to affect its ability to support neurite growth [88]. Compared to laminin, FN was a very weak promoter of growth for adult DRG neurons in culture [55], and it should be noted though that after peripheral nerve injury, the FN gene undergoes a dramatic change in splicing and several different isoforms are produced, some with a higher potential to support growth [39, 89], indicating that caution should be exercised when extrapolating results from cell-culture studies. Using a culture system where adult DRG neurons are grown on slices of CNS white matter, Tom and colleagues [90] found that blocking antibodies raised towards FN decreased neurite growth indicating that this molecule could be of importance for regeneration in the CNS. This hypothesis has been further supported by a recent report showing that cultured adult CNS neurons (from cortex and hippocampus) grew better on FN than laminins-111 or -112 [13]; however, laminin-511 (the most potent stimulator of CNS neurite growth [78]) was not included in this comparison. In addition, a single injection of FN (at the lesion site) after dorsal crush injury led to an increase in sprouting of descending serotonergic fibres and alleviated injury-induced allodynia [91]. Finally, fibronectin has some other interesting qualities. Using a culture system where FN is attached to conducting polymers, Svennersten and colleagues [92] showed that when the molecule is a reduced state (but not in oxidized), RGD sites are exposed and effect cell attachment and proliferation. On a different, but related, note, stretching of FN has also been shown to cause exposure of cryptic binding sites [93]. How these processes might be exploited for neuronal outgrowth remains to be explored.

*4.3. Osteopontin.* Osteopontin (OPN) is a glycosylated phosphoprotein of 44 kDa. It also goes by the names secreted phosphoprotein 1 (SPP1), bone sialoprotein 1 (BSP-1), and early T-lymphocyte activation-1 (ETA-1). It was originally identified as a component of the ECM of bone tissue (hence its name derived from the Latin words "osteo" bone and "pons" bridge). The OPN protein harbours the RGD motif [94].

Upregulation of OPN has been detected after cerebral ischemia [95], focal brain injury [96, 97], spinal cord injury [98], and optic nerve crush [99]. *In vitro* studies of OPN's effect on DRG neurite growth are somewhat inconclusive with either no reported effect [100] or inhibitory effect [101]. Concerning the CNS, there is evidence suggesting a supportive role of OPN: cultured retinal ganglion cells grow well on OPN [43, 102], and a recent report by the author of this review shows that cultured hippocampal neurons also grow well on this substrate [97]. Further, Hashimoto and colleagues reported impaired regenerative growth of corticospinal fibres after spinal cord injury in OPN −/− mice compared to wild type [103]. The authors also demonstrated impaired functional recovery in these mice, but the underlying mechanism is not completely understood. Finally, OPN also shows promise as a neuroprotective agent, as administration of this molecule protects neuronal cultures from ischemic injury *in vitro* [104], decreases infarct size after experimental stroke in mice [104], and promotes recovery after intracerebral haemorrhage [105].

*4.4. Vitronectin.* In terms of neurite growth, vitronectin (VN) has mainly been studied in regard to the visual system. Retinal ganglion cells extend neurites when cultures on VN [106, 107] and VN are upregulated after optic nerve crush in adult animals [108]. Also VN supports neurite growth from DRG neurons, on par with laminin and to a higher degree than fibronectin [109]. Further, postnatal cerebellar granule cells extend neurites in response to VN, via interaction with the RGD site [110]. In contrast, a recent study by Previtali and colleagues [111] showed that VN was upregulated in nerves from patients with defective regeneration due to peripheral neuropathy (whereas the levels of laminin and collagen IV were similar), and when compared for outgrowth-stimulating properties, VN was a poor substrate compared to collagen and fibronectin.

*4.5. Thrombospondins.* Thrombospondin-1 (TSP-1) supports growth from several types of neurons in culture: retinal ganglion cells [106], superior cervical ganglion neurons [106, 112, 113]. It is upregulated in peripheral nerve after injury and appears to support neuronal growth [114]. In addition, TSP-1 is upregulated at the injury site after spinal cord injury [115] and experimental stroke in mice [116] and rats [117]. TSP knockout mice display deceased neuronal sprouting and impaired functional recovery after stroke [116]. Another TSP family member, thrombospondin-4, has also been shown to support growth from several types of neurons, using a coculture with cells over expressing this molecule [118]. Later studies by Dunkle and coworkers showed that TSP-4 is expressed in the developing retina, and although it does not by itself support growth of retinal ganglion cells, it does potentiate the outgrowth-promoting properties of laminin [119]. TSP-4 is also produced in the dorsal horn of the spinal cord (likely by reactive astrocytes), in neuropathic pain models, and infusion of TSP-4 caused allodynia in rats [120], possibly by stimulating aberrant sprouting or synaptic plasticity.

*4.6. Tenascin-C.* Tenascins (Tn) are a family consisting of five members (Tenascins-C, -R, -X, -Y, and -W), where tenascin-C is the most extensively studied with regard to neuronal outgrowth and regeneration [121]. *In vitro* studies using embryonic sensory and motor neurons [122] and cerebellar granule neurons [123] indicate that TnC could be beneficial for outgrowth. *In vivo*, tenascin-C is upregulated after ventral funiculus lesion [124], and studies using knockout mice, forced overexpression, and protein infusion indicate a beneficial effect on regeneration after spinal cord injury [125]. In contrast Andrews and coworkers [126] did not observe extensive outgrowth from cultured DRG neurons on TnC, but having observed an increase in TnC in CNS scar tissue they overexpressed integrin $\alpha 9\beta 1$ in DRG neurons and observed regeneration of fibres after dorsal root injury.

## 5. Conclusion

This review summarizes the supportive functions of various ECM molecules from the perspective of neurite outgrowth. Needless to say, ECM molecules also influence other aspects of nervous system regeneration such as synapse formation [127, 128] and migration of neural stem cells [129] or cells transplanted to the CNS [130] that are not addressed in this review.

Further, in addition to the aforementioned nanofibres [81], novel developments in material design, using either incorporated ECM motives [131] or coupled intact molecules such as laminin [132], will hopefully be able to provide the research community with new tools to develop strategies to improve neural regeneration.

Further, the complex interplay between supportive and inhibitory molecules is not yet fully understood. Although laminin can block the inhibitory effect of myelin-associated glycoprotein (MAG) [133], it can also switch the influence of netrin from attractive to repulsive [134] and mediate the inhibitory effect of ephrin5 [135]. These examples show that the practise of segregating molecules into either "inhibitory" or "stimulating" may be to oversimplify the situation. Perhaps improved knowledge about this will increase our chances of designing successful therapies for CNS injuries.

Finally, when considering regeneration *in vivo*, it is important to remember that within both the CNS and the PNS there is a great heterogeneity concerning regenerative ability. There are several examples of CNS neurons that do regenerate given the right circumstances [136–138], and some PNS neurons seem to possess less of a regenerative capacity than others [139]. From this perspective, it seems unlikely that one ECM molecule will be able to support growth from all types of neurons and that increased knowledge on different subgroups of neurons and their preferred substrate for growth is highly desirable.

## Abbreviations

CNS: Central nervous system
DRG: Dorsal root ganglion
ECM: Extracellular matrix
FAK: Focal adhesion kinase

FN: Fibronectin
EHS: Engelbreth-Holm-Swarm sarcoma
ICAM: Intercellular adhesion molecule
ILK: Integrin linked kinase
LM: Laminin
MAG: Myelin-associated glycoprotein
NGF: Nerve growth factor
OPN: Osteopontin
PNS: Peripheral nervous system
Tn: Tenascins
TSP: Thrombospondin
VN: Vitronectin.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 125469, 10 pages
http://dx.doi.org/10.1155/2013/125469

*Review Article*

# MicroRNAs in Kidney Fibrosis and Diabetic Nephropathy: Roles on EMT and EndMT

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Received 14 May 2013; Revised 12 July 2013; Accepted 2 August 2013

Academic Editor: Achilleas D. Theocharis



MicroRNAs (miRNAs) are a family of small, noncoding RNAs that regulate gene expression in diverse biological and pathological processes, including cell proliferation, differentiation, apoptosis, and carcinogenesis. As a result, miRNAs emerged as major area of biomedical research with relevance to kidney fibrosis. Fibrosis is characterized by the excess deposition of extracellular matrix (ECM) components, which is the end result of an imbalance of metabolism of the ECM molecule. Recent evidence suggests that miRNAs participate in the fibrotic process in a number of organs including the heart, kidney, liver, and lung. Epithelial mesenchymal transition (EMT) and endothelial mesenchymal transition (EndMT) programs play vital roles in the development of fibrosis in the kidney. A growing number of the extracellular and intracellular molecules that control EMT and EndMT have been identified and could be exploited in developing therapeutics for fibrosis. This review highlights recent advances on the role of miRNAs in the kidney diseases; diabetic nephropathy especially focused on EMT and EndMT program responsible for the development of kidney fibrosis. These miRNAs can be utilized as a potential novel drug target for the studying of underlying mechanism and treatment of kidney fibrosis.

## 1. Introduction

MicroRNAs (miRNAs) are short noncoding RNAs that modulate fundamental cellular processes such as differentiation, proliferation, death, metabolism, and pathophysiology of many diseases by inhibiting target gene expression via inhibition of protein translation or by inducing mRNA degradation. By recent estimates, nearly 1000 human miRNAs target and downregulate at least 60% of human protein coding genes expressed in the genome [1]. The understandings of miRNAs in molecular mechanisms on various disease processes are now expanding day by day. In the current scenario, miRNAs play the role of conductors in the pathogenesis of fibrosis diseases. There are many literatures that organ-specific miRNAs alterations cause fibrotic disorders [2]. Fibrosis is the leading cause of organ dysfunction in diseases, either as outcome of an uncontrolled reaction to chronic tissue injury or as the primary disease itself in predisposed individuals [3]. Fibrosis of the kidney is caused by prolonged injury and dysregulation of normal wound healing process in association with an excess deposition of extracellular matrix. In such fibrotic process, kidney fibroblasts play important roles but the origin of fibroblasts remains elusive. In addition to the activation of residential fibroblasts, other important sources of fibroblasts have been proposed such as pericytes, fibrocytes, and fibroblasts originated from epithelial mesenchymal transition, endothelial mesenchymal transition. The two main loci for fibrosis in the kidney are the tubulointerstitial space and the glomerulus. Recent studies using transgenic mice have demonstrated that primary changes in glomeruli can lead to progressive glomerulosclerosis and renal failure [4]. For these reasons and knowing the multitude of pathways that miRNAs can affect, it is envisaged that investigating the roles of miRNAs in fibrosis could not only advance our understanding of the pathogenesis of this common condition but might also provide new targets for therapeutic intervention. In this review we focused on roles of miRNA biology in the kidney disease especially in epithelial mesenchymal transition (EMT) and endothelial mesenchymal transition (EndMT) programs.

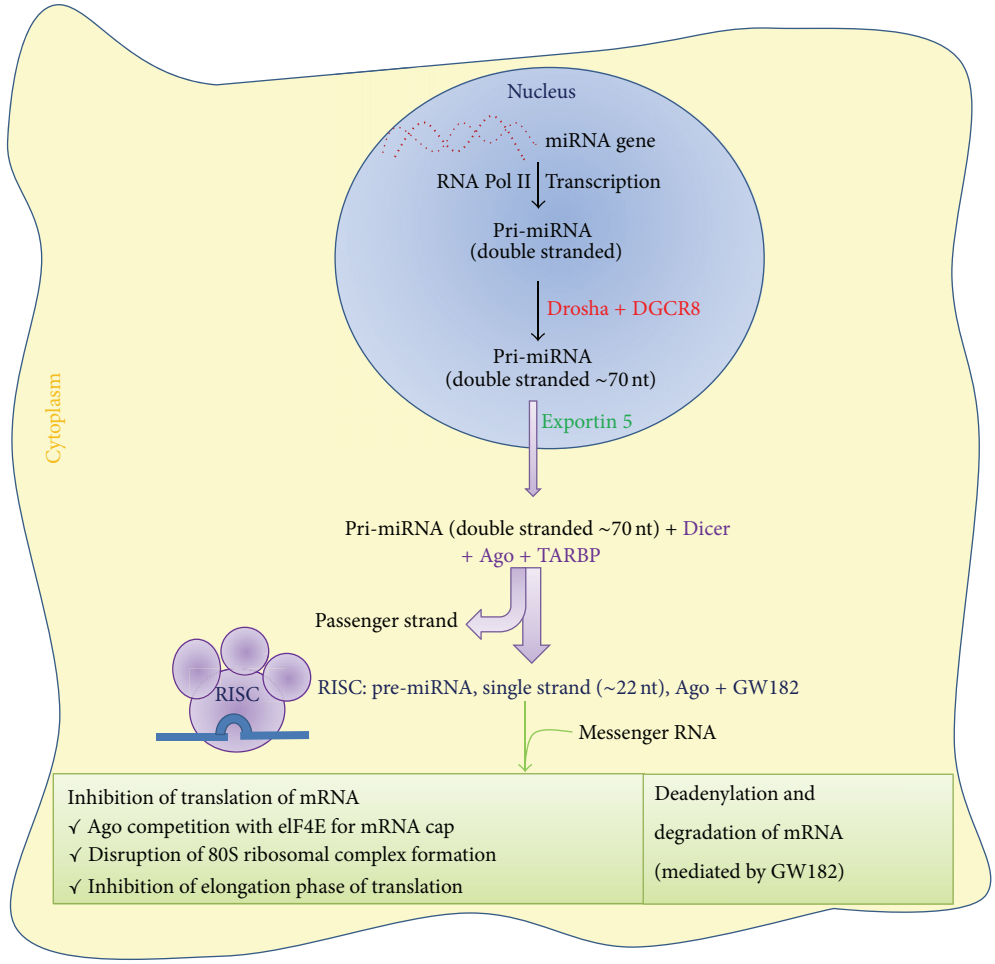


Figure 1: Schematic presentation of biogenesis and action of miRNAs. Ago: Argonaute; DGCR8: DiGeorge syndrome critical region 8; eIF4E: eukaryotic initiation factor 4E; GW182: glycine-tryptophan protein-182; nt: nucleotides; RISC: miRNA-induced silencing complex; TARBP: transactivation-responsive RNA-binding protein.

## 2. miRNA Gene and Transcription

miRNAs are single-stranded RNAs (ssRNAs) of ~22 nt in length that are generated from endogenous hairpin-shaped transcripts [5]. miRNAs function as guide molecules in posttranscriptional gene regulation by base-pairing with the target mRNAs, usually in the 3′ untranslated region (UTR). Binding of a miRNA to the target mRNA typically leads to translational repression and exonucleolytic mRNA decay, although highly complementary targets can be cleaved endonucleolytically. Over one-third of human genes are predicted to be directly targeted by miRNAs.

The 1st step in miRNAs biogenesis is nuclear processing by Drosha; the primary transcripts (pri-miRNAs) that are generated by Pol II are usually several kilobases long and contain local stem-loop structures (Figure 1). The first step of miRNA maturation is cleavage at the stem of the hairpin structure, which releases a small hairpin that is termed a pre-miRNA. This reaction takes place in the nucleus by the nuclear RNase III-type protein Drosha. Drosha requires a cofactor, the DiGeorge syndrome critical region gene 8 (DGCR8) protein in humans (Pasha in *D. melanogaster* and *C. elegans*) [6]. Together with DGCR8 (or Pasha), Drosha forms a large complex known as the microprocessor complex, which is ~500 kDa in *D. melanogaster* and ~650 kDa in humans [6]. Drosha and DGCR8 are conserved only in animals. The 2nd step in biogenesis is the nuclear export by the exportin 5. The trimmed precursor (pre-miRNA) hairpins from both canonical and noncanonical miRNA pathways are then transported by an exportin 5 (EXP 5, member of nuclear transport family). As with the other nuclear transport receptor, EXP 5 binds cooperatively to its cargo and the GTP-bound form of the cofactor Ran in the nucleus and releases the cargo following the hydrolysis of GTP in the cytoplasm. EXP 5 recognizes the >14 bp dsRNA stem along with a short 3′ overhang (1–8 nt) [7]. The 3rd step is cytoplasmic processing by the Dicer, pre-miRNA in the cytoplasm is typically further processed by the Dicer and transactivation-response RNA-binding protein (TRBP) RNase III enzyme complex to form the mature double-stranded ~22-nucleotide miRNA. Finally, the 4th step is argonaute loading, Argonaute proteins then unwind the miRNA duplex and facilitate incorporation of the miRNA-targeting strand (also known as the guide strand) into the AGO-containing RNA-induced silencing complex

(RISC). The RISC-miRNA assembly is then guided to specific target sequences in mRNAs. The initial recognition of mRNAs by the RISC-miRNA complex is driven primarily by Watson-Crick base-pairing of nucleotides 2 to 8 in the mature miRNA (seed sequence) with specific mRNA target sequences chiefly located in the $3'$ untranslated region, and additional base-pairing affords greater affinity and targeting efficiency [8].

## 3. Regulation of miRNAs Biogenesis

Precise control of miRNA levels is crucial to maintain normal cellular functions, and dysregulation of miRNA is often associated with human diseases, such as cancer [9].

*3.1. Regulation at Transcriptional Level.* Transcription is a major point of regulation in miRNA biogenesis. Numerous Pol II-associated transcription factors are involved in transcriptional control of miRNA genes. For instance, myogenic transcription factors, such as myogenin and myoblast determination 1 (MyoD1), bind upstream of miR-1 and miR-133 loci and induce the transcription of these miRNAs during myogenesis. Some miRNAs are under the control of tumour-suppressive or oncogenic transcription factors. The tumour suppressor p53 activates the miR-34 family of miRNAs [10], whereas the oncogenic protein MyC transactivates or represses a number of miRNAs that are involved in the cell cycle and apoptosis [11].

*3.2. Regulation at Posttranscriptional Level.* Drosha processing is also another important point of regulation. miR-21 is induced in response to bone morphogenetic protein (BMP)/transforming growth factor-$\beta$ (TGF-$\beta$) signaling without transcriptional activation [12]. It was proposed that SMAD proteins activated by BMP/TGF-$\beta$ interact with Drosha and DDX5 (also known as p68) to stimulate Drosha processing, although the detailed mechanism for this remains unclear. The let-7 miRNAs show interesting expression patterns. The primary transcript of let-7 (pri-let-7) is expressed in both undifferentiated and differentiated ES cells, whereas mature let-7 is detected only in differentiated cells, indicating that let-7 might be posttranscriptionally controlled [13]. Recent studies show that an RNA-binding protein, LIN28, is responsible for the suppression of let-7 biogenesis. Several different mechanisms of LIN28 action have been proposed: blockage of Drosha processing interference [14] with Dicer processing and terminal uridylation of pre-let-7 [14]. RNA editing is another possible way of regulating miRNA biogenesis. The alteration of adenines to inosines, a process that is mediated by adenine deaminases (ADARs), has been observed in miR-142 [15] and miR-151 [16]. Because the modified pri-miRNAs or pre-miRNAs become poor substrates of RNase III proteins, editing of the precursor can interfere with miRNA processing. Editing can also change the target specificity of the miRNA if it occurs in miRNA sequences [17].

*3.3. Feedback Circuits in miRNA Networks.* Two types of feedback circuits are frequently observed: single-negative feedback and double-negative feedback. The levels of Drosha and Dicer are controlled by single-negative feedback to maintain the homeostasis of miRNA production [18]. Drosha constitutes a regulatory circuit together with DGCR8; Drosha downregulates DGCR8 by cleaving *DGCR8* mRNA, whereas DGCR8 upregulates Drosha through protein stabilization. Double-negative feedback control is also often used as an effective genetic switch of specific miRNAs during differentiation. An interesting example is the conserved loop that involves let-7 and LIN28. miRNA let-7 suppresses LIN28 protein synthesis, whereas LIN28 blocks let-7 maturation. The miR-200 family and the transcriptional repressors Zeb1 and Zeb2 also constitute a double-negative feedback loop that functions in epithelial-mesenchymal transition program (EMT) [19].

## 4. EMT in Renal Fibrosis

EMT involves a series of changes through which epithelial cells lose their epithelial characteristics and acquire properties typical of mesenchymal cells. EMT facilitates cell movement and the generation of new tissue types during development and also contributes to the pathogenesis of disease. Earlier the role of EMT in renal fibrosis was discussed in the review [20]. Figure 2 displayed unique phenotypes of epithelial and mesenchymal cells. Epithelial cells are normally associated tightly with their neighbors, which inhibit their potential for movement and dissociation from the epithelial layer. Epithelia contour the cavities and surfaces of organs throughout the body and also form many glands. In contrast, mesenchymal cells do not form a regular layer of cells or specialized intercellular adhesion complexes. Mesenchymal cells are elongated in shape relative to epithelial cells and exhibit end-to-end polarity and focal adhesions, allowing for increased migratory capacity. Although mesenchymal cells may be polarized when migrating or interacting with neighboring cells, they lack the typical apical-basal polarity seen in epithelia. Moreover, mesenchymal cells migrate easily within tissues individually or collectively by forming a chain of migrating cells. Mesenchymal cells are essential for development as they can migrate large distances across the embryo to give rise to a particular organ. In the adult, the main function of fibroblasts, prototypical mesenchymal cells that exist in many tissues, is to maintain structural integrity by secreting extracellular matrix (ECM). Dr. Kalluri proposed classification of EMT into following three subtypes based on context [21]. Type 1 EMT involves the transition of primordial epithelial cells into motile mesenchymal cells and is associated with the generation of diverse cell types during embryonic development and organogenesis. These type 1 EMTs neither cause fibrosis nor induce invasion, and, in many cases, the mesenchymal cells that are generated later undergo MET to give rise to secondary epithelia. Type 2 EMT involves transition of secondary epithelial cells to tissue fibroblasts and is associated with wound healing, tissue regeneration, and organ fibrosis. In contrast to type 1, type 2 EMT is induced in response to inflammation but stops once inflammation is attenuated, especially during wound healing and tissue

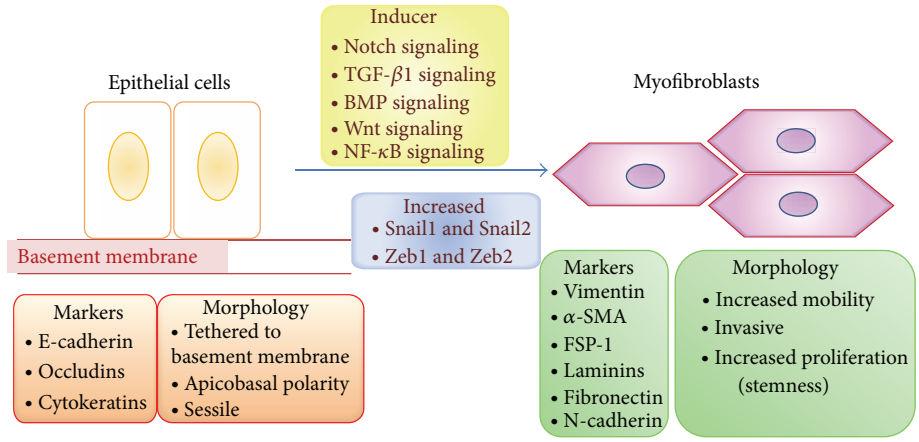


Figure 2: Biochemical changes during EMT in fibrosis. Repression of the transcription factors Snail1, Snail2, Zeb1, and Zeb2 is important for the maintenance of epithelial morphology. Several factors that are upregulated in the context of inflammation, including nuclear factor-κB (NF-κB), TGF-β1, bone morphogenetic proteins (BMPs), Wnt, and Notch signaling proteins, can activate the Snail-Zeb pathway, leading to mesenchymal differentiation in these cells. FSP-1: fibroblast-specific protein-1.

regeneration [22]. During organ fibrosis, type 2 EMT continues to respond to persistent inflammation, resulting in tissue destruction [22]. Type 3 EMT occurs in carcinoma cells that have formed solid tumors and is associated with their transition to metastatic tumor cells that have the potential to migrate through the bloodstream and, in some cases, form secondary tumors at other sites through mesenchymal epithelial transition (MET) [23]. Fibroblast-specific protein 1 (FSP-1; also known as S100A4 and MTS-1), an S100 class of cytoskeletal protein, α-SMA, and collagen I have provided reliable markers to characterize the mesenchymal products generated by the EMTs that occur during the development of fibrosis in various organs [21]. These markers, along with discoidin domain receptor tyrosine kinase 2 (DDR2), vimentin, and desmin, have been used to identify epithelial cells of the kidney, liver, lung, and intestine that are in the midst of undergoing an EMT associated with chronic inflammation. Such cells continued to exhibit epithelial-specific morphology and molecular markers, such as cytokeratin and E-cadherin, but showed concomitant expression of the FSP-1 mesenchymal marker and α-SMA. Such cells are likely to represent the intermediate stages of EMT, when epithelial markers continue to be expressed, but new mesenchymal markers have already been acquired. The behavior of these cells provided one of the first indications that epithelial cells under inflammatory stresses can advance to various extents through an EMT, creating the notion of "partial EMTs." Eventually, these cells leave the epithelial layer, negotiate their way through the underlying basement membrane, and accumulate in the interstitium of the tissue, where they ultimately shed all of their epithelial markers and gain a fully fibroblastic phenotype. Inflammatory injury to the mouse kidney can result in the recruitment of a diverse array of cells that can trigger an EMT through their release of growth factors, such as TGF-β, PDGF, EGF, and FGF-2 [21]. Most prominent among these cells are macrophages and activated resident fibroblasts that accumulate at the site of injury and release these growth factors. In addition, these cells release chemokines and MMPs, notably MMP-2, MMP-3, and MMP-9. The significance of TGF-β-induced EMT for progression of organ fibrosis has been demonstrated in studies using BMP-7, an antagonist of TGF-β signaling, in mouse models of kidney, liver, billiard tract, lung, and intestinal fibrosis [24]. BMP-7 functions as an endogenous inhibitor of TGF-β-induced EMT [24]. Among other effects, it reverses the TGF-β-induced loss of the key epithelial protein, E-cadherin. Restoration of E-cadherin levels by BMP-7 is mediated via its cognate receptors, activin like kinase-2/-3/-6 (ALK-2/-3/-6), and downstream transcription factors smads [24]. Systemic administration of recombinant BMP-7 to mice with severe fibrosis resulted in reversal of EMT and repair of damaged epithelial structures, with repopulation of healthy epithelial cells, all presumably mediated via an MET. This reversal was also associated with restoration of organ function, a substantial decrease in FSP-1+ and α-SMA+ interstitial fibroblasts, and the de novo activation of BMP-7 signaling [24]. However, these different EMT programs may be induced and regulated by a common set of stimuli, signal transduction pathways, transcription factors, and posttranslational regulations [22].

*4.1. miRNAs in EMT.* Genome-wide analysis for miRNAs has revealed that the miR200 family and miR205 are highly associated with EMT [25]. This change is reflected in a strong correlation between the expression of the miR200 family and E-cadherin across numerous cell lines and epithelial tissues [25, 26]. The miR200 family binds to the 3′ UTRs of RNA and suppresses the expression of Zeb1 and SIP1, which repress E-cadherin. The miR200 family is thereby capable of enforcing epithelial phenotypes. Additional EMT-related downstream targets of the miR200 family have been identified: miR141 inhibits TGF-β2 [26] and miR200a suppresses β-catenin (CTNNB1) [27]. miRNAs are also associated with the TGF-β signaling pathway. The expression of miR155 increases during TGF-β-induced EMT in mammary epithelial cells through smad4-mediated transcriptional upregulation and facilitates

loss of cell polarity and tight junctions [28, 29]. Moreover, epithelial cells expressing miR155 responded more rapidly to TGF-$\beta$. A key downstream target of miR155 is RhoA, which plays a role in the formation and stabilization of cell junctions. RhoA contains three conserved regions that may serve as binding sites for miR155 [28]. These data suggest that miR155 may provide further inhibitory effects on RhoA during EMT, in addition to TGF-$\beta$-mediated ubiquitination and degradation. The expression levels of miR29a and miR21 also are increased upon TGF-$\beta$-induced EMT in mammary epithelial cells [28], although their role in EMT has not been completely elucidated. Overexpression of miR29a suppresses the expression of tristetraprolin (known as zinc finger protein 36 homolog, ZFP36) and leads to EMT in cooperation with the Ras signaling pathway [29].

*4.2. Regulation of EMT.* It was recently shown that miR9 directly targets the mRNA encoding E-cadherin [30]. Ectopic expression of miR9 led to EMT in human mammary epithelial cells [31]. Moreover, a significant number of breast carcinoma cells located at the edge of miR9-expressing tumors expressed mesenchymal markers including vimentin, whereas few cells located in intratumoral regions were vimentin-positive, suggesting that miR9 may sensitize cells to EMT-inducing signals from the tumor microenvironment [30]. The EMT-inducing transcription factors have recently emerged as transcriptional regulators of miRNAs. miR21 is highly expressed in various tumors and known to induce metastasis through EMT. The promoter regions of miR21 include consensus E-box sequences that serve as binding sites for Zeb1 [31]. Binding of Zeb1 induces transcription of miR21 and also blocks bone morphogenetic protein- (BMP-)6-mediated inhibition of EMT in breast cancer cells [31].

## 5. EndMT in Renal Fibrosis

Vascular endothelial cells share several common traits with epithelial cells and can generate fibroblasts by undergoing a phenotypic transition similar to EMT, referred to as endothelial-mesenchymal transition (EndMT). EndMT is characterized by the loss of endothelial markers including CD31 and vascular endothelial cadherin (VE-cadherin) and the expression of mesenchymal proteins including $\alpha$-smooth muscle actin ($\alpha$SMA) [32]. EndMT contributes to cardiac fibrogenesis which results in progressive stiffening of the ventricular walls, loss of contractility, and abnormalities in cardiac conductance [32]. EndMT is also involved in pulmonary fibrosis, idiopathic hypertension [33], and corneal fibrosis [34]. Many growth factors and signaling pathways that govern EMT also regulate EndMT in the embryonic heart and during cardiac fibrosis. However, as compared to EMT, relatively little is known about EndMT. Earlier role of EndMT in renal fibrosis was discussed and reviewed by many researchers [35, 36]. In the adult organism, pathological conditions such as injury, inflammation, or aging can awaken EndMT and induce the fibrosis of the involved organs. The EndMT program has also been suggested to contribute to the development and progression of cardiac fibrosis, pulmonary fibrosis, hepatic fibrosis, corneal fibrosis, intestinal fibrosis, and wound healing in addition to renal fibrosis [37]. Zeisberg et al., 2008, designed and conducted a landmark experiment that first confirmed the contribution of EndMT in renal fibrosis in three mouse models, unilateral ureteral obstruction (UUO; a model used to study progressive tubulointerstitial fibrosis), streptozotocin- (STZ-) induced diabetic nephropathy, and $\alpha$3 chain of collagen type 4 (COL4A3) knockout mice (a mouse model for Alport syndrome). They found that a considerable proportion of myofibroblasts coexpress the endothelium marker CD31, also known as platelet endothelial cell adhesion molecule-1, and the (myo) fibroblast markers $\alpha$SMA and fibroblast-specific protein-1 (FSP-1, also known as S100A4) in all three models [38]. Furthermore, they analyzed the kidneys 6 months after a single injection of STZ in CD1 mice, which exhibited progressive glomerular sclerosis and tubulointerstitial fibrosis. A double-immunolabeling experiment demonstrated that approximately 40% of all FSP-1 (+) and 50% of $\alpha$SMA (+) stromal cells in STZ kidneys were also CD31-positive [38]. In the kidneys of 22-week-old COL4A3 knockout mice, 45% of all $\alpha$SMA-positive fibroblasts and 60% of all FSP-1-positive fibroblasts were CD31-positive, suggesting that these fibroblasts are likely of endothelial origin and that EndMT may contribute substantially to the accumulation of fibroblasts in the development and progression of renal fibrosis [38]. Li et al., 2009, also confirmed that EndMT occurs and contributes to the generation of myofibroblasts in early diabetic renal fibrosis. Using endothelial-lineage tracing with Tie2-cre, LoxP-enhanced green fluorescent protein (EGFP) transgenic mice, they identified a significant number of interstitial $\alpha$SMA-positive cells (myofibroblasts) of an endothelial origin in the fibrotic kidneys from mice with STZ-induced diabetic nephropathy [39]. ECs line the entire circulatory and lymphatic system, forming the inner lining of blood vessels and lymphatic vessels. These cells, which are anatomically similar to squamous epithelium, express apical-basal polarity and are tightly bound by adherens junctions and tight junctions [40]. These cells demonstrate a disparate set of biomarkers including VE-cadherin, CD31, TIE1, TIE2, von Willebrand factor (vWF), and cytokeratins. Similar to EMT, during EndMT, ECs lose their adhesion and apical-basal polarity to form highly invasive, migratory, spindle-shaped, elongated mesenchymal cells. Biochemical changes accompany these distinct changes in cell polarity and morphology, including the decreased expression of endothelial markers and the acquisition of mesenchymal markers (FSP-1, $\alpha$SMA, SM22$\alpha$, N-cadherin, fibronectin, vimentin, types I and III collagen, nestin, CD73, MMP-2, and MMP-9) [40] (Figure 3).

*5.1. miRNAs in EndMT.* Indirectly, miRNAs also upregulate many genes via suppression of their repressor molecules. The expression of both primary and mature miRNA-21 (miR-21), especially the latter, was upregulated by TGF-$\beta$ by silencing phosphatase and tensin homolog (PTEN) and activating the Akt pathway in EndMT *in vitro* (HUVECs) and *in vivo* (heart) [41]. smad3 signaling increases the expression of miR-21 in the kidney to promote renal fibrosis in response to TGF-$\beta$ whereas smad2 negatively regulates the posttranscriptional

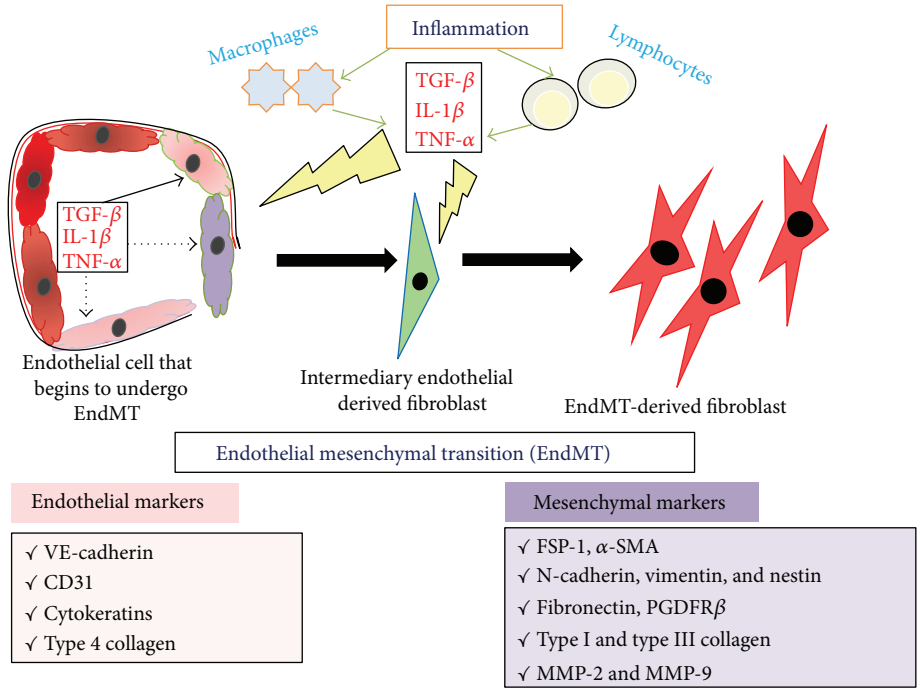


Figure 3: Biochemical changes during EndMT program. The EndMT program causes decreased expression of endothelial markers VE-cadherin, CD31, cytokeratins, and type 4 collagen and a gain of mesenchymal markers FSP-1, αSMA, N-cadherin, vimentin, fibronectin, type I and type III collagen, and MMP-2 and MMP-9. FSP-1: fibroblast-specific protein-1; α-SMA: α-smooth muscle actin; and MMP: matrix metalloproteinase.

modification of miR-21 [42]. miR-23 inhibits TGF-β-induced EndMT in mouse ECs, and miR-23 in the embryonic heart is required to restrict endocardial cushion formation by inhibiting hyaluronic acid synthase 2 (Has2) expression and extracellular hyaluronic acid production in Zebrafish dicer mutants [43]. Using the miRNA array analysis, Ghosh et al. [44] found that although miR-125b, let-7c, let-7g, miR-21, miR-30b, and miR-195 were significantly elevated during EndMT, the levels of miR-122a, miR-127, miR-196, and miR-375 were significantly downregulated. MiR-125b is approximately 4-fold higher in EndMT-derived fibroblast compared with MCECs [44]. The level of cellular p53, the major target of miR-125b and a known negative modulator of TGF-β-induced profibrotic signaling, was significantly suppressed with an elevated level of αSMA [44]. Blockade of FGF signaling induced EndMT program can be mimicked by the let-7b or let-7c miRNA inhibition. Although these studies were mostly performed in the heart, MCECs, or HUVECs, they still suggest that the specific suppression of upregulated miRNAs, such as miR-21, or the specific overexpression of downregulated miRNAs, such as miR-125b, may be a viable approach to blocking the induction of EndMT in a wide variety of organs.

## 6. miRNAs in Kidney Disease and Diabetic Nephropathy

Diabetic nephropathy is a progressive kidney disease and a major debilitating complication of both type 1 and type 2

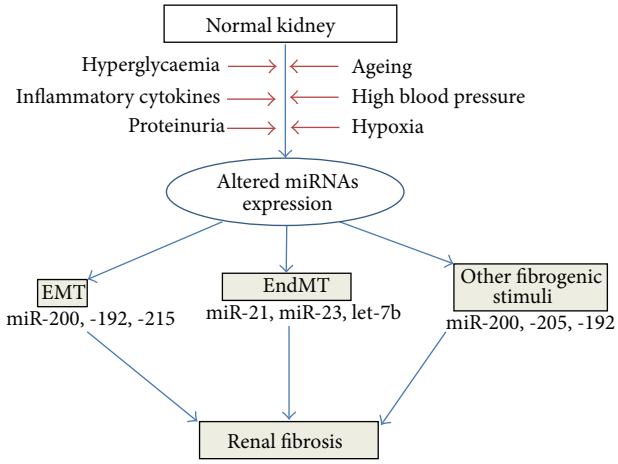


Figure 4: Implications of miRNAs in renal fibrosis.

diabetes that can lead to end-stage renal disease (ESRD) and related cardiovascular disorders. Absence or lower levels of particular miRNAs in the kidney compared with other organs may permit renal specific expression of target proteins that are important for kidney functions [45]. Figure 4 depicts the connection between the role of miRNAs and kidney fibrosis. Altered expression of miRNAs causes renal fibrosis by inducing EMT, EndMT, and other fibrogenic stimuli. The accumulative effects of hyperglycaemia, inflammatory cytokines, proteinuria, ageing, high blood pressure, and hypoxia result

into alteration of miRNAs expression profiles. The altered miRNAs level causes the initiation of such transition program in normal kidney, finally fibrosis. Some of the miRNAs that are more abundant in the kidney compared with other organs include miR-192, miR-194, miR-204, miR-215, and miR-216. A critical role of miRNA regulation in the progression of glomerular and tubular damage and the development of proteinuria been suggested by studies in mice with podocyte-specific deletion of Dicer [46]. There was a rapid progression of renal disease with initial development of albuminuria followed by pathological features of glomerulosclerosis and tubulointerstitial fibrosis. It is likely that these phenotypes are due to the global loss of miRNAs because of Dicer deletion, but, given multiple miRNAs and their myriad targets, the precise pathways responsible require identification. These investigators also identified specific miRNA changes, for example, the downregulation of the miR-30 family when Dicer was deleted. Of relevance, the miR-30 family was found to target connective tissue growth factor, a profibrotic molecule that is also downstream of transforming growth factor (TGF)-$\beta$ [47]. Thus, the targets of these miRNAs may regulate critical glomerular and podocyte functions. These findings have also been complemented by an elegant study revealing a developmental role for the miR-30 family during pronephric kidney development in *Xenopus* [48]. Sun et al. [49] identified five miRNAs (-192, -194, -204, -215, and -216) that were highly expressed in human and mouse kidney using miRNA microarray. A recent report using new proteomic approaches to profile and identify miRNA targets demonstrated that miRNAs repress their targets at both the mRNA and translational levels and that the effects are mostly relatively mild [50]. The role of miR-192 remains controversial and highlights the complex nature of miRNA research. Kato et al. [51] observed increased renal expression of miR-192 in streptozotocin-(STZ-) induced diabetes and in the db/db mouse and demonstrated that transforming growth factor (TGF-$\beta$1) upregulated miR-192 in mesangial cells (MCs). miR-192 repressed the translation of Zeb2, a transcriptional repressor that binds to the E-box in the collagen 1$\alpha$2 (col1$\alpha$2) gene. They proposed that miR-192 repressed Zeb2 and resulted in increased col1$\alpha$2 expression *in vitro* and contributed to increased collagen deposition *in vivo*. These data suggest a role for miR-192 in the development of the matrix accumulation observed in DN. It is interesting that the expression of miR-192 was increased by TGF-$\beta$ in mouse MCs (mesangial cells), whereas, conversely, the expression of its target, Zeb2, was decreased [51]. This also paralleled the increased Col1 $\alpha$2 and TGF-$\beta$ expression [51]. These results suggested that the increase in TGF-$\beta$ *in vivo* in diabetic glomeruli and *in vitro* in MCs can induce miR-192 expression, which can target and downregulate Zeb2 thereby to increase Col1 $\alpha$2. This is supported by the report showing that miR-192 is upregulated in human MCs treated with high glucose [51]. TGF-$\beta$ induced downregulation of Zeb2 (*via* miR-192) and Zeb1 (*via* potentially another miRNA) can cooperate to enhance Col1 $\alpha$2 expression *via* de-repression at E-box elements [51]. In contrast to the above, other reports suggest the relationship between miR-192 and renal fibrosis may be more complicated. Krupa et al. [52] identified two miRNAs in human renal biopsies, the expression of which differed by more than twofold between progressors and nonprogressors with respect to DN, the greatest change occurring in miR-192 which was significantly lower in patients with advanced DN, correlating with tubulointerstitial fibrosis and low glomerular filtration rate. They also reported, in contrast to the Kato et al. [51] study in MCs, that TGF-$\beta$1 decreased expression of miR-192 in cultured proximal tubular cells (PTCs). These investigators concluded that a decrease in miR-192 is associated with increased renal fibrosis *in vivo*. Interestingly, connective tissue growth factor (CTGF) treatment also resulted in fibrogenesis but caused the induction of miR-192/215 and, consequently, decreased Zeb2 and increased E-cadherin. The contrasting findings above highlight the complex nature of miRNA research. Some of the differences may relate to models and/or experimental conditions; however, one often overlooked explanation is that some effects of miRNAs and inhibitors are likely to be indirect in nature. A recent report also showed that BMP6-induced miR-192 decreases the expression of Zeb1 in breast cancer cells [53]. Thus, TGF-$\beta$ induced increase in the expression of key miRNAs (miR-192 and miR-200 family members) might coordinately downregulate E-box repressors Zeb1 and Zeb2 to increase Col1$\alpha$2 expression in MCs related to the pathogenesis of DN. The proximal promoter of the *Col1a2* gene responds to TGF-$\beta$ *via* smads and SP1. Conversely, the downregulation of Zeb1 and Zeb2 by TGF-$\beta$ *via* miR-200 family and miR-192 can affect upstream E-box regions. Because E-boxes are present in the upstream genomic regions of the miR-200 family, miR-200 family members may themselves be regulated by Zeb1 and Zeb2 [54]. It is possible that the miR-200 family upregulated by TGF-$\beta$ or in diabetic glomeruli under early stages of the disease can also regulate collagen expression related to diabetic kidney disease by targeting and downregulating E-box repressors. miR-192 might initiate signaling from TGF-$\beta$ to upregulate miR-200 family members, which subsequently could amplify the signaling by further regulating themselves through down regulation of E-box repressors. Such events could lead to progressive renal dysfunction under pathologic conditions such as diabetes, in which TGF-$\beta$ levels are enhanced. Conversely, there are several reports that miR-200 family members and miR-192 can be suppressed by TGF-$\beta$, and this promotes epithelial-to-mesenchymal transition (EMT) in cancer and other kidney-derived epithelial cell lines *via* subsequent upregulation of targets Zeb1 and Zeb2 to repress E-cadherin [54, 55].

## 7. Prospective

The discovery of miRNAs in 1993 in the nematode made the tremendous revolution in the field of RNA world. There are several major challenges in exploring the role of miRNAs in kidney diseases. Now miRNA-based therapeutics has already entered Phase 2 clinical trials. miR-122 antagonists are the indicator of hepatitis C virus and now in the Phase 2 clinical trials [56]. miR-208/499 antagonists are the indicator for chronic failure and now in the preclinical development. Likewise miR-195 antagonists are also in preclinical development, used for the indicator of postmyocardial infarction

remodeling. Some of the miRNAs (miR-34 and miR-7) are in preclinical development for the miRNA replacement therapy of cancer [57, 58]. This rapid progress from discovery to development reflects the importance of miRNAs as critical regulators in human disease and holds the promise of yielding a new class of therapeutics that could represent an attractive addition to the current drug pipeline of Big Pharma. Most importantly many fundamental questions remain regarding miRNA biology. The mechanism of regulation of miRNA is not completely clear. While many miRNAs are located within the intron of the host gene, their expression does not correlate perfectly with that of host genes suggesting further, posttranscriptional regulation [59]. Furthermore, the use of the miRNAs as therapeutic agents is attractive but faces considerable challenges, including development of safe and reliable organ and cell-specific delivery system, avoidance of toxicity derived from off-target effects and from activation of the innate and adaptive immune response. Current health statistics suggest that nearly 45% of all deaths in the western world can be attributed to some types of chronic fibroproliferative disease [60]. EMT and EndMT have become a key topic in the study of organ fibrosis, since stressed and injured epithelium can give rise to myofibroblasts and thereby contribute to fibrogenesis therapeutics for fibrosis. The participation of EMT and EndMT in the pathogenesis of various fibrotic disorders requires confirmation and validation from further studies of human clinical pathological conditions. Future efforts should also be devoted to further understanding of the molecular mechanisms and the regulatory controls involved in these processes including miRNA regulation. These efforts would eventually lead to the development of novel therapeutic approaches for these incurable and often devastating disorders by targeting miRNAs.

## Abbreviations

ADARs: Adenine deaminases
$\alpha$SMA: $\alpha$-Smooth muscle actin
COL4A3: $\alpha$3 chain of collagen type 4
BMP: Bone morphogenetic protein
CTGF: Connective tissue growth factor
DN: Diabetic nephropathy
DGCR8: DiGeorge syndrome critical region gene 8 protein
EndMT: Endothelial mesenchymal transition
EMT: Epithelial mesenchymal transition
ECM: Extracellular matrix
EXP 5: Exportin 5 (member of nuclear transport family)
EGFP: Enhanced green fluorescent protein (EGFP)
ESRD: End-stage renal disease
FSP-1: Fibroblast-specific protein-1
FGF: Fibroblast growth factor
microRNAs: miRNAs
MyoD1: myoblast determination 1
MET: Mesenchymal epithelial transition
MCs: Mesangial cells
MMP: Matrix metalloproteinase
PTEN: Phosphatase and tensin homolog
PTCs: Proximal tubular cells
RISC: RNA-induced silencing complex
TRBP: Transactivation-response RNA-binding protein
TGF-$\beta$: Transforming growth factor-$\beta$
UTRs: Untranslated regions
VE-cadherin: Vascular endothelial cadherin.

## Conflict of Interests

The authors declare there is no conflict of interests in this work.

## Acknowledgments

The authors' laboratory is supported by a Grant from the Japan Society for the Promotion of Science to Keizo Kanasaki (23790381), and Daisuke Koya (25670414, 25282028) and research grants from the Japan Research Foundation for Clinical Pharmacology to Keizo Kanasaki In addition, this work was partially supported by Grants for Promoted Research to Keizo Kanasaki (S2012-5, S2013-13) from Kanazawa Medical University. Keizo Kanasaki was currently also supported by several foundation grants from the following: the Novartis Foundation (Japan) for the Promotion of Science, the Takeda Science Foundation, and the Banyu Foundation. The authors declare that no financial conflict of interests exists. Swayam Prakash Srivastava is supported by the Japanese Government MEXT (Ministry of Education, Culture, Sports, Science, and Technology) Fellowship Program.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 929531, 12 pages
http://dx.doi.org/10.1155/2013/929531

*Review Article*

# The Roles of Hyaluronan/RHAMM/CD44 and Their Respective Interactions along the Insidious Pathways of Fibrosarcoma Progression

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Received 24 April 2013; Accepted 2 August 2013

Academic Editor: Achilleas D. Theocharis



Fibrosarcomas are rare malignant mesenchymal tumors originating from fibroblasts. Importantly, fibrosarcoma cells were shown to have a high content and turnover of extracellular matrix (ECM) components including hyaluronan (HA), proteoglycans, collagens, fibronectin, and laminin. ECMs are complicated structures that surround and support cells within tissues. During cancer progression, significant changes can be observed in the structural and mechanical properties of the ECM components. Importantly, hyaluronan deposition is usually higher in malignant tumors as compared to benign tissues, predicting tumor progression in some tumor types. Furthermore, activated stromal cells are able to produce tissue structure rich in hyaluronan in order to promote tumor growth. Key biological roles of HA result from its interactions with its specific CD44 and RHAMM (receptor for HA-mediated motility) cell-surface receptors. HA-receptor downstream signaling pathways regulate in turn cellular processes implicated in tumorigenesis. Growth factors, including PDGF-BB, TGF$\beta$2, and FGF-2, enhanced hyaluronan deposition to ECM and modulated HA-receptor expression in fibrosarcoma cells. Indeed, FGF-2 through upregulation of specific HAS isoforms and hyaluronan synthesis regulated secretion and net hyaluronan deposition to the fibrosarcoma pericellular matrix modulating these cells' migration capability. In this paper we discuss the involvement of hyaluronan/RHAMM/CD44 mediated signaling in the insidious pathways of fibrosarcoma progression.

## 1. Introduction

Cancer is a lethal disease characterized by uncontrolled cell growth, tumor formation, and loss of tissue organization. Primary tumors can be either caused by genetic alterations or by environmental factors. These alterations involve abnormalities in the regulation of basic cell functions, such as proliferation, differentiation, and apoptosis caused by genetic damage in oncogenes and tumor suppressor genes [1]. Within tumors, cancer cells often gain the ability to migrate, escaping from the normal mechanisms of control and thus invade surrounding tissues, leading to the formation of metastases via various tumor cell matrix interactions [2]. These interactions are considered continuous features of the metastatic cascade and play key roles in cell differentiation mechanisms.

Fibrosarcomas are rare malignant mesenchymal tumors originating from fibroblasts. The characteristic aspects of these tumors are the presence of immature proliferating fibroblasts or undifferentiated anaplastic spindle cells in a storiform pattern. Fibrosarcomas are usually localised in soft tissues, for example, muscles, connective tissues, blood vessels, and in lipid tissues [3]. Based on the presence and frequency of certain cellular and subcellular characteristics associated with malignant biological behaviour, sarcomas are also assigned a grade (low, intermediate, or high) [3]. Aetiology for sarcoma development has not been fully established; however, variations between ethnic groups in the incidence of rhabdomyosarcoma and fibrosarcoma, together with their occurrence in a number of heritable syndromes, suggest that genetic predisposition is important [4]. Comparative genomic hybridization further established the participation

Table 1: Common fibrosarcoma types.

| Childhood | Adult |
|---|---|
| 1,5% of childhood malignancies | 0,7% of adult malignancies |
| Spindle shaped malignant cells often interdigitating in a "herringbone" pattern | Spindle shaped malignant cells often interdigitating in a "herringbone" pattern |
| Less aggressive | More aggressive |
| Good prognosis | Poor prognosis |
| Genetic alterations may be involved | Genetic alterations may be involved |

of genetic factors in sarcoma tumorigenesis [5]. Congenital fibrosarcoma is a paediatric spindle cell tumor of the soft tissues that usually presents before the age of 2 years. These tumors have a relatively good prognosis and only rarely metastasize even though they display histological features of malignancy and frequently recur. Therefore it is imperative to differentiate congenital fibrosarcoma from more aggressive spindle cell sarcomas that occur during childhood, particularly adult-type fibrosarcoma which can have an identical morphology but poor prognosis (Table 1) [6, 7]. Classic pathology defined any sarcoma with fibroblasts a fibrosarcoma, and as a result the diagnosis "fibrosarcoma" represented two-thirds or more of all sarcomas diagnoses. Due to improved methodology in tissue study, such as immunohistochemistry (testing of specific proteins within tumors) and cytogenetics (analysis of chromosomes), during the last 20 years the diagnosis of fibrosarcoma has become much rarer [8]. However, in spite of these methodological advances due to lack of positive diagnostic markers, fibrosarcoma is in some cases a diagnosis of exclusion, that is, once the possibility of other soft tissue tumors has been ruled out [9]. Mesenchymal tissues can also develop fibromas, benign tumors that are formed of fibrous or connective tissue. It is important to note that there is presently no specific "targeted" therapy against fibrosarcoma due to lack of identification of molecular targets [10].

The extracellular matrices (ECMs) are complicated structures that surround and support cells within tissues. Their main components are proteoglycans, fibrillar proteins including collagens, elastins, fibronectins and laminins as well as glycosaminoglycans (GAGs) [11–13]. Matrix proteins determine a varying degree of matrices organization. On the other hand, both bound GAG chains and free GAGs such as hyaluronan (HA) bestow voluminosity to the ECM, due to negative charges they carry and to their subsequent water binding ability. Cells interact with the ECM components not only through specific receptors, such as the integrin family members, but also through syndecans, CD44, and RHAMM receptors [14–16]. The attachment of these receptors to the ECM induces specific signal transduction pathways that lead to a variety of functional responses directing cellular organization of both the cytoskeleton and chromatin structures [17]. As a result the ECM participates in the regulation of almost all cellular functions and is thus indispensable for morphogenesis, tissue homeostasis, and different pathological processes [18–21].

It is noteworthy that the ECM provides a physical scaffold to which tumor cells attach and migrate and thus is required for key cellular events such as cell motility, adhesion, proliferation, invasion, and metastasis. The alterations of ECM components, cell shape, and changes at the cell-ECM interface are considered as important hallmarks of cancer [22–27]. Abnormal ECM also indirectly affects cancer cells by influencing the behaviour of stromal cells, including endothelial cells, immune cells, and fibroblasts, which are the main initial culprits that cause abnormal ECM production [28, 29]. Moreover, altered expression of ECM molecules also deregulates the behavior of stromal cells and promotes tumor-associated angiogenesis and inflammation, leading to generation of a tumorigenic microenvironment [25]. ECM remodelling and turnover are considerably increased most often due to modulation in the expression of degrading enzymes [30, 31]. Modification of the ECM can also be capable of reactivating dormant tumor cells, for example, mediated by integrin-FAK signaling [32]. As a result, abnormal ECM further perpetuates the local niche and promotes the formation of a tumorigenic microenvironment [33] and subsequent tumor metastasis.

The above reports clearly show that the intrinsic structure of the tumor matrix has a key role on the insidious pathways of tumorigenesis. Importantly, fibrosarcoma cells were shown to have a high content and turnover of ECM components including hyaluronan, proteoglycans, collagens, fibronectin, and laminin [34–36]. In this paper we critically present and discuss how hyaluronan and its respective receptor for hyaluronan mediated motility (RHAMM) and CD44 receptors participate in the processes of fibrosarcoma tumorigenesis and dissemination.

## 2. Hyaluronan Function and Synthesis

Hyaluronan is a high molecular weight ($10^5$ to $10^7$ Da) GAG composed of alternating N-acetyl-glucosamine (GlcNAc) and glucuronic acid (GlcA) units [37]. It differs from the other members of the GAG family in that it neither contains sulfate groups nor is covalently attached to a core protein [38]. There are three different, but related, hyaluronan synthases (HAS), denominated HAS1, HAS2, and HAS3, that synthesize different hyaluronan sizes, with HAS1 and HAS2 producing high molecular weight HA (2000 kDa) [39]. The cleavage of hyaluronan on the other hand is performed by enzymes known as hyaluronidases (HYALs). The best studied mammalian HYALs are the HYAL1 and HYAL2. HYAL2 is located at the cell surface and cleaves the high molecular weight hyaluronan (HMWHA) into fragments of 20 kDa, whereas HYAL1 is intracellular and degrades the products of HYAL2 to small disaccharides [40].

Hyaluronan has remarkable physicochemical properties, such as the capacity to bind large amounts of water and form viscous gels, which are crucial in tissue homeostasis and biomechanical integrity. It also interacts with proteoglycans and other extracellular macromolecules forming

a template that is important in the assembly of extracellular and pericellular matrices [41]. These properties bestow to hyaluronan the ability to act like a filter, allowing only small molecules to penetrate [42]. The extraordinary characteristics of hyaluronan have paved the way for its frequent use in tissue engineering [43].

The roles of hyaluronan *in vivo* are extremely heterogenous including the regulation of tissue repair, such as the activation of inflammatory cells in order to induce immune response [44–46] as well as the specific responses of epithelial cells and fibroblasts to injury [47–50]. Hyaluronan has also been correlated to pathological processes as high levels of hyaluronan on the surface of different cancer cells have been suggested to be connected with the pathophysiological conditions of cancer [51].

Importantly, the numerous biological functions of hyaluronan are size dependent. HMWHA (1,000 kDa) is present in intact tissues and is antiangiogenic as well as immunosuppressive, whereas low molecular hyaluronan (LMWHA) has been speculated to act as an endogenous signal for T-cell activation and has the ability to induce the processes of inflammation and angiogenesis [44, 52–54].

Certain biological roles of hyaluronan result from its interactions with a large number of HA-binding proteins, called hyaladherins [55–57]. Thus, hyaluronan binds to its specific cell-surface receptors, including CD44, RHAMM, and ICAM-1, to induce the transduction of a wide range of intracellular signals [58], which in turn regulate various cellular processes including morphogenesis, wound healing, and inflammation as well as being implicated in pathological conditions [57, 59–61].

## 3. Hyaluronan Expression in Tumor Cells and Its Role in Cancer Progression: Focus on Fibrosarcoma Cells

Importantly, hyaluronan deposition is usually higher in malignant tumors as compared to benign tissues [51, 62, 63], and in some tumor types the level of hyaluronan can predict tumor progression [62]. Furthermore, activated stromal cells adjacent to the cancer cells are able to produce a tissue structure rich in hyaluronan in order to promote tumour growth as well as to secrete factors that enhance cancer cell migration into the new matrix [64, 65]. Indeed, a striking difference in hyaluronan stromal expression was reported between the benign dermatofibroma and the malignant dermatofibrosarcoma protuberans. Thus, whereas the dermatofibroma specimens show just a faint hyaluronan staining of the tumor stroma, the dermatofibrosarcoma protuberans specimens exhibit high HA deposition [66].

The specific roles of hyaluronan metabolism in cancer cell function remain to be elucidated. In physiological *in vivo* systems it has been determined that hyaluronan synthesis is directly linked to the level of HAS mRNA [67–69]. Indeed, it has been suggested that the expression of HAS enzymes is the first and perhaps the most important determinant of the hyaluronan synthesis rate in a given cell type under specific circumstances [70]. Accordingly, HAS mRNA levels are known to influence the content of hyaluronan in fibrosarcoma [34, 36] and other mesenchymal type tumors [71]. Importantly, the expression profile and the activity of the HAS enzymes can stimulate tumor progression as has been shown in clinical studies on ovarian and colon carcinomas [72, 73]. Emerging data, however, strongly correlate the action of HA-degrading HYALs with the increase in the permeability of connective tissues and with the decrease in the viscosity of body fluids characteristic of various disease processes including cancer [74, 75]. Moreover, elevated extracellular levels of partially catabolized hyaluronan oligomers are found in certain malignancies [76].

It is noteworthy that in different tumor types there is a distinct regulation model for the expression of respective HAS and HYAL isoforms and their activities. Thus, it is indicative that the overexpression of HAS2 human fibrosarcoma HT1080 cells promoted anchorage-independent growth and tumorigenicity of the cells [77]. Furthermore, increased HAS1 and -2 expressions promoted migration abilities of these cells [34]. HYAL also seems to induce the tumor resistance of L929 fibrosarcoma cells to tumor necrosis factor and Fas cytotoxicity, in the presence of actinomycin D [78]. Interestingly intralesional injection of HYAL in a case of dermatofibrosarcoma protuberans was correlated to decreased margin width, and a postoperative wound size less than was expected [79, 80]. Moreover, decreasing HYAL-2 expression significantly attenuated migratory activity of HT1080 cells [34]. This emerging complex pattern as regarding HYAL expression and activity is corroborated with data obtained from other cancer tissues [81]. Thus, aggressiveness of human cancers including breast cancer [82], laryngeal cancer [83], tumors of the male genitourinary tract, and prostate [84] and urinary bladder cancers is correlated to increased HYAL1 expression [85]. In contrast an overexpression of HYAL1 suppressed tumorigenicity in a model for colon carcinoma [86].

These seeming contradictions of hyaluronan and respective fragments actions could be explained by taking into account the myriad of different hyaluronan molecular sizes. Studies have shown that the mass of the actual amount of the hyaluronan polymer determine its physiological function. Whereas, HMWHA is an established marker of intact, healthy tissues, the fragmented forms, which are indicators of distress signals and occur abundantly in tumors. Importantly, these fragments have been suggested to promote angiogenesis, stimulate production of inflammatory cytokines, and activate signaling pathways that are critical for cancer progression. LMWHA fragments may be truncated products of the synthetic reaction, the result of hyaluronidase activities [80] or degradation products of chemical reactions triggered by reactive oxygen species (ROS) [87].

Growth factors have been demonstrated to regulate the production of hyaluronan through the modulation of hyaluronan metabolic enzymes expressions under both pathological and physiological conditions [88]. This is also the case in fibrosarcoma cells, as FGF-2 stimulates in a cell-specific manner the migration capability of fibrosarcoma cells by decreasing HYAL-2 expression in HT1080 cells and by increasing HAS1 and -2 expressions [34]. In B6FS

fibrosarcoma cells hyaluronan production was increased by TGFB2 and PDGF-BB actions [36].

Hyaluronan and derivatives can also support tumorigenesis by promoting tumor angiogenesis [89]. Firstly, hyaluronan accumulation in cancer tissues has been established to enhance the recruitment of monocytes and macrophages, which are important for angiogenesis [90, 91]. Secondly, hyaluronan seems to affect the binding ability of immunomodulatory cells. Thus, in inflamed colon tissues cell membranes were shown to form specific hyaluronan structures (cables) responsible for mediating leukocyte adhesion [92]. Thirdly, hyaluronan has been shown to maintain vascular integrity through endothelial glycocalyx modulation and caveolin-enriched microdomain regulation and interaction with endothelial hyaluronan binding proteins [93]. In vascular disease, also characterized by increased HYAL activity and ROS generation, HMWHA is broken down to LMWHA causing damage to the endothelial glycocalyx. Consecutively, LMWHA fragments can activate specific hyaluronan binding proteins to enhance actin cytoskeletal reorganization and inhibition of endothelial cell-cell contacts leading to decreased vascular integrity [94]. It is noteworthy that a decrease of vascular integrity is important both for tumor cell intravasation and tumor-associated angiogenesis.

Interestingly, experimental evidence showed that the progression and vascularization of carcinomas may be dependent on the hyaluronan production by epithelial or stromal cells. It appears that in the absence of the stromal cells, respective tumors progressed more slowly because of their fewer stroma and lymphatic vessels content [94, 95]. Indeed, the important role of stroma-derived hyaluronan on tumor vascularization was demonstrated when the implantation of HAS2 null fibroblasts with epithelial tumor cells into nude mice resulted in attenuated tumor angiogenesis and lymphangiogenesis with impaired macrophage activation [96].

Hyaluronidases and HAS may also act as tumour suppressors or oncogenes [85, 97, 98]. These data strongly suggest that the definition of quantity as well as the quality of hyaluronan chains in tumors is fundamental for the regulation of cancer cell processes during the different stages of the metastatic cascade.

## 4. Receptor for Hyaluronan Mediated Motility (RHAMM)

RHAMM receptor was originally isolated from subconfluent fibroblasts in culture [99] and subsequently cloned from mesenchymal cells [100]. Various RHAMM isoforms are produced due to alternative splicing, and these transcript variants are suggested to be expressed in a specific cell type manner [101]. This receptor is unique among the hyaladherins due to its variable distribution on the cell surface, within the cytoplasm, in the nucleus, or secreted to the ECM [102, 103]. Namely, RHAMM belongs to a heterogeneous group of proteins that lack signal peptides and are traditionally predicted to be cytoplasmic proteins, but they also have a cell surface presentation by being GPI-anchored to the cell membrane [100, 104, 105]. The cell surface display of these proteins modifies the roles of tumor suppressors and promoters, and tumor cells commonly use this adaptive mechanism [15]. On the other hand, intracellular RHAMM binds to actin filaments, podosomes, the centrosome, microtubules and the mitotic spindle [58, 102, 106], thereby affecting crucial cellular processes in tumorigenesis [106]. Indeed, Telmar et al. [107] have recently proposed that intracellular RHAMM can bind directly to ERK1 to form complexes with ERK2, MEK1, and ERK1,2 substrates and suggested a model whereby RHAMM's function is as a scaffold protein, controlling activation and targeting of ERK1,2 to specific substrates [107]. Therefore, the function of RHAMM appears to be strictly linked to its expression and cellular localisation.

Reports suggest that RHAMM expression is differentially regulated during the cell cycle and can be downregulated by the tumor suppressor p53 [108]. RHAMM protein expression during the cell cycle fits well into the picture proposed by several other studies that RHAMM binds to the mitotic spindle [109] and that, through interaction with HA, RHAMM affects microtubule spacing and stability [110]. These results underline the role of RHAMM as an important regulator of the cell cycle.

RHAMM appears to be a key mediator of fibroblastoid cell functions. It has been proposed in fibroblasts that RHAMM targets and anchors MEK1/ERK1/2 to tubulin and that these MAPKs phosphorylate the tubulin-associated proteins that regulate microtubule dynamics [111]. The dynamic nature of microtubules has been linked to functions associated with cancer progression, including cell cycle progression and motility/invasion. Therefore, these results raise the possibility that microtubules are an important oncogenic target of transforming RHAMM protein forms, such as RHAMMΔ163, and are relevant targets of investigation in fibrosarcoma tumorigenesis. The importance of RHAMM for fibroblast motility is illustrated by a study which shows that RHAMM(−/−) fibroblasts fail to resurface scratch wounds >3 mm or invade HA-supplemented collagen gels in culture [112]. Furthermore, RHAMM is shown to be necessary for the localization of CD44 to the cell surface, formation of CD44-ERK1,2 complexes, and activation/subcellular targeting of ERK1,2 to the cell nucleus [112]. It was likewise shown that restricting cell surface RHAMM to the extracellular compartment by linking recombinant protein to beads, combined with expression of mutant active mitogen-activated kinase 1 (Mek1), rescued aberrant signalling through CD44-ERK1,2 complexes in resurface scratch wounds of RHAMM(−/−) fibroblasts. ERK1,2 activation and fibroblast migration/differentiation are also defective during repair of Rh(−/−) excisional skin wounds and results in aberrant granulation tissue *in vivo*. Therefore, Tolg et al. [112] identify RHAMM as an essential regulator of CD44-ERK1,2 fibroblast motogenic signaling required for wound repair. Moreover, a separate study demonstrated that native hyaluronan activated NF-$\kappa$B and activated protein 1, in fibroblasts during wound repair. Use of CD44 siRNA suggests that this hyaluronan receptor is partly implicated in the effects, although it does not rule out the involvement of other receptors including RHAMM [113].

## 5. The Role of RHAMM in Fibrosarcoma Tumorigenesis

Cell surface RHAMM is not highly expressed in normal tissues but is usually overexpressed in many advanced cancers [58, 102, 114]. The potential oncogenetic role of RHAMM is supported by various studies demonstrating an overexpression of RHAMM during tumor development and a prognostic significance of its expression in breast, colon, brain, prostate, endometrial, and pancreatic cancers, as well as in leukemia, aggressive fibromatosis, multiple myeloma, and melanoma [115–117].

RHAMM/hyaluronan mediated signaling appears to be important in the process of fibrosarcoma tumorigenesis. Early studies have demonstrated that the overexpression of RHAMM in fibroblasts is transforming [100] and required for H-ras transformation [118], implying thus that RHAMM has a unique role in orchestrating events that are essential for transformation to occur. These events include the ability of RHAMM to alter focal adhesions in the cytoskeleton and elevate cell locomotion [118]. In an early report RHAMM/hyaluronan signaling was found to be obligatory for the stimulation of fibrosarcoma cell migration which is induced by transforming growth factor-beta 1 (TGF$\beta$1). Indeed, signaling is perpetrated through the formation of the RHAMM-HA complex because antibodies that inhibit RHAMM-HA binding simultaneously suppress TGF$\beta$1-induced increases in fibrosarcoma cell motility rate [119]. On the other hand, TGF$\beta$1 was found to stimulate multiple protein interactions at a unique cis-element in the $3'$-untranslated region of RHAMM mRNA to stimulate its expression [120]. It was demonstrated that the treatment of fibrosarcoma (HT1080) cells with various molecular weight hyaluronan preparations resulted in regulation of their migration capacity in a manner strictly dependent on HA size [34]. In continuation, when the effects of hyaluronan on fibrosarcoma cell adhesion and the respective mechanism of its action were examined, it was demonstrated that HA regulates fibrosarcoma cell adhesion through interaction with its RHAMM receptor and consecutive activation of FAK and ERK1/2 signaling pathways (Figure 1) [121]. This is well explained by aprevious study reporting that in ras transformed fibroblasts, but not in the original cells, hyaluronan regulates cell motility via RHAMM by signaling transient protein-tyrosine phosphorylation within focal adhesions [122]. In this signaling pathway, FAK is transiently phosphorylated, followed by net dephosphorylation and focal adhesion turnover, which initiates cell locomotion [122]. Indeed, cells overexpressing RHAMM resemble ras-transformed fibroblasts and have elevated cell locomotion and focal adhesion loss, as well as tumorigenic and metastatic potential leading to fully metastatic fibrosarcoma [123]. Furthermore, it appears that RHAMM targets focal adhesions downstream of ras or via a parallel pathway that converges at the level of ras because expression of a dominant suppressor mutant of RHAMM was shown to reverse the transformation induced by ras and to stabilise focal adhesions [123]. Further, Hall and Turley (1995) proposed that tyrosine kinase pp60c-src is associated with RHAMM in cells and is required for RHAMM mediated cell motility. The established correlation between ras signaling and RHAMM-dependent mechanisms could be a key point in fibrosarcoma development in view of the previously reported correlation between specific ras mutations and the fibrosarcoma phenotype [124]. Specifically, the incidences of K-ras mutations have been described at a variable frequency in this tumor type, and an association has been reported between specific sarcoma types and mutations in codon 13 [125–127] and in codon 12 [128, 129]. Interestingly, K-ras 13-derived tumors were shown to resemble malignant fibrous histiocytomas (MFH), whereas K-ras 12-derived tumors were shown to resemble fibrosarcomas [128]. A further distinction has been reported in that the K12 tumors show differences in the expression or activation of other Ras downstream pathways, JNK, MAPK, AKT, Bcl-2, FAK, and cyclin B1, which could be correlated to their functional differences [124]. These studies highlight the importance of the ras-signaling pathways in mesenchymal tumors development.

RHAMM-HA binding is implicated during the process by which soluble RHAMM arrests ras transformed fibroblasts at G2/M without affecting their progression through S-phase [130]. Because RHAMM can regulate expression and regulation of cell cycle mediators, the reports demonstrating correlation between cell cycle mediator expression and fibrosarcoma development deserve due attention. In H-ras transformed fibroblasts soluble RHAMM induces mitotic arrest by suppressing Cdc2 and cyclin B1 expression [131]. Importantly, Oda et al. [132] demonstrated that variations of cell cycle regulators in related myxofibrosarcoma have specific prognostic implications. A comparison of conventional clinicopathological and immunohistochemical features and the assessment of the immunohistochemical expression of MIB-1, cyclin E, p21 and p27 may be helpful to distinguish low-grade myxofibrosarcoma (MFS) from low-grade fibromyxoid sarcoma (LGFMS) which have different metastatic properties [132]. Microarray analysis identified a novel set of AP-1 target genes, including the tumor suppressor TSCL-1, and regulators of actin cytoskeletal dynamics, including the gelsolin-like actin capping protein CapG. The demonstration that AP-1 regulates the expression of genes involved in tumor cell motility and cytoskeletal dynamics in a clinically derived HT1080 human tumor cell line identifies new pathways of control for tumor cell motility [133]. Skp2 and cyclin-dependent kinase subunit 1 (Cks1) are involved in posttranscriptional degradation of p27 (Kip1) tumor suppressor. The prognostic utility of p27 (Kip1) and its interacting cell cycle regulators in myxofibrosarcomas were analyzed: Skp2 overexpression is highly representative of the biological aggressiveness of myxofibrosarcomas and plays an important prognostic role [134]. The participation of RHAMM in the development of fibromatosis, the aggressive mesenchymal tumor, has also been demonstrated [135]. Furthermore, it is reported that RHAMM regulates proliferation of cells with sparse cell-cell contacts, indicating RHAMM blockade as a potential therapeutic target for this otherwise difficult-to-treat neoplasm [135]. Likewise, the overexpression of RHAMM in osteoblastic MC3T3-E1 cells induces proliferation and suppresses differentiation through phosphorylation of ERK1/2. It is therefore suggested that the rupture of balance

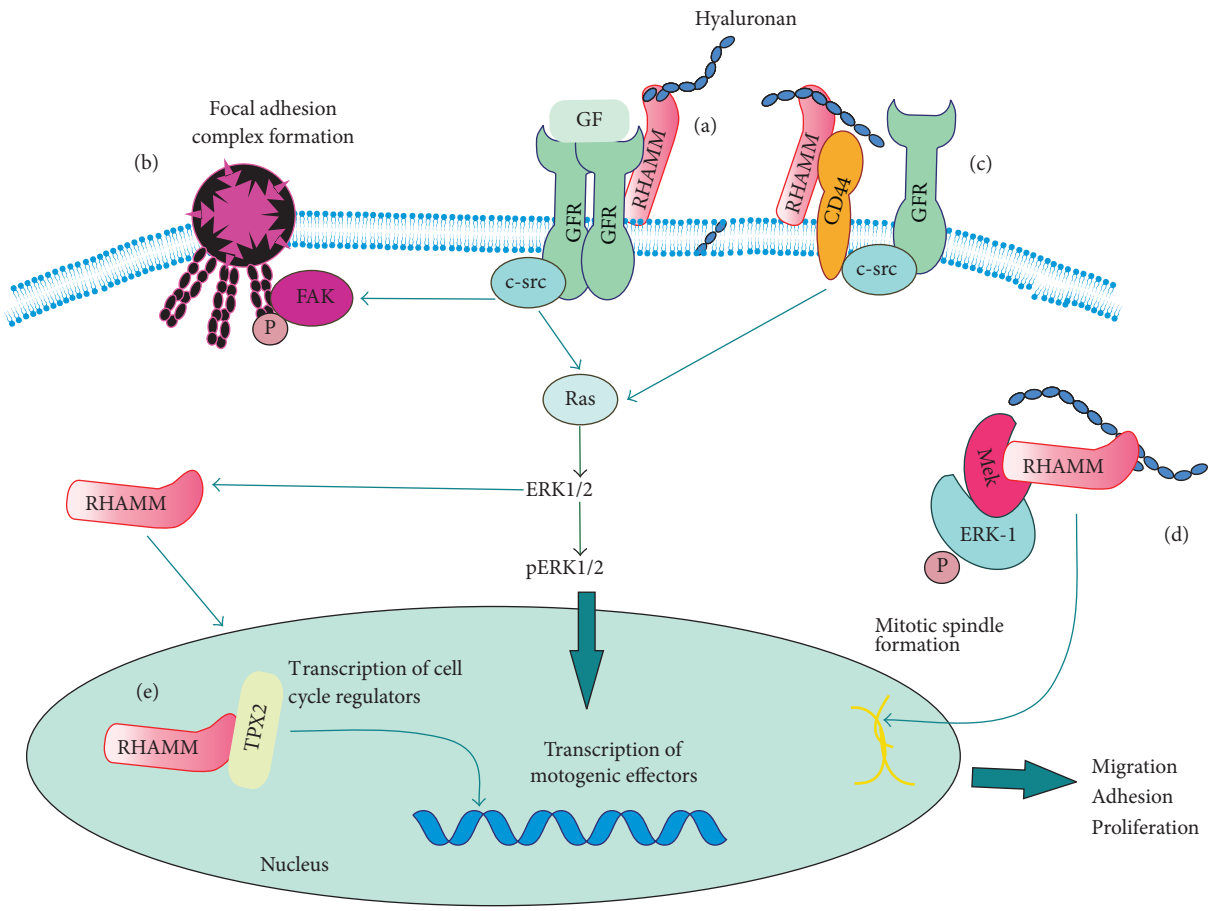


Figure 1: Hyaluronan/RHAMM/CD44-dependent signaling affects fibrosarcoma cell functions. (a) Interactions of cell membrane RHAMM with growth factor receptors (GFR) in a c-src/ERK1,2 dependent manner modulate transcription of motogenic effectors, and RHAMM/GFR interactions through c-src signaling induce FAK phosphorylation and focal adhesion complex formation. Interactions of cell membrane RHAMM with CD44 and GFRs in a c-src/ras/ERK1,2 dependent manner modulate transcription of motogenic effectors to regulate fibrosarcoma motility. (d) Cytoplasmatic RHAMM through RHAMM/MEK/ERK1,2 complex formation regulates mitotic spindle formation affecting cell growth. (e) Activated RHAMM positioned to nucleus forms complexes with transcription facors, for example, TPX2 to regulate expression of cell cycle mediators.

from differentiation to proliferation induced by RHAMM overexpression may be linked to the pathogenesis of bone neoplasms such as human cementifying fibroma [136].

Importantly, RHAMM has been indicated as a specific target in cancer. Thus, TCR-transgenic lymphocytes specific for RHAMM limit tumor outgrowth *in vivo* in various solid and leukemia tumor models [137]. It has been suggested that immunotherapies like peptide vaccination or adoptive transfer of RHAMM-specific T cells might improve the immune response and the outcome of acute myeloid leukemia patients [138]. Moreover, it has been shown *in vivo* that sulfated hyaluronan augmented tumor growth due to a blockade in complex formation between phosphoinositide 3-kinase (PI3K) and hyaluronan receptors and to a transcriptional downregulation of HA receptors, CD44, and RHAMM, along with PI3K inhibition [139]. In *in vitro* prostate cancer models the antitumor activity of hyaluronan synthesis inhibitor 4-methylumbelliferone was shown to be perpetrated through the downregulation of prostate cancer cells' proliferation, motility, and invasion [140].

Cell surface RHAMM can interact with the second specific hyaluronan receptor [141, 142], CD44, and modulate cell motility, wound healing, and signal transduction. More importantly, cell surface RHAMM can have invasive functions similar to CD44 and can even substitute for CD44 functions [143].

## 6. Role of CD44 in Cancer Progression: Focus on Fibrosarcoma

CD44 is a well-characterized transmembrane glycoprotein that has the ability to specifically bind to hyaluronan as well as to participate in the regulation of cell-cell contacts and cell-matrix interface [134, 135]. CD44 binds to hyaluronan through its extracellular domain, whereas its cytoplasmic tail acts as an intracellular signaling pathway activator that is involved in the association of signaling complexes with the actin cytoskeleton [62, 144–146]. The cytoplasmic tail of CD44 interacts with various molecules regulating different

signaling pathways [147, 148]. This receptor is encoded by a single gene but can exist in multiple isoforms that are generated both by alternative splicing of its 20 exons and through posttranslational modifications [149]. The altered splice variants expressed in cancer cells generally increase the ability of cancer cells to bind hyaluronan which ultimately results in an induction of tumorigenicity [62]. The most commonly expressed CD44 isoform is the standard CD44s, an 85 kDa protein that contains none of the variable exons. This CD44 isoform acts as a mediator of HA-promoted motility in breast cancer cell lines [58, 114, 150]. Alternative CD44 isoforms that can also bind hyaluronan and transduce its signaling are the so-called variable (v) isoforms [151]. The expression of discrete CD44 splice variants seems to be tumor specific. Thus, the dermatofibroma cells are negative for CD44v3, CD44v4, CD44v6, CD44v7, and CD44v7v8 but show a strong reactivity for CD44v5 and CD44v10. In contrast, CD44s' expression was significantly reduced or absent in all dermatofibrosarcoma protuberans lesions [66].

CD44 appears to be a mediator of fibrosarcoma development and metastatic dissemination. Importantly, CD44 was the only adhesion-related molecule consistently expressed among the early metastatic colonies derived from tumor clones of a murine fibrosarcoma [152]. Thus, hCD44s overexpression and possibly its ability to bind HA are critical for conveying metastatic competence but are antagonistic or selected against during aggressive primary tumor or overt metastasis outgrowth of fibrosarcoma cells [153]. Specifically, overexpression of human CD44s promotes lung colonization during micrometastasis of murine fibrosarcoma cells and facilitates their retention in the lung vasculature [154]. The described plasticity of CD44 gene expression in fibrosarcoma during metastasis could be relevant to discrete metastasis stages [155]. These studies suggest that CD44s may be a critical component of the fibrosarcoma metastatic phenotype induced by specific oncogenes [154].

Furthermore, CD44 expressed by HT1080 cells was established to be mainly activated which distinguishes its ability to bind hyaluronan and to mediate downstream signaling [156]. Upon binding to CD44 isoforms, HA initiates tumor cell activities including tumor cell adhesion, growth, survival, migration, invasion, and tumour progression through the activation of intracellular signaling pathways. Specifically, results have revealed that HA-CD44 interactions activate the c-Src kinase, which, in turn, induces twist phosphorylation, leading to the stimulation of miR-10b expression. This sequence of events evokes a reduction of a tumor suppressor protein (HOXD10), upregulates RhoA/RhoC, activates Rho-kinase (ROK), promoting breast tumor cell invasion [157].

Regulated uptake of hyaluronan via a CD44 receptor-mediated endocytosis pathway and subsequent degradation by HYAL2 may be important for tumor growth and progression either through the stimulation of angiogenesis or through degradation of HA around blood vessels promoting tumor metastasis [158–160]. Interestingly, CD44 can mediate the adhesion of platelets to hyaluronan secreted by fibrosarcoma cells [161].

Results have indicated that CD44 coimmunoprecipitates and colocalizes with cell surface RHAMM in invasive breast cancer cells, acting together in a HA-dependent autocrine mechanism to regulate signaling through ERK1,2, leading to an increase cell migration [162]. Moreover coexpression of CD44 and RHAMM is associated with poor prognosis in B-cell lymphomas implicating that the interaction of these two proteins may have a clinical significance. These two hyaluronan receptors connect and are involved in many common signaling pathways such as the ones that include VEGF, HGF, HA, Src, ERK1/2, and Fos that regulate cell migration. Other CD44/RHAMM networks that are associated with proliferation, growth, and cancer include MAFG, DYNLL1, MAFK, and FAM83D mediators, which are known to regulate the formation of cell mitotic spindle [163]. It has been proposed that RHAMM and CD44 receptors cooperate in order to induce the cell growth of cementifying fibroma cells. More specifically RHAMM interacts with ERK increasing the proliferative ability of these cancer cells through a mechanism that involves the interaction of CD44 with the epidermal growth factor receptor (EGFR) [164].

## 7. Conclusions

Hyaluronan/RHAMM/CD44 signaling can affect key cellular functions (Figure 1) and is strongly indicated in fibroblastoid cell malignant transformation and concomitant disease progression. Importantly, this signaling mediates fibrosarcoma cell behavior and regulates their specific cell-matrix interactions. Unraveling the complex characteristics of the hyaluronan/RHAMM/CD44 signaling axis in fibrosarcoma may reveal specific targets of pharmacological interventions.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 284873, 10 pages
http://dx.doi.org/10.1155/2013/284873

*Review Article*

# The Role of Changes in Extracellular Matrix of Cartilage in the Presence of Inflammation on the Pathology of Osteoarthritis

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Received 3 June 2013; Revised 27 July 2013; Accepted 29 July 2013

Academic Editor: Martin Götte



Osteoarthritis (OA) is a degenerative disease that affects various tissues surrounding joints such as articular cartilage, subchondral bone, synovial membrane, and ligaments. No therapy is currently available to completely prevent the initiation or progression of the disease partly due to poor understanding of the mechanisms of the disease pathology. Cartilage is the main tissue afflicted by OA, and chondrocytes, the sole cellular component in the tissue, actively participate in the degeneration process. Multiple factors affect the development and progression of OA including inflammation that is sustained during the progression of the disease and alteration in biomechanical conditions due to wear and tear or trauma in cartilage. During the progression of OA, extracellular matrix (ECM) of cartilage is actively remodeled by chondrocytes under inflammatory conditions. This alteration of ECM, in turn, changes the biomechanical environment of chondrocytes, which further drives the progression of the disease in the presence of inflammation. The changes in ECM composition and structure also prevent participation of mesenchymal stem cells in the repair process by inhibiting their chondrogenic differentiation. This review focuses on how inflammation-induced ECM remodeling disturbs cellular activities to prevent self-regeneration of cartilage in the pathology of OA.

## 1. Introduction

Osteoarthritis (OA) is a debilitating disease, which primarily affects joints, especially load-bearing areas such as hips and knees. It is characterized by pain and degenerative changes in the tissues surrounding those areas. There are no current therapies which can completely prevent the progression of the disease. Some of the main factors that drive the progression of OA are chronic inflammation and gradual structural changes within the joint tissues [1]. Unlike the general concept of OA being a degenerative disease, the remodeling processes are highly active throughout each stage of the disease [2]. During the active remodeling, however, the quality of extracellular matrix (ECM) is compromised due to the quick turnover rate and atypical composition of the newly synthesized ECMs [3]. Among many factors, inflammatory cytokines and proteases are main contributors which mediate the changes in the quality of ECM [2]. As a consequence of the microenvironmental changes, the altered ECM synthesis in the presence of inflammation, in turn, further disturbs the functions of the cells. Therefore, there is a constant cycle of evolution between the cells and their newly synthesized ECM, forming a positive feedback loop, which drives the progression of OA. In this review, we will focus on the interplay between ECM and cellular functions under inflammation, and how these factors are responsible for the progression of OA. An understanding of the complexity of the interplay between the cells and their microenvironment may provide a sound basis for developing suitable therapies to treat osteoarthritis.

## 2. Changes in Extracellular Matrix Synthesis during Osteoarthritis

Progression of OA can be characterized by changes in ECM composition and structure. Natural, healthy cartilage matrix is mainly composed of collagen type II which provides tensile support for the tissue. Aggrecan, a negatively charged

proteoglycan that attracts water molecules, provides the compressive resistant and shock absorbing capability of cartilage under loading [2]. It has been shown that during OA, there are sequential events that affect the integrity of homeostatic ECM; aggrecan content is decreased, while collagen content is increased [2, 3, 5]. This change in ECM composition predisposes the tissue for mechanical fault resulting in significantly altered mechanical environments of the cells within the cartilage matrix.

In the initial stages of OA, proliferative chondrocytes form clusters in order to adjust to the changing microenvironments [2]. This alteration of cellular configuration also changes the quantity and composition of the ECM secreted by the cells. It has been shown that there is a significant downregulation of aggrecan gene expression at the onset of OA in a rat model [1], and this finding agrees with markedly low proteoglycan synthesis, observed in human OA samples with normal appearance [6]. The changes of aggrecan, which exists in a nonaggregated form in OA, alter the permeability and thus mechanical compliance of the matrix [2, 7]. The reduced proteoglycan content decreases compressive modulus of cartilage and consequently exposes the tissue to greater strains when exposed to mechanical stress.

Unlike the decreased production of proteoglycan, collagen synthesis rate increases in the early stages of OA and remains elevated [8]. In addition to the increased ratio of collagen/aggrecan synthesis, the composition of collagen type has been also shown to change from collagen type II to type I [9]. Healthy cartilage matrix mainly contains collagen type II, while collagen type I is mainly found in subchondral bone tissue [2, 3, 10]. The compositional change affects the mechanical stability of the ECM network [10]. Compared to collagen type I, type II chains contain a higher content of hydroxylysine as well as glucosyl and galactosyl residues which mediate the interaction with proteoglycans [11]. Therefore, the decreased collagen type II content during OA inevitably undermines the integrity of ECM networks formed by collagen and proteoglycan. Furthermore, Silver et al. showed that the elastic modulus, due to shortened collagen fibril lengths, decreases with an increased extent of OA [12]. As a result of these changes, the osteoarthritic cartilaginous tissues exhibit a reduced ability to store elastic energy, and this, in turn, leads to fibrillation and fissure formation [12]. Figure 1 shows the structural and compositional changes in cartilage in a monoiodoacetate- (MIA-) induced arthritis model in rats. Although the animal model induces significantly accelerated cartilage degeneration as compared to typical human osteoarthritis, it depicts similar structural and compositional changes in cartilage exhibited in the pathogenesis of OA [13]. On day 11 post-MIA injection, the overall cartilage damage was assessed at Grade 2-3 according to Osteoarthritis Research Society International's (OARSI's) histopathology grading system showing cartilage lesion formation, articular surface fissurization, subchondral bone advancement, and bone marrow edema/cyst [14]. An area exhibiting chondrocyte disorientation without vertical fissure development was chosen to observe changes in cartilage matrix. In this area, nonchondrocytic collagen type I is present in the cartilage matrix of the OA tissue, whereas it is negligible in the control (Figure 1(B)). These changes in the structure and composition of ECM progressively alter the biological and mechanical microenvironments that significantly modulate cellular activities as described later in this review.

## 3. Inflammation-Induced Extracellular Matrix Changes in Osteoarthritis

ECM changes in cartilage can be attributed to multiple factors during the progression of OA. Among them, inflammation plays an active role affecting both quantity and quality of ECMs. Mechanical damage and/or age-related wear/tear are thought to trigger systematic inflammatory responses in all tissues surrounding the joint including articular cartilage, synovial membrane, subchondral bone, and ligaments [2, 15]. Chondrocytes, the only cell type residing in cartilage, respond to such inflammatory conditions and participate in the catabolic activities that ultimately lead to the degradation of cartilaginous ECM [16]. An animal model of MIA-induced arthritis showed that the sequential upregulation of inflammatory genes is associated with all levels of cartilage damage throughout the progression of OA [1]. These upregulated inflammatory genes form a positive feedback loop, mainly through the NF-$\kappa$B signaling pathway, as the severity of the cartilage damage progresses [17]. In fact, it was observed that chondrocytes in human arthritic cartilages also constitutively exhibit elevated activities of NF-$\kappa$B [18]. Factors that contribute to the catabolic processes in OA include interleukin 1$\beta$ (IL-1$\beta$), tissue necrosis factor-$\alpha$ (TNF-$\alpha$), IL-12, IL-15, and various associated chemokines [19–23]. These inflammatory factors were shown to significantly increase the expression of matrix degrading proteins including matrix metalloproteinases (MMPs) (i.e., MMP-1 and MMP-13) and various types of a disintegrin and metalloproteinase with a thrombospondin type 1 motif (ADAMTS) (i.e., ADAMTS 1,4,5) in chondrocytes [1, 24–30]. For example, an increase in cell clustering, a typical morphological feature of chondrocytes in the early stage of OA, was observed with an increase in MMP-13 expression [31]. The receptor for advanced glycation end products (RAGE), which is increased in OA articular chondrocytes, was also shown to stimulate MAP kinase and NF-$\kappa$B activities that, in turn, increased the production of MMP-13 and propagated the catabolism of the cartilage matrix [32, 33].

The degenerative activities of matrix degrading proteins are intensified by the elevated level of nitric oxide (NO), a molecule which is also upregulated by inflammatory proteins in chondrocytes. NO, upregulated by the transcriptional activity of NF-$\kappa$B, perpetuates the chronic inflammation that enhances matrix degradation and mediates apoptosis of chondrocytes by creating oxidative environments [34–36]. In a canine model of OA, the use of a NO inhibitor reduced the degenerative changes in cartilage, possibly demonstrating the critical role of NO in the progression of OA [34].

Concurrently with matrix degradation, the inflammation-mediated downregulation of chondrogenic growth/

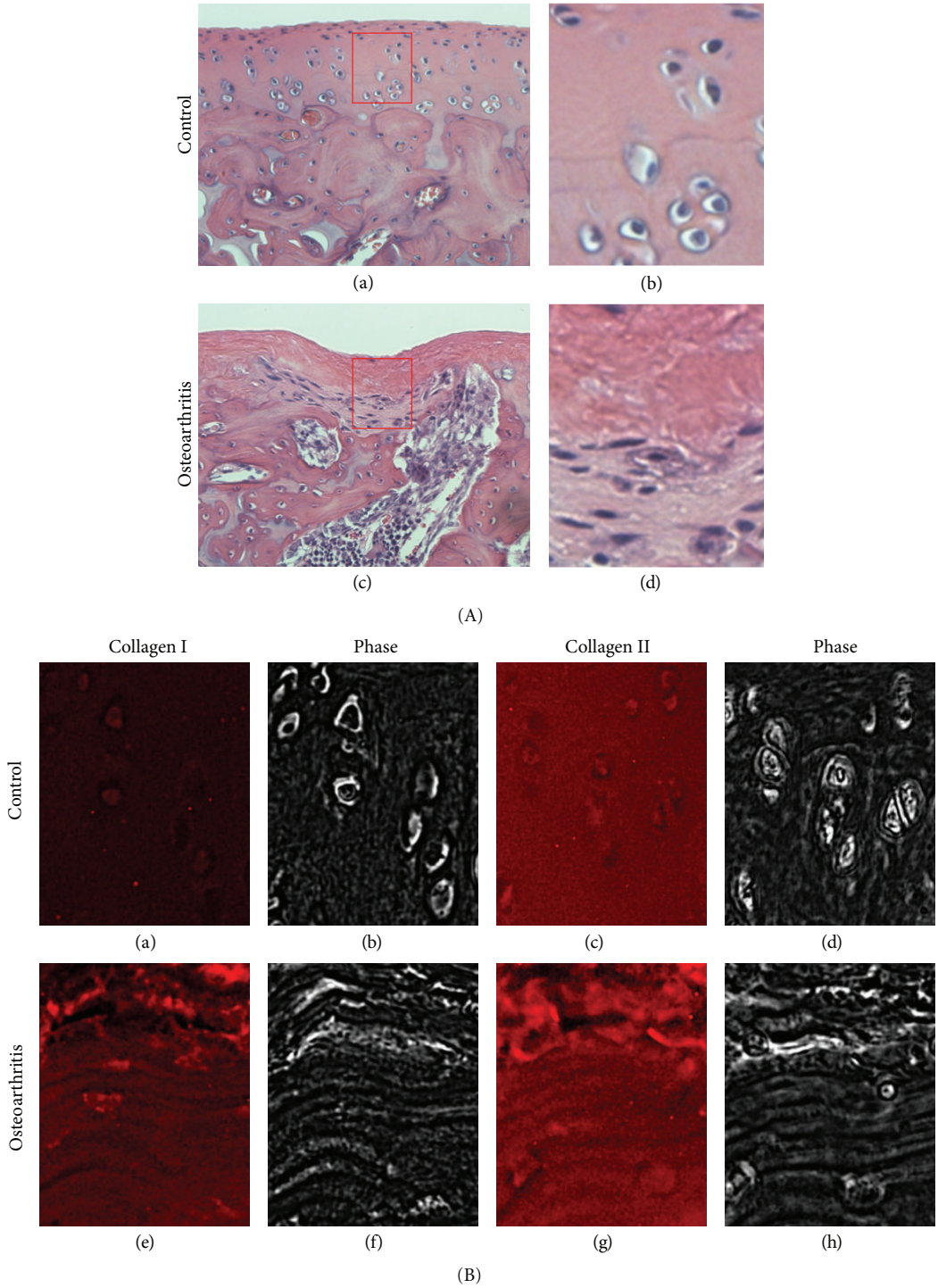


Figure 1: Changes in the extracellular matrix structure and composition of cartilage afflicted by osteoarthritis (OA). Experimental OA was induced by intra-articular injection of monoiodoacetate (MIA) similar to the previously described protocol using a rat model [4]. OA induced rats were sacrificed at day 11, and the medial condyles of the arthritic knees (A (c-d); B (e–h)) were histologically (H&E staining (A)) and immunohistologically (collagen type I (B (a) and (e)) and type II (Figure B (c) and (g)) compared to that of the saline-injected sham control ((A (a-b); B (a–d)). (A) Microscopic features of OA cartilage (grade 2-3) show cartilage lesion formation, articular surface fissurization, subchondral bone advancement, and bone marrow edema/cyst. In addition, cell clustering and fibrocartilage formation is apparent in OA samples. (A) (b and d) are magnified images of the area indicated in (A) (a and c), respectively, to reveal the changes in cellular morphology. (B) Consecutive sections of the healthy and OA cartilages were stained using monoclonal antibodies for collagen type I or type II. An increase in intensity for collagen type I is observed in the OA cartilage, while it is not present in the control cartilage. Collagen type II is readily observed for both the healthy and OA cartilage. (B) (b, d, f, and h) are phase-contrast images of (B) (a, c, e, and g), respectively, to reveal tissue morphologies.

transcription factors that mediate chondrocytic ECM synthesis, such as transforming growth factor $\beta$ (TGF-$\beta$), sex determining region Y-box 9 (SOX9), insulin-like growth factor (IGF), and connective tissue growth factor (CTGF), is also responsible for suppressing the anabolic activities of chondrocytes [1, 37, 38]. Taken together, these results demonstrate the significant influence of inflammatory mediators in the progression of OA by altering the homeostasis of cartilage ECM.

Another matrix component which is found in increased concentrations in synovial fluid during OA is Tenascin-C (TN-C), an ECM glycoprotein. Elevated levels of TN-C have been suggested to induce inflammatory mediators and promote ECM degradation in OA patients [39]. Although TN-C is highly expressed during embryogenesis, its presence is minimal in healthy adult tissues. Its expression during OA is, however, highly upregulated [40, 41]. The elevated concentration of TN-C causes a significant effect in the catabolism of the cartilage, resulting in degradation of ECM [39, 40]. Additionally, biglycan fragments in articular cartilage and meniscus and fibronectin fragments in hip and knee synovia have also been found in elevated levels as OA progresses [42–44]. Both fragmented biglycan and fibronectin exhibit proinflammatory effects through the activation of toll-like receptors [45, 46]. Overall, the combination of inflammation-induced upregulation of matrix-degrading proteins, downregulation of chondrocytic ECM synthesis, and accelerated matrix degradation due to fragmented inflammatory ECMs, promotes the progression of disease.

## 4. Alteration in Biomechanical Environments during Osteoarthritis

The changes in altered ECM synthesis and elevated activities of matrix degrading proteins drastically change the mechanical properties of cartilage, which further intensifies the destructive processes associated with OA [47]. Initially, an increase in cartilage thickness is observed by hyperproliferative chondrocytes before noticeable surface fibrillation occurs [48]. The highly proliferating chondrocytes produce greater amount of aggrecan that leads to cartilage thickening in dimensions as well as softening of extracellular matrix [2]. At this stage, a lower shear modulus was observed in the cartilage from an OA model when compared to normal articular cartilage [49, 50]. In a mouse model, a reduction in tensile stiffness in articular cartilage is also accompanied by the tentative cartilage thickening [51]. These biomechanical changes expose chondrocytes to an environment more susceptible to greater strains, as compared to physiological levels, thus altering their cellular functions.

As the disease progresses, however, the tissue gradually loses aggrecan content, which has provided compliance of local mechanical environments due to its ability to interact with water molecules. In addition to aggrecan loss, it has been recently shown that collagen fibril stiffens in osteoarthritic cartilage [52]. Furthermore, another possible mechanism through which the mechanical microenvironment changes is the accumulation of advanced glycation end products (AGEs) which can crosslink to the collagen network [53]. In vitro, the increased AGE crosslinking to the collagen network was shown to increase the stiffness of human adult articular cartilage [53]. The combination of aggrecan loss and collagen network stiffening results in increased overall stiffness of the tissue. Consequently, as OA advances, the cartilage layer becomes thinner and stiffer transmitting greater load to the underlying subchondral bones. The change in mechanical conditions induces the advancement of subchondral bones towards the articular surface leading to the development of bone marrow edema/subchondral bone cysts and the propagation of periarticular osteophytes [2, 54, 55]. Recent studies suggest that these changes in subchondral bone structure may precede the articular cartilage thinning [56].

Nevertheless, due to changes in the mechanical properties of the cartilage via altered homeostasis of ECM, its residing cells, chondrocytes, are exposed to vastly different biomechanical microenvironments that further intensify the progression of OA by altering cellular behaviors. Ultimately, this leads to the formation of fibrocartilaginous tissues that exhibit more bone-like properties replacing the completely degenerated cartilage in addition to osteophyte formation at the periphery of the articular surface [2, 54].

## 5. The Effects of Inflammation on Cartilage Extracellular Matrix Homeostasis by Articular Chondrocytes

Global inflammation in synovium during OA affects chondrocytes that are responsible for ECM turnover and thus cartilage homeostasis [57]. Inflammation which is persistent in OA has shown to directly induce the catabolic activities of chondrocytes. IL-1$\beta$, a highly upregulated cytokine during OA, has shown to induce upregulation of matrix degrading enzymes such as MMP-1, 3, and 13 in chondrocytes [58]. Dozin et al. also showed that when exposed to inflammatory cytokines, chondrocytes, regardless of patient age or OA status of human donors, enhance their production of proinflammatory cytokines such as IL-6 and IL-8 [59]. TNF-$\alpha$, another critical cytokine that is highly upregulated in OA, has been shown to induce MMP-13 expression, mediated by ERK, p38, JNK MAP kinases, and AP-1 and NF-$\kappa$B transcriptions factors [24, 60, 61]. At the same time, the presence of inflammatory cytokine IL-1$\beta$ has been shown to play a role in suppressed ECM synthesis through downregulation of SOX9 [62]. This, in turn, decreases the expression of collagen type II and aggrecan in articular chondrocytes. The activation/suppression of such signaling cascades autoregulates chondrocytes to further upregulate the synthesis of matrix degrading enzymes and downregulate the production of chondrocytic ECMs [63]. Nitric oxide (NO) and cyclooxygenase-2 (COX-2), two components which have active roles in perpetuating inflammation, were also endogenously expressed at high levels in chondrocytes from OA tissues even when cultured in vitro in the absence of inflammatory cytokines [64, 65]. These changes in metabolism may demonstrate a possible permanent phenotypical change in the OA chondrocytes.

In this regard, one notable alteration of chondrocytes in arthritic joints is their production of nonchondrocytic ECM. In addition to the increase in the production of collagen type I replacing type II as previously described, chondrocytes isolated from OA diseased tissues have shown to produce collagen type X, a marker for hypertrophic chondrocytes, as compared to undetectable expression of the protein in healthy cartilage [66]. Collagen type X is typically synthesized by hypertrophic chondrocytes that also produce collagen type I. The emergence of these nonarticular chondrocytic proteins may indicate the change of phenotype in chondrocytes as the disease progresses. The morphological change of chondrocytes with abnormal nonround morphology in arthritic cartilages could be related to a phenotypical change such as an increase in IL-1$\beta$ production and a decrease in pericellular collagen type VI synthesis [67]. When the cells from arthritic knees are subject to a chondrogenic in vitro culture condition, they are not able to fully recover normal tissue phenotype as evident by low cellularity and decreased chondrocytic ECM production as compared to chondrocytes from healthy joints [66, 67]. This demonstrates that damages in OA cartilage may not be able to be fully recovered by autologous chondrocytes.

One possible cause of the phenotype change of OA chondrocytes is inflammation as inflammatory synovial fluid has shown to activate chondrocytes and dramatically affect the normal processes of the cells. When healthy chondrocytes are subjected to inflammation, simulated by inflammatory cytokines such as IL-1$\beta$, TNF-$\alpha$, CXCL1, or 8, all of which are upregulated during OA, the cells exhibit hypertrophic differentiation [68]. This differentiation is shown to be mediated by RAGE signaling through the p38 MAPK pathway [69]. Interestingly, the activation of the p38 MAPK signaling pathway has also shown to promote the synthesis of MMP-13 possibly linking the change in phenotype to the facilitated rate of matrix turnover [32]. In addition to the synthesis of nonchondrocytic ECM and enhancement in matrix degradation, chronic inflammation also induces cell death. When healthy chondrocytes were subject to synovial fluids from osteoarthritic patients, the cells not only upregulated the expression of cytokines, such as IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1), and vascular endothelial growth factor (VEGF), but also underwent apoptosis [16].

## 6. The Effects of Changes in Extracellular Matrix on Articular Chondrocytes

The altered microenvironments by ECM changes, in the presence of inflammation, further drive catabolic/nonreparative activities of chondrocytes, ultimately leading to cartilage destruction/achondrocytic ECM formation. As previously described, the mechanical properties of cartilage are dynamically altered during the progression of OA due to imbalanced matrix turnover (greater matrix degradation versus synthesis) and noncartilaginous ECM formation. The increase in local matrix stiffness due to changes in ECM appears to suppress chondrocytic activities of the cells. Recent studies show that chondrocytes sense the stiffness of the matrix and differentially respond to it by altering their phenotype, resulting in production of different types of ECM (i.e., ratio of collagen type II to type I) [70–72]. An optimal stiffness has been shown to promote greater SOX9, COL2A1, and aggrecan gene expression in chondrocytes and either above or below this stiffness induced dedifferentiation of the cells towards fibrochondrocytic phenotype [70]. This effect of matrix stiffness on modulating chondrogenic phenotype has been shown to occur through the regulation of the TGF-$\beta$ signaling pathway [70]. In addition, the mechanosensitive behavior of chondrocytes may explain the fact that typical in vitro 2D culture of chondrocytes on stiff tissue culture plastics results in the dedifferentiation of the cells [73–75].

The changes in matrix composition during OA not only affect the mechanical environments of chondrocytes but also alter interactions of matrix proteins with the cells. Matrilin-3 (MATN3) is a matrix protein that is highly upregulated during OA [76, 77]. Although the protein is a part of healthy cartilage matrix, the soluble form of MATN3 is upregulated and released to synovial fluid in OA [78]. When human chondrocytes were cultured in the presence of soluble MATN3, there was a decrease in ECM anabolism and increased catabolism only at concentrations higher than those found in OA patients. On the other hand, when soluble MATN3 was immobilized, ECM synthesis and accumulation was enhanced [78]. These results show how MATN3, which is found in synovial fluid of OA patients, can change the behavior of chondrocytes, demonstrating the direct involvement of ECM in the progression of OA by interacting with the cells as well as indirectly by changing the cells' mechanical environments.

The presence of calcium crystals in cartilage has been shown to increase with severity of OA, and these changes have a strong correlation with hypertrophic chondrocyte differentiation [79]. Interestingly, bovine articular chondrocytes within cartilage explants, when exposed to basic calcium phosphate crystals, had significant increases in intracellular calcium content, which is correlated with cartilage matrix degradation [80]. Another ECM component that affects chondrocyte metabolism is fibronectin, which showed a significant positive correlation between chondrocyte apoptosis and fibronectin content [81]. Overall, these multifaceted effects by changes in ECM, including dysregulation of matrix synthesis (reduction in collagen type II and aggrecan, increase in collagen type I and X), upregulation of matrix degradation, and induction of cell apoptosis, promote the progression of OA by altering the cellular behaviors of chondrocytes.

## 7. The Effects of Inflammation on Chondrogenic Differentiation of Mesenchymal Stem Cells during Osteoarthritis

The mechanisms involving the initiation of OA are still elusive as some argue it is mechanical damage-induced and others inflammation-induced. Nevertheless, once the disease is initiated, the degeneration of cartilage matrix progresses due to the combination of chronic inflammation and altered

mechanical loading as discussed earlier. A part of the progressive degenerative processes is due to the limited regenerative capability of chondrocytes. These cells are typically quiescent in healthy cartilage [2]. When they are exposed to proliferating conditions to repair the cartilage damage, they often dedifferentiate to a phenotype that produces nonchondrocytic ECM [2]. This atypical ECM synthesis further drives chondrocyte dedifferentiation and nonhomeostatic ECM synthesis by altered mechanical environments. In addition to chondrocytes, the repair of the damaged tissue is attempted by another cell type, mesenchymal stem cell (MSC), that can differentiate to all mesenchymal lineage cells including chondrocyte, osteoblast, and adipocyte [82]. MSCs often participate in the repair of bone damage since they constitute bone marrow. Due to its close proximity to the cartilage layer in the subchondral marrow and their ability to differentiate into chondrocytes, MSCs have been considered as a possible cell source involved in cartilage repair.

For this reason, microfracture (or microperforation) surgery is often used to treat a localized cartilage lesion. Small fractures are created in the subchondral bone, and this causes new cartilage formation mainly due to the regenerative activities of MSCs from the bone marrow [83]. Although this technique has shown some benefits repairing damaged cartilage, the neotissue contains fibrocartilage that exhibits different mechanical properties, leading to question its long-term stability [84, 85]. These studies may provide clues for why endogenous MSCs cannot fully rescue damaged cartilage during the progression of osteoarthritis, unlike the positive healing response after bone fractures. Typically, subchondral bone advances towards the cartilage surface as the articular surface degrades [86]. In this condition, MSCs are subjected to a milieu of inflammation, altered ECM composition, and vastly different mechanical loading profiles in the injured cartilage, all of which affect the differentiation of MSCs to chondrocytes.

As described earlier, the native cartilage is exposed to chronic inflammation conditions by increased levels of inflammatory mediators including IL-1$\beta$, TNF-$\alpha$, and prostaglandin $E_2$ ($PGE_2$) [87, 88]. These inflammatory cytokines not only affect the homeostatic functions of residential chondrocytes but also impact the chondrogenic differentiation of MSCs [87, 89–91]. Treatment of IL-1$\beta$ during chondrogenic differentiation of bone marrow-derived MSCs suppresses Sox9 expression, a critical transcription factor that controls chondrogenesis [90]. The suppression of Sox9 subsequently leads to a decrease in collagen type II and aggrecan expression. In addition, TNF-$\alpha$, in combination with IL-1$\beta$, has been shown to transform embryonic chondroprogenitor cells into fibroblast-like cells, further suggesting the inhibitory effects of inflammatory cytokines on chondrogenesis [87]. Similarly, when human MSCs are exposed to conditioned medium derived from osteoarthritic synovium, chondrogenesis is inhibited [92]. These antichondrogenic effects of inflammatory cytokines were shown to be caused by the activation of the NF-$\kappa$B signaling pathway [93]. Overall, inflammatory conditions present in OA cartilage prevent chondrocytic differentiation of MSCs, thus inhibiting regeneration of damaged cartilage with appropriate chondrocytic ECMs.

## 8. The Effects of Changes in Extracellular Matrix on Chondrocytic Differentiation of Mesenchymal Stem Cell

The changes in the composition of ECM also affect chondrogenic differentiation of MSCs. In a study by Bosnakovski et al., MSCs cultured in collagen type II hydrogels exhibited greater gene expression levels of chondrocytic markers as compared to those cultured on typical tissue culture plates [94]. As OA progresses, residential chondrocytes start to produce collagen type I instead of type II. This change can affect the subsequent chondrogenesis of MSCs as it has been shown that collagen type II favors chondrogenic induction by modulating cell shape, as compared to collagen type I [95]. It was demonstrated that collagen type II promotes a more rounded cell shape, similar to that of the native chondrocyte in healthy cartilage, through the $\beta$1 integrin-mediated Rho A/Rock signaling pathway.

In addition to the compositional effect, mechanical changes of ECMs (become stiffer due to the loss of hydrating aggrecan in OA) affect chondrogenesis of MSCs by regulating cell morphology [96]. A softer mechanical environment enhances chondrogenesis of MSCs, evident by greater gene and protein expression of chondrogenic markers including SOX9, collagen type II, and aggrecan by inhibiting stress fiber formation, as compared to the stiffer environment. Similarly, using polyacrylamide hydrogels with varying stiffnesses, Xue et al. showed that human mesenchymal stem cells are differentiated towards a chondrocytic phenotype on softer gels, regardless of initial cell seeding density [97]. The study highlights the importance of cell-matrix interactions during chondrogenic differentiation of MSCs.

Along with the direct influence of local stiffness change on MSC differentiation, the altered mechanical profiles under loading also affect the differentiation process. Bone marrow derived MSCs seeded onto fibrin hydrogels developed a spread out morphology and differentiated towards a myogenic lineage [98]. In the presence of long-term, dynamic compression, myogenic differentiation was inhibited, while markers for chondrogenic phenotype were upregulated. However, the magnitude of loading is an important factor determining chondrocytic differentiation of MSCs and thus synthesis of proper ECMs. Under the same loading regimen, a stiffer ECM induces less strain on the cells. In this regard, Michalopoulos et al. have recently shown that physiological compressive loading (15% strain) on MSC-laden scaffolds induces greater chondrogenesis as compared to a smaller strain of 10% that led to greater osteogenesis [99]. Similarly, stiffer agarose gels inhibited cartilage matrix production and gene expression of MSCs under hydrostatic pressure as compared to those in softer microenvironments [100]. These studies demonstrate that changes in the mechanical properties of cartilage during OA may favor the differentiation of MSCs towards nonchondrocytic lineages further intensifying the degeneration of cartilage. Overall, altered environments

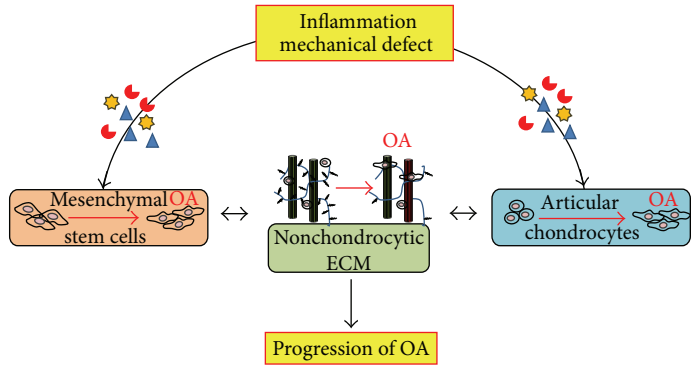


Figure 2: Schematic of the interplay between the extracellular matrix and cellular activities under inflammation during the progression of osteoarthritis (OA). Wear and tear or trauma induces inflammation and mechanical defects in cartilage, which initiate OA. These altered microenvironments affect the residential chondrocytes to produce nonchondrocytic extracellular matrix (ECM) that, in turn, further drives the dedifferentiation of the chondrocytes. The changes in microenvironments also negatively affect the chondrogenic differentiation of mesenchymal stem cells that originate from subchondral bone marrow, preventing the self-regeneration of cartilage. The positive feedback loop between mal-formed ECM and cellular activities drives the progression of OA.

in ECM composition and mechanical properties during the progression of OA significantly limit the chondrogenesis of MSCs inhibiting the regeneration process of cartilage damage.

## 9. Summary

Both inflammatory factors and compositional/structural changes of ECM drive the progression of OA by affecting residential articular chondrocytes as well as MSCs that migrate from bone marrow in the underlying subchondral bone to repair the cartilage defect (Figure 2). Due to chronic inflammation and altered microenvironments, chondrocytes change their phenotype towards more hypertrophic cells resulting in achondrocytic ECM synthesis. These changes in ECM, in combination with cartilage matrix degradation under inflammation, further fuel the degeneration process resulting in the alteration of biomechanical conditions, which disturb the surrounding tissues in the joint. The ECM changes in the presence of inflammation also negatively affect chondrogenic differentiation of MSCs, limiting self-regeneration of cartilage. Overall, the interplay between changes in ECM and changes in cellular function under inflammation forms a positive feedback loop that drives the pathology of OA.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 546318, 8 pages
http://dx.doi.org/10.1155/2013/546318

*Review Article*

# The Role of Epidermal Growth Factor Receptor in Cancer Metastasis and Microenvironment

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Received 3 May 2013; Accepted 25 June 2013

Academic Editor: Davide Vigetti



Despite significant improvements in diagnosis, surgical techniques, and advancements in general patient care, the majority of deaths from cancer are caused by the metastases. There is an urgent need for an improved understanding of the cellular and molecular factors that promote cancer metastasis. The process of cancer metastasis depends on multiple interactions between cancer cells and host cells. Studies investigating the TGF$\alpha$-EGFR signaling pathways that promote the growth and spread of cancer cells. Moreover, the signaling activates not only tumor cells, but also tumor-associated endothelial cells. TGF$\alpha$-EGFR signaling in colon cancer cells creates a microenvironment that is conducive for metastasis, providing a rationale for efforts to inhibit EGFR signaling in TGF$\alpha$-positive cancers. In this review, we describe the recent advances in our understanding of the molecular basis of cancer metastasis.

## 1. Introduction

Epidermal growth factor receptor (EGFR) is a key factor in epithelial malignancies, and its activity enhances tumor growth, invasion, and metastasis [1]. EGFR is a member of the ErbB family of tyrosine kinase receptors that transmit a growth-inducing signal to cells that have been stimulated by an EGFR ligand (e.g., TGF$\alpha$ and EGF) [2, 3]. In normal tissues, the availability of EGFR ligands is tightly regulated to ensure that the kinetics of cell proliferation precisely match the tissues' requirements for homeostasis. In cancer, however, EGFR is often perpetually stimulated because of the sustained production of EGFR ligands in the tumor microenvironment [4, 5] or as a result of a mutation in EGFR itself that locks the receptor in a state of continual activation [6]. Aberrant expression of TGF$\alpha$ or EGFR by tumors typically confers a more aggressive phenotype and is thus often predictive of poor prognosis [7–10]. Not surprisingly, EGFR has emerged as a principal target for therapeutic intervention.

## 2. EGF-Like Ligands and EGFR

Receptor tyrosine kinases (RTKs) are primary mediators of many of these signals and thus determine the fate of the cell: growth, differentiation, migration, or death. The ErbB family of RTKs consists of four receptors: ErbB-1 (EGFR), ErbB-2 (HER2 or Neu), ErbB-3, and ErbB-4 [11, 12]. The mature EGF receptor is composed of a single polypeptide chain of 1186 amino acid residues and a substantial amount of N-liked oligosaccharide. A single hydrophobic membrane anchor sequence separates an extracellular ligand-binding domain from a cytoplasmic domain that encodes an EGF-regulated tyrosine kinase [13–15]. The hallmark of the cytoplasmic protein of this receptor is the sequence defining the tyrosine kinase domain.

Ligand binding induces receptor hemo- or heterodimerization that is essential for activation of the tyrosine kinase. Six mammalian ligands that bind to EGFR have been characterized, including epidermal growth factor (EGF), transforming growth factor-$\alpha$ (TGF$\alpha$), amphiregulin,

heparin-binding EGF-like growth factor, betacellulin, and epiregulin [16, 17]. Tyrosine kinase activity following ligand binding is essential and is the first step in the EGF signal transduction pathway [18], once the ligand binds the receptor and further stimulates multiple signal pathways including Ras/mitogen-activated protein kinase, phosphatidylinositol 3-kinase/Akt, nuclear factor-$\kappa$B, and others [19–22].

## 3. Colorectal Cancer and TGF$\alpha$/EGFR Signaling

Studies investigating the signaling pathways that promote the growth and spread of cancer cells suggest that the information transmitted by means of TGF$\alpha$-EGFR signaling is particularly important for progression of tumors that develop in the colon [23–26].

Overexpression of the EGFR and its ligands, TGF$\alpha$, has been correlated with poor prognosis [27–29]. Colon cancer cells secrete TGF$\alpha$ in response to hypoxia and the ligand signals, the cell surface EGFR, to initiate a sequence of cell survival programs [30]. This activation of the EGFR signaling pathways stimulates downstream signaling cascades involved in cell proliferation (Ras/mitogen-activated protein kinase [MAPK]) and antiapoptosis (phosphatidylinositol 3-kinase [PI3K]/Akt) [20, 31, 32]. In addition, the overexpression of TGF$\alpha$ and EGFR by many carcinomas correlates with the development of cancer metastasis, resistance to chemotherapy and poor prognosis [27, 32, 33].

## 4. Metastatic Colorectal Cancer

The expression levels of TGF$\alpha$, EGF, and EGFR have been shown to correlate with progressive tumor growth, development of metastasis, and resistance to chemotherapy [27, 32, 34]. Measurements of EGFR expressed on human colon cancer cells *in vitro* indicate that metastatic cells may express as much as five-times more EGFR in comparison to non-metastatic cells [35]. Reports examining the distribution of EGFR and TGF$\alpha$ on colorectal biopsies also conclude that the receptor-ligand pair is a characteristic feature of more advanced tumors [27, 36–38].

## 5. Microenvironment of Colon Cancer for Metastasis

The concern of the microenvironment of tumors has been growing. The process of cancer metastasis is sequential and selective and contains stochastic elements. The growth of metastases represents the endpoint of many lethal events that few tumor cells can survive. Angiogenesis refers to the development of new blood vessels from the preexisting vasculature. Angiogenesis plays a key role in the initiation of metastases. Tumor cell proliferation and survival depend on the vasculature to supply adequate oxygen and nutrients [39]. The extent of angiogenesis depends on the balance between proangiogenic and antiangiogenic factors released by tumor cells and host cells [40, 41]. The communication networks that are established between tumor cells and the nonneoplastic cells in the microenvironment of primary tumors play a critical role in tumor growth and development of metastasis [42, 43].

Data derived from examinations of human lung cancer brain metastases indicate that tumor cell division takes place within 75 $\mu$m of the nearest blood vessel, whereas tumor cells residing beyond 150 $\mu$m from a vessel undergo programmed cell death [44]. The turnover rate of endothelial cells within the tumor-associated vessels is 20 to 2,000 times faster than the rates of the vessels in normal organs [45]. One recent detailed study of the multiple clinical specimen of human neoplasms reported that proliferation rate of endothelial cells within the vasculature of normal human organs has been reported to be <0.01%, whereas 2% to 9% of endothelial cells in tumor-associated vessels divide daily [46].

Expression of EGF, VEGF, or their respective receptors has been shown to correlate with angiogenesis and progressive growth of human carcinomas of the colon [47]. Furthermore, the expression of EGFR, VEGFR, and the phosphorylated receptors was observed on tumor-associated endothelial cells. These receptor and phosphorylated receptor were expressed on tumor-associated endothelial cells only when the tumor cells expressed the relevant ligands. These findings suggest that ligands released by tumor cells can upregulate the expression of receptors on tumor-associated endothelial cells in a paracrine manner [48–50] (Figure 1).

The angiogenic proteins, VEGFA and IL-8, were strongly expressed in the microenvironment of tumors that produced TGF$\alpha$. In contrast, expression levels of VEGFA and IL-8 were considered unremarkable in TGF$\alpha$-deficient tumors. VEGFA is often regarded as the prototypical angiogenic protein in that it can stimulate each of the cellular responses required for the generation of a new vascular bed (e.g., migration, proliferation, protease production, and cell survival) [51, 52]. There are also several lines of evidence suggesting that some cells rely on TGF$\alpha$-induced stimulation of EGFR to enhance their production of IL-8. These data demonstrate that the extensive EGFR network (autocrine and paracrine) generated by TGF$\alpha$-expressing colon cancer cells leads to a greater production of proangiogenic proteins (TGF$\alpha$, VEGFA, and IL-8) in the microenvironment of primary tumors (Figure 2).

Several other factors that promote angiogenesis and tumor cell invasion were also preferentially expressed in the microenvironment of TGF$\alpha$-positive tumors. Specifically, we noted robust expression of two members of the MMP family, MMP-2 and MMP-9, in tumors that were positive for TGF$\alpha$ [50] (Figure 2). These proteolytic enzymes perform several key functions during angiogenesis (e.g., increase the bioavailability of angiogenic proteins, degrade basement membrane barriers, and promote endothelial cell migration) and metastasis (e.g., invasion and extravasation) [53].

Macrophages are also capable of creating structural and biochemical imbalances in the extracellular matrix. A closer inspection of the tumor-infiltrating macrophages in TGF$\alpha$-positive tumors showed that these cells express the lymphangiogenic growth factor VEGFC. The few macrophages

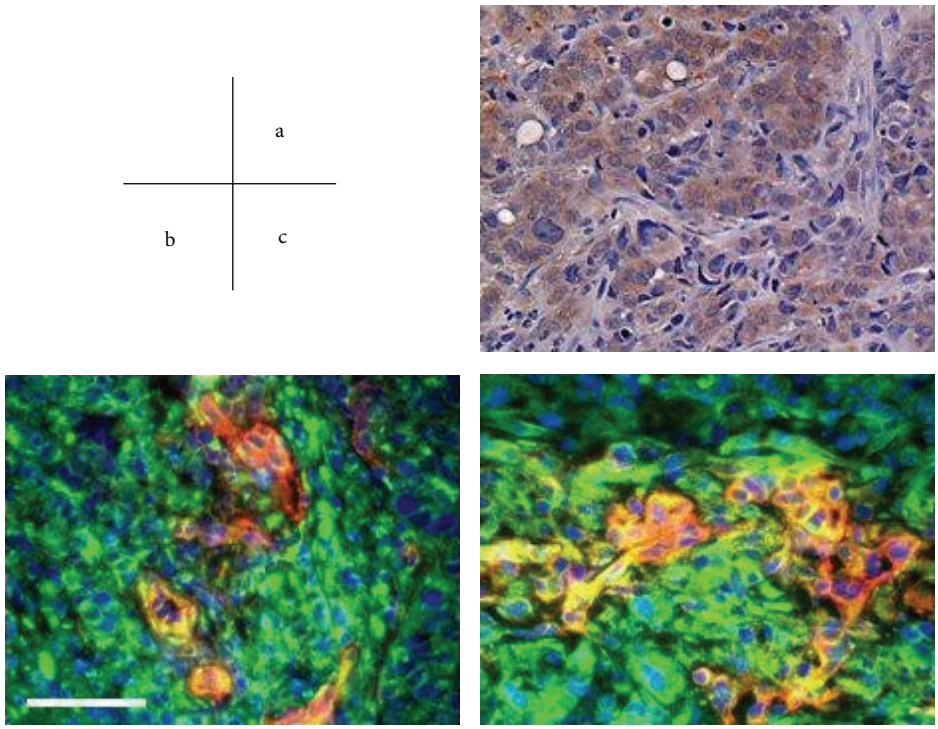


Figure 1: Immunohistochemical analyses of expression of TGF$\alpha$, EGFR, and phosphorylated EGFR on tumor cells and tumor-associated endothelial cells in orthotopically implanted colon tumors. (a) TGF$\alpha$ expression in the tumor cells. (b) EGFR was present on tumor cells (green) and was also detected on the tumor-associated vasculature (yellow). (c) Expression of phosphorylated EGFR was localized to both tumor cells (green) and the supporting vascular network (yellow). Scale bars = 100 $\mu$m [50].

present in the TGF$\alpha$-negative tumors in our study did not express VEGFC, but they did so when tumor cells were transfected with TGF$\alpha$ transgenes and then implanted into the cecal walls of mice [50] (Figure 3). These results add to the growing evidence that suggests that macrophages are a major source of VEGFC in pathological tissues and, therefore, function as central regulators of the lymphatic vascular surface area [54, 55].

The number of tumor-associated lymphatic vessels in the different tumors was determined by counting the number of vessels that were positive for LYVE-1. LYVE-1 is an integral membrane protein that functions as the receptor for the glycosaminoglycan hyaluronan. LYVE-1 is also expressed by sinusoidal endothelial cells in the liver and spleen and by some macrophages [32]. We found that the number of lymphatic vessels in EGFR-expressing tumors was fourfold higher than that observed in EGFR-deficient tumors, demonstrating that TGF$\alpha$-EGFR signaling is an important cofactor for expansion of the tumor-associated lymphatic vascular network [56] (Figure 4).

Supportive evidence for the involvement of TGF$\alpha$ in metastasis comes from a recent study that identified TGF$\alpha$ as a member of the gene set that that identifies colorectal cancer cells that metastasize to the liver [57]. Alternatively, it has been known for some time now that a high vascular density increases the likelihood that tumor cells will enter the systemic circulation and reach distal organs of metastasis [58], and we found that the activation of autocrine and paracrine TGF$\alpha$/EGFR signaling networks affects the tumor microenvironment in colon cancer and determines its impact on the formation of metastases.

## 6. Microenvironment of Biliary Tract Cancers for Metastasis

Biliary tract cancers express EGFR in 60.7% of cases [59]. The EGFR-overexpressing gallbladder cancer (GBC) cases show poorly differentiated histology and decreased survival of 1.5 years in median survival [60]. Amplification and point mutations of the EGFR gene have been reported to be 1% and 15%–26.5%, respectively, in GBC [61–63]. The HGF receptor c-Met is involved in the early carcinogenesis of biliary tract cancers [64]. c-Met is expressed in 74% of invasive GBC and is associated with invasive depth [65]. Because HGF is secreted from fibroblasts, c-Met activation depends on the cancer-host interaction [66]. Transforming growth factor-b is widely expressed in advanced GBC and is associated with angiogenesis and tumor-associated macrophage infiltration as well as with stromal fibrosis [67, 68]. Epidermal growth factor receptor, c-Met, and TGF-b have recently been implicated in the process of epithelial-mesenchymal transition (EMT) [69–71]. EMT comprises a switch in cell differentiation from polarized epithelial cells to contractile and motile mesenchymal cells [72]. In EMT-type cells, the reduction of the epithelial marker E-cadherin (ECD) occurs in parallel with the induction of the mesenchymal marker

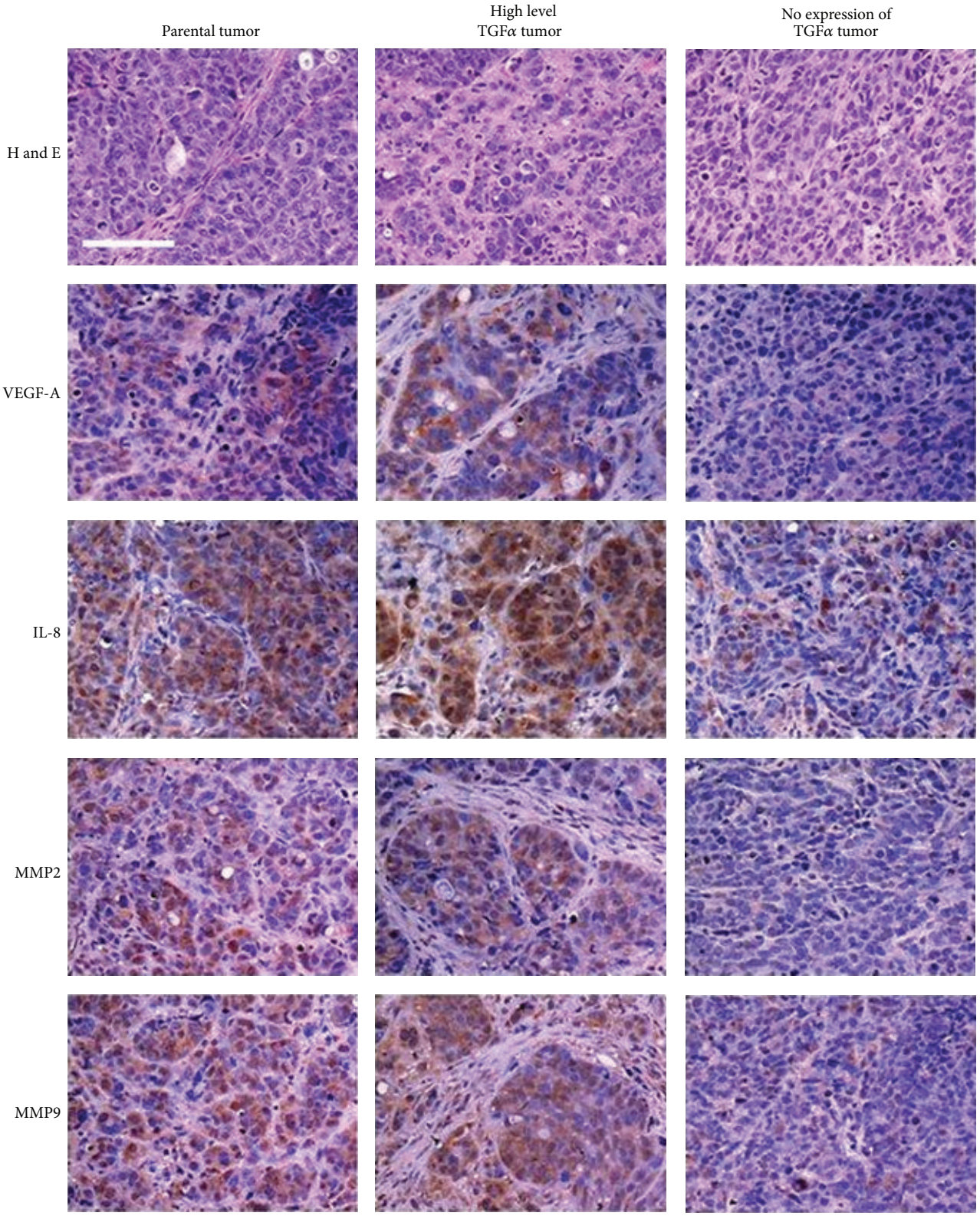


Figure 2: Immunohistochemical analyses of expression of VEGFA, IL-8, MMP-2, and MMP-9 in orthotopically implanted colon tumors. The parental colon cancer cell line originates from a primary human colon carcinoma. The clones were expanded, and the resulting populations were screened for production of TGF$\alpha$. The microenvironment of selected high level TGF$\alpha$ tumors is enriched in VEGFA, IL-8, MMP-2, and MMP-9. Expression of the angiogenic proteins in tumors that do not express TGF$\alpha$ is significantly attenuated. Scale bars = 100 $\mu$m [50].

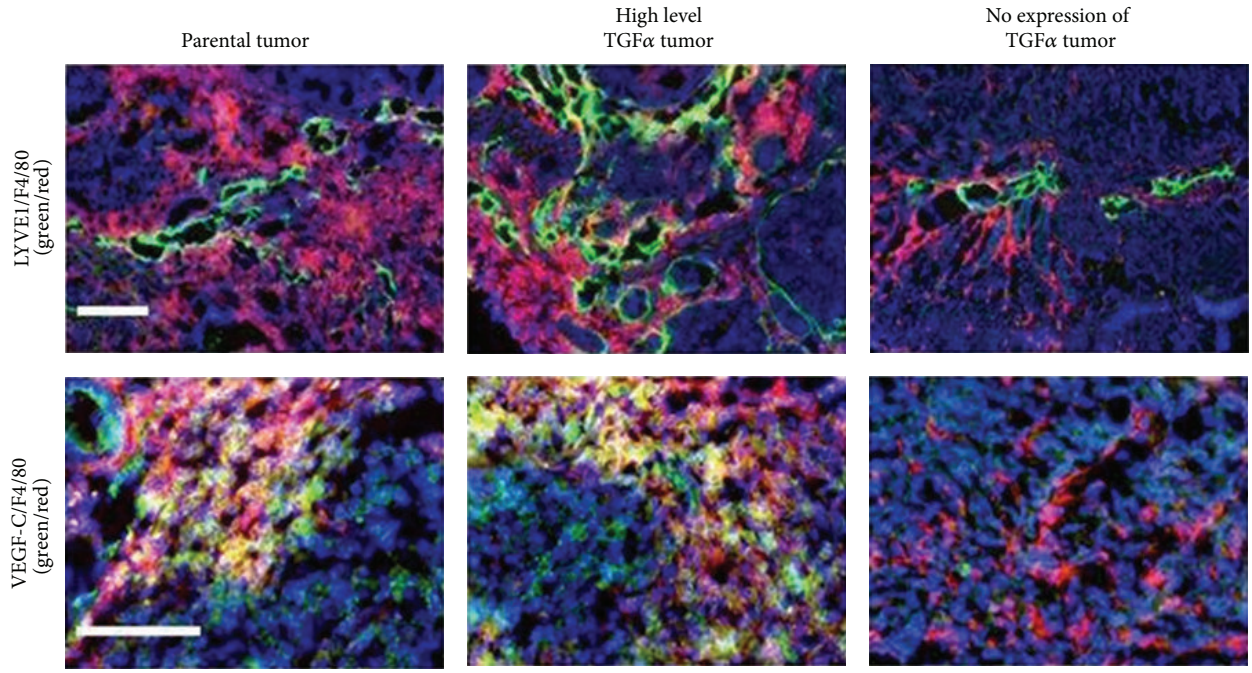


Figure 3: Immunofluorescent staining of LYVE-1, F4/80, and VEGFC in human colon carcinoma cells expressing different levels of TGF$\alpha$. Lymphatic vessels are labeled with LYVE-1 (green) and macrophage cells with F4/80 (red). The number of tumor-associated lymphatic vessels was greatest in selected high-level TGF$\alpha$ tumors and fewest in tumors that do not express TGF$\alpha$. Tumor recruitment of macrophages was also fewest in tumors that do not express TGF$\alpha$. Macrophage cells localized to selected high level TGF$\alpha$ tumors also expressed LYVE-1. The macrophage population recruited to TGF$\alpha$-expressing tumors also produced abundant levels of the lymphatic endothelial cell growth factor VEGFC. Scale bars = 100 $\mu$m [50].

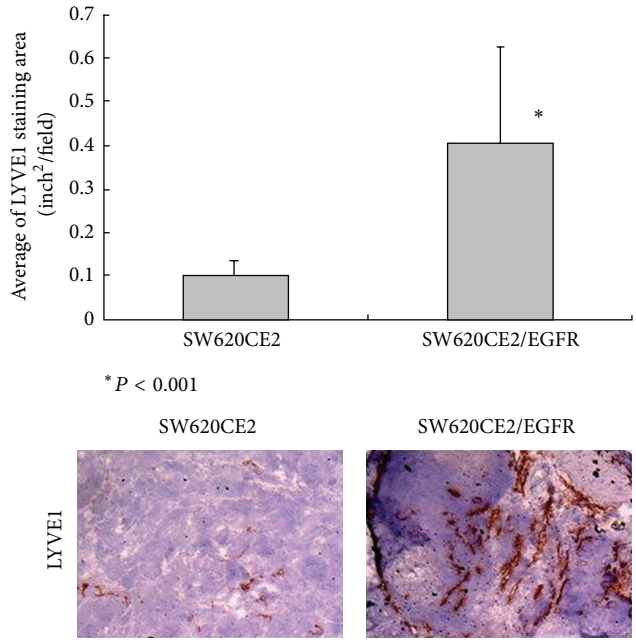


Figure 4: Mean density of LYVE-1 on orthotopic colon tumors expressing different levels of EGFR. The SW620CE2 is human colon cancer cell line. SW620 cells were injected into the cecal wall of nude mice. Three months after the injection, cecal tumors were harvested. Cells were established in culture. Primary cultures were passaged *in vitro* two or three times, and then, cells were injected into the cecum of another set of nude mice. The selection cycle was repeated two times to yield cell lines designated SW620CE2. SW620CE2 did not produce detectable levels of EGFR. SW620CE2/EGFR was established from SW620CE2 which was transfected sense EGFR plasmids. Cells ($5 \times 10^5$) in 50 $\mu$L of Hanks' buffered saline solution were injected into the cecal wall of nude mice. The number of lymphatic vessels in SW620CE2/EGFR tumors was fourfold higher than that observed in SW620CE2 tumors [56].

vimentin (VIM) [73]. EMT occurs during cancer progression and enhances invasion and metastasis [72].

## 7. Strategy of Treatment

Inhibiting signaling pathways through EGFR represents a good strategy for therapeutic intervention. Gefitinib inhibits EGF-stimulated EGFR autophosphorylation in a broad range of EGFR-expressing human cancer cell lines [74]. Cetuximab, a monoclonal antibody targeting EGFR, has been shown to induce apoptosis of colorectal cancer cells [75–77]. TGF$\alpha$-EGFR signaling in both tumor-associated endothelial cells and the tumor cells themselves is important in the progression of colon cancer. Abrogating the signaling activation by a dual tyrosine kinase inhibitor in combination with conventional therapy can induce a significant decrease in proliferation of tumor cells and significant apoptosis of both tumor cells and endothelial cells. Targeting the EGFR and VEGFR signaling in tumor vasculature with antineovascular therapy provides a new approach to the treatment of colon cancer.

In cholangiocellular carcinoma cell lines, the anti-EGFR antibody cetuximab is partially effective in EGFR-expressing cells [78]. KRAS mutations affect the efficacy of cetuximab in these cells. Gefitinib, a selective EGFR tyrosine kinase inhibitor, inhibits the phosphorylation of EGFR, ERK, and AKT and induces G1 arrest and apoptosis by upregulating p21 and p27 and BAX activation in GBC cells [79]. Epidermal growth factor receptor targeting is, therefore, critical in the treatment of GBC.

## 8. Conclusion

The activation of TGF$\alpha$-EGFR signaling in primary colon tumors contributes to the spread of tumor cells to lymph nodes and the liver. TGF$\alpha$-expressing tumors cells are more proficient in their ability to initiate metastases by virtue of their ability to communicate with the resident nontumor cell population. Therapeutic interventions that are designed to block EGFR signaling in TGF$\alpha$-positive colon tumors will likely have a negative impact on a number of processes that are essential for metastasis formation.

## Abbreviations

EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
TGF$\alpha$: Transforming growth factor-$\alpha$
RTKs: Receptor tyrosine kinases
VEGF: Vascular endothelial growth factor
VEGFR: Vascular endothelial growth factor receptor
IL-8: Interleukin-8
MMP: Matrix metalloproteinase
LYVE-1: Lymphatic vessel endothelial hyaluronate receptor 1
GBC: Gallbladder cancer
EMT: Epithelial-mesenchymal transition
ECD: E-cadherin
VIM: Vimentin.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 852093, 11 pages
http://dx.doi.org/10.1155/2013/852093

*Review Article*

# Heparan Sulfate and Heparanase as Modulators of Breast Cancer Progression

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Received 10 June 2013; Accepted 4 July 2013

Academic Editor: Davide Vigetti



Breast cancer is defined as a cancer originating in tissues of the breast, frequently in ducts and lobules. During the last 30 years, studies to understand the biology and to treat breast tumor improved patients' survival rates. These studies have focused on genetic components involved in tumor progression and on tumor microenvironment. Heparan sulfate proteoglycans (HSPGs) are involved in cell signaling, adhesion, extracellular matrix assembly, and growth factors storage. As a central molecule, HSPG regulates cell behavior and tumor progression. HS accompanied by its glycosaminoglycan counterparts regulates tissue homeostasis and cancer development. These molecules present opposite effects according to tumor type or cancer model. Studies in this area may contribute to unveil glycosaminoglycan activities on cell dynamics during breast cancer exploring these polysaccharides as antitumor agents. Heparanase is a potent tumor modulator due to its protumorigenic, proangiogenic, and prometastatic activities. Several lines of evidence indicate that heparanase is upregulated in all human sarcomas and carcinomas. Heparanase seems to be related to several aspects regulating the potential of breast cancer metastasis. Due to its multiple roles, heparanase is seen as a target in cancer treatment. We will describe recent findings on the function of HSPGs and heparanase in breast cancer behavior and progression.

## 1. Introduction

Breast cancer is defined as a cancer that originates in tissues of the breast, more frequently in the ducts and lobules. It prevails in women, although male breast cancer is also observed. In 2013, the National Cancer Institute estimates 232,340 (female) and 2,240 (male) new cases of breast cancer in the United States with 39,620 female and 410 male deaths, respectively.

Ductal carcinoma in situ (DCIS) is the most common type of noninvasive breast cancer. This cancer begins in cells of the milk ducts. Approximately, 7 out of 10 women with breast cancer have ductal carcinoma. The most common treatment for DCIS consists of lumpectomy, a procedure where most of the breast is conserved, followed by radiation therapy. However, in some cases, removal of the breast or mastectomy is recommended. Alternatively, when DCIS is hormone receptor-positive, hormonal therapy to lower the amount of estrogen in the body is recommended after surgery.

Invasive ductal carcinoma (IDC) is the most common type of invasive breast cancer, representing about 80% of all breast cancers. Two broad categories are employed for treating IDC: local treatment, consisting of surgery and radiation, or systemic treatment, consisting of chemotherapy, hormonal therapy and targeted therapies.

Invasive lobular carcinoma (ILC) is the second most common type of breast cancer following IDC. Each year, according to the American Cancer Society, more than 180,000 women in the United States are diagnosed with ILC. Other women have a mixture of ductal and lobular type or they have a less common type of breast cancer.

Similar to IDC, treatment for ILC consists of local surgery and radiation, or systemic chemotherapy, hormonal therapy, and targeted therapies. Both in IDC and ILC,

the chemotherapy usually follows similar drugs: doxorubicin, epirubicin, cyclophosphamide, docetaxel, paclitaxel, capecitabine, ixabepilone, methotrexate, and 5-FU.

Breast cancer is a complex, heterogeneous, tissue-specific disease. It is one of the most important malignancies affecting women and the leading cause of cancer-related deaths worldwide. During the last 30 years, several basic and clinical studies to understand the biology of breast tumor cells and to treat breast cancer have improved survival rates of patients [1]. These studies have been historically focused on genetic components involved in tumor progression and tumor microenvironment. The latter is a complex entity composed of several cell types immersed in extracellular matrix (ECM) composed by molecules, such as laminin, fibronectin, collagen, proteoglycans, and matrix metalloproteinases and heparanase.

Heparan sulfate proteoglycans (HSPG), predominantly found in the ECM and cell surface, are involved in cell signaling, cell adhesion, extracellular matrix assembly, and growth factors storage [2]. As a central molecule, HSPG can regulate cell behavior and tumor progression. Indeed, it has been reported that high levels of the cell surface HSPG syndecan are associated with an aggressive phenotype and poor prognosis in breast cancer patients [3]. Glypican, a GPI-anchored cell surface HSPG, is also highly expressed in human breast cancer cells and regulates mitogenic cell response to heparin-binding growth factors [4].

Despite the central role of HSPG in eukaryotic organisms, there is only one enzyme able to act upon heparan sulfate (HS) chains, named heparanase. This enzyme is an endo-$\beta$-D-glucuronidase, which cleaves heparan sulfate in fragments of 4 to 7 kDa [5]. In order to display enzymatic activity, heparanase needs to be activated by cleavage, yielding a heterodimer of 8 and 50 kDa subunits [6]. Heparanase is more expressed in tumors, compared to normal tissues [7]. Moreover, this glucuronidase regulates angiogenesis, metastasis, and tumor growth [8], contributing to an aggressive behavior of breast tumor cells and to poor prognosis in breast cancer patients [9]. In this review, we will describe recent findings on the function of HSPGs and heparanase in breast cancer behavior and progression.

## 2. Heparan Sulfate in Breast Cancer

Heparan sulfate is a linear glycosaminoglycan composed by repeating disaccharide unities of uronic acid (glucuronic acid or iduronic acid), 1,4 linked to glucosamine. Sulfate substitutions can occur at carbon 2 of the iduronic acid units, and at carbon 3 and/or 6 of the glucosamine, which can also be N-deacetylated followed by N-sulfation (Figure 1). HS has been described to participate in numerous processes during cancer progression [10]. The main importance of this glycosaminoglycan in tumor growth is its ability to bind key growth factors and stromal signaling molecules that activate tumor cells, influencing signaling, cell-cell interactions, uncontrolled proliferation, microenvironment modulation, and migration. HS is synthesized onto different protein cores, forming cell surface proteoglycans (syndecan and glypican) or extracellular matrix proteoglycans (perlecan, agrin, collagen XVIII) [2]. During cancer, HSPGs are usually differentially expressed in comparison to healthy tissue and cells, and drugs that affect HSPGs expression commonly affect other malignant aspects of the tumor [11, 12].

*2.1. Mammary Development and Heparan Sulfate.* The embryonic development of mammary gland becomes evident during midgestation, when placodes are formed and invaginate, following the milk line, to form buds. Bud formation then initiates gland growth, resulting in the structure of a rudimentary gland by the end of the gestational period. Only later, during puberty, mammary gland continues to develop, forming branches [13].

Mammary development depends on numerous cellular events such as cell-cell and cell-matrix interactions. In these events, ECM and cell surface proteoglycans play a central role. The HSPGs, syndecans, perlecans, and glypicans, for example, are frequently present during mammary formation, [14–19]. The activity of these HSPGs may rely on the core protein or on the HS glycan chain. It has been described that HS chains play an important role during branching morphogenesis. Garner et al. showed that lack of HS primary central core, after deletion of Ext1, the enzyme responsible for building up the central core of 1,4-linked uronic acid (D-glucuronic acid or L-iduronic acid) and D-glucosamine, results in highly defective mammary development [20]. It was also shown that N-sulfation is very important for mammary gland development, since N-deacetylase/N-sulfotransferase (NDST) 1 and 2 depletion results in hyperbranching [21], and specific deletion of NDST1 inhibits lobuloalveolar expansion [22]. Additionally, HSPGs expression seems to fluctuate along menstrual cycle [23], an indication that these molecules also have a role in the maintenance of the adult tissue.

*2.2. Heparan Sulfate in Breast Cancer.* HSPGs have been described as tumor biomarkers [24–29], being upregulated in aggressive phenotype, or downregulated when tumorigenesis is attenuated in tumor tissues [30–33]. For example, syndecan-2 and syndecan-4, as well as glypican-1, are overexpressed in breast cancer cell lines, compared to normal mammary cells [34], and are mediators of growth factor signaling. EGF/IGF-mediated upregulation of HSPG gene expression enhances breast tumor cell proliferation [35]. Recently, a direct relationship between growth factor signaling and estrogen receptors (ER) has been shown. Breast cancer cells that express ER can be directly stimulated via estrogen, or indirectly stimulated via epidermal growth factor receptor (EGFR) or insulin growth factor receptor (IGFR). Activation of these pathways is crucial for tumor establishment and development and lead to specific modulation of HSPGs, such as syndecans-2 and -4 and glypican-1, in addition to other ECM-modulating molecules [36, 37].

Syndecan-1 has been thoroughly described as a protumorigenic agent during breast cancer development [38–41], especially in the shed form. Glypicans display different activities in tumor development, while glypican-1 overexpression in tumor cells triggers mitogenic response [4], absence

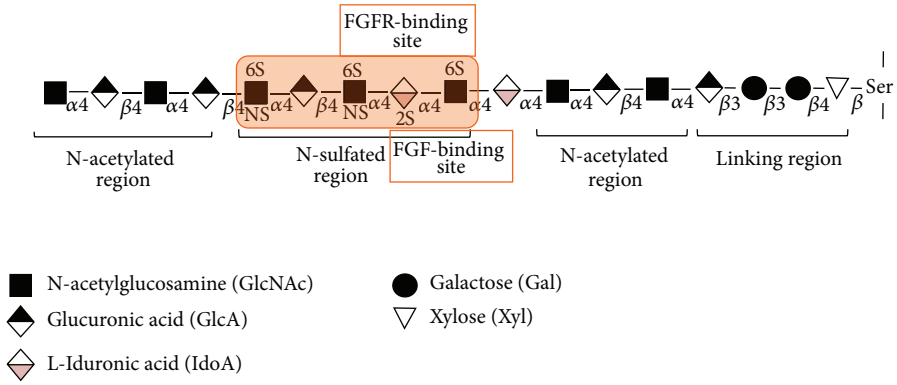


Figure 1: Heparan sulfate structure. Heparan sulfate glycosaminoglycan is linked to specific serine residues on heparan sulfate proteoglycans by a tetrasaccharide sequence of glucuronic acid, galactose, galactose, and xylose. The HS chains contain clusters of N-acetylated unmodified domains and N-sulfated modified domains. Specific sequences in the N-domains bind to different growth factors and their receptors, for example, fibroblast growth factor-2 (FGF-2) and its receptor (FGFR).

of glypican-3 expression in breast cancer cell lines inhibits mammary tumorigenesis [19]. The work by Buchanan et al. showed that glyplican-3 reexpression in murine mammary adenocarcinoma inhibits the PI3/Akt antiapoptotic pathway [42]. Glypican-3 is overexpressed in hepatocellular carcinoma (HCC) and a valued serum diagnostic marker of the disease [43]. More recently, glypican-3 also became a potential and reliable biomarker for predicting tumor recurrence and overall survival in HCC patients after curative resection [44]. Similar to glypican-3, perlecan has also been shown to be downregulated or absent in breast tumors [30, 45].

HSPGs at the surface of breast cancer cells act as growth factor coreceptors, especially for FGF-2 [46, 47]. HS chains bind to different FGFs with reasonable specificity according to its sulfation pattern. For example, a trisaccharide sequence of 2-O-sulfated iduronic acid flanked by N-sulfated glucosamines has recently been shown to be the minimum binding motif and N-sulfation was found to be critical for the binding of HS to FGF-2 [48] (Figure 1). This provides useful information for further development of more potent compounds towards FGF-2 binding, which can have potential applications in wound healing and anticancer therapy.

Disruption of HS sulfation inhibits tumor cell migration, while addition of specific exogenous HS recovers tumor behavior [32]. Growth factor binding specificity leads to different responses according to cell status and the type of HS chain presented by the cells [49–52] and for that function, a balance between cell surface and shed HSPGs, such as syndecan, is crucial. Early reports on syndecan-1 depletion in normal mammary epithelial cells show that these cells acquire a mesenchymal phenotype, losing epithelial markers and presenting a fibroblastoid morphology [53]. The work by Nikolova et al. shows that expression of a shed form of syndecan-1 in MCF-7 cells lowers proliferation rate and enhances migration features, such as Matrigel invasiveness potential. On the other hand, expression of an uncleavable membrane-bound syndecan-1 accelerates proliferation and inhibits matrigel invasiveness [54]. In addition, it has been shown that expression of active heparanase also promotes shedding of syndecan-1, and heparitinase treatment substitutes heparanase activity, indicating that breaking HS chains promotes syndecan shedding [55, 56]. Syndecan-1 shedding also influences FGF-2 signaling via glypican-1, as early shedding promotes glypican-1-dependent FGF-2 signaling. However, maintenance of syndecan-1 on the cell surface promotes glypican-1 independent FGF-2 signaling [57].

The use of HS-related compounds as antitumor agents has been reported. For example, low anticoagulant heparin reduces P-selectin adhesion in breast cancer cells, leading to attenuation of metastasis [58]. HS and heparin oligosaccharides have also been tested to inhibit HS-dependent tumor behavior. The inhibitory activity is achieved during different aspects of cancer development, such as migration, metastasis formation, and tumor growth [59]. In addition, a protective role of glypican-3 has been reported by Peters et al., showing that expression of rat Glypican-3, on mouse breast cancer cell line LM3, inhibited aggressive behavior by maintaining adequate levels of protective molecules [60]. On the other hand, compounds that interfere with HSPGs expression also have a positive effect on tumor inhibition. The bisphosphonate zoledronate, for example, is able to downregulate syndecans-1 and -2 and glypican-1, while upregulating syndecan-4 in a cell line model [11]. This effect is accompanied by inhibition of growth, invasion, and adhesion of tumor cells, in addition to inhibition of osteoclast activation in a cellular model of breast cancer bone metastasis [61].

*2.3. Heparan Sulfate 6-O-Endosulfatases in Breast Cancer.* HS 6-O-endosulfatases, also known as Sulfs, are deeply involved in the metastatic behavior of breast tumor cells [62]. It has been shown that the two isoforms Sulf1 and Sulf2 seem to present different activities during tumor growth. Lai et al. showed that Sulf1 expression is low in breast and ovarian cancer, and induction of enzyme expression inhibits tumor behavior in cells [63]. Narita et al. showed that high expression of Sulf1 in tumor cells fails to develop vessels, leading to marked necrosis and apoptosis, and this probably

occurs due to the inability of tumor endothelial cells to bind FGF-2 [64]. On the other hand, Sulf2 has been shown to be proangiogenic [65], and depletion of this enzyme leads to reduced tumor size [66]. Bret et al. showed SULF1 and SULF2 mRNA overexpression in breast cancer cohorts from different parts of the world [67]. On the other hand, Peterson et al. reported that transfection of human Sulf1 and Sulf2 in MDA-MB-231, a human breast cancer cell line, affected xenografts growth, while a single injection of purified sulfotransferases did not have the same effect [68]. Overall, Sulfs1 and 2 seem to have opposite effects, but compensation mechanisms on tissue response to tumor still need to be explored in order to unveil the activities of Sulfs on breast cancer development.

*2.4. Heparan Sulfate in Breast Cancer Environment.* Other cell types, such as endothelial, immune cells, and fibroblast, surround breast cancer cells. HS-dependent crosstalking between these cell types and tumor cells also plays an important role during breast cancer development. Lines of evidence suggest that conditioned media from endothelial cells inhibit breast cancer cells invasiveness, and depletion of perlecan may disrupt this ability [69]. Also, lymphocytes derived from breast cancer patients affect healthy lymphocytes, turning them into tumor-inducing cells via heparanase expression [70]. Fibroblasts derived from breast carcinoma tissue produce MT1-MMP, which leads to syndecan-1 shedding from tumor cells, an important step in tumor invasion, while fibroblasts derived from healthy mammary tissue do not possess the same effect [71].

*2.5. Other Sulfated Glycosaminoglycans in Breast Cancer.* Other sulfated glycosaminoglycans, such as chondroitin sulfate (CS) and dermatan sulfate (DS), are also involved in mammary gland development [23] and may, consequently, be involved in breast cancer development. The activity of CS in breast cancer is still contradictory. In one hand, CS overexpression by tumor cells is associated with a poor prognostic phenotype. On the other hand, downregulation of the glycosaminoglycan is described in aggressive tumors [72]. CS has been reported as an aggressive tumor molecule, showing increased levels in tumor tissue samples compared to normal tissue [73]. The role of CS during metastasis still needs to be explored. CS participates in P-selectin binding in a cell line model, which suggests that this glycosaminoglycan is involved in the metastatic process [74, 75]. Controversially, chondroitinase ABC treatment in tumors triggers metastasis [76]. It is still unclear whether CS is relevant to breast cancer development; however, its expression and overall activity seem to correlate with a more aggressive tumor phenotype and, consequently, poor prognosis.

DS chains are reduced in breast tumor samples compared to healthy neighbor tissue, both glycan chains and core proteins had their levels altered [77–79]. Nevertheless, DSPGs expression were described to be increased in breast cancer fibroadenoma compared to healthy tissue [80], and, although the DSPG decorin was present in both healthy and tumor tissue, versican was exclusively detected in tumor samples.

In conclusion, significant data have been generated over decades correlating HSPGs expression and modulation with EMT and metastasis (Figure 2). The literature shows that HS accompanied by its glycosaminoglycan counterparts regulate tissue homeostasis and cancer development. In different circumstances, these molecules present opposite effects according to tumor type or cancer development model. Further studies in this area may contribute to unveiling sulfated glycosaminoglycan activities on cell dynamics during breast cancer and explore these polysaccharides as antitumor agents.

## 3. Heparanase and Its Regulatory Function on Breast Cancer

Heparanase is a potent modulator of tumor behavior due to its protumorigenic, proangiogenic, and prometastatic activities. Several lines of evidence indicate that heparanase is upregulated in all human sarcomas and carcinomas [81] and occurs at elevated levels in body fluids of breast cancer patients [82]. Indeed, evaluation of heparanase expression in breast cancer cells reveals that more aggressive lines, such as MDA-MB-231 and MDA-MB-435, present high levels of heparanase, whereas MCF-7, a nonmetastatic and poorly invasive luminal breast cancer cell, presents low levels of the enzyme [7]. The enzymatic activity of heparanase has been assessed, and the results show that aggressive lines present high activity, while nonmetastatic cells present low or no activity. Moreover, in an orthotopic model of MCF-7 cells transfected with the HPSE gene, tumors are able to grow faster, presenting increased vascularization and a higher degree of vessel maturation in comparison with tumors formed by control cells [8]. On the other hand, heparanase gene silencing reduces invasive ability of MDA-MB-435 [83].

*3.1. Heparanase Function in Metastasis.* Heparanase seems to be related to several aspects that regulate the potential of breast cancer metastasis. It has been shown that breast cancer cells from brain metastatic sites exhibited high levels of heparanase, compared to parental cells [84]. In this case, metastatic cells respond to EGF by phosphorylation of EGF and HER-2 receptors and by increasing heparanase levels. EGF also induces heparanase translocation to the nucleus [85]. DNA topoisomerase I, a key player during DNA replication, is regulated by nuclear heparanase, thus affecting cell proliferation of breast cancer cells in brain metastases [84]. Moreover, colocalization of heparanase and DNA topoisomerase I in the nucleus was found only in slices obtained from metastatic brain that overexpress HER2, confirming the idea that heparanase is a downstream molecule arisen from HER2-induced signaling [84]. Heparanase has also been shown to play a role in bone metastasis. Tumors formed by a variant of bone-colonizing MDA-MB-231 cells, which overexpress heparanase, are capable of inducing bone reabsorption without detectable bone metastasis, indicating that heparanase may have a role prior to the establishment of macrometastasis [86].

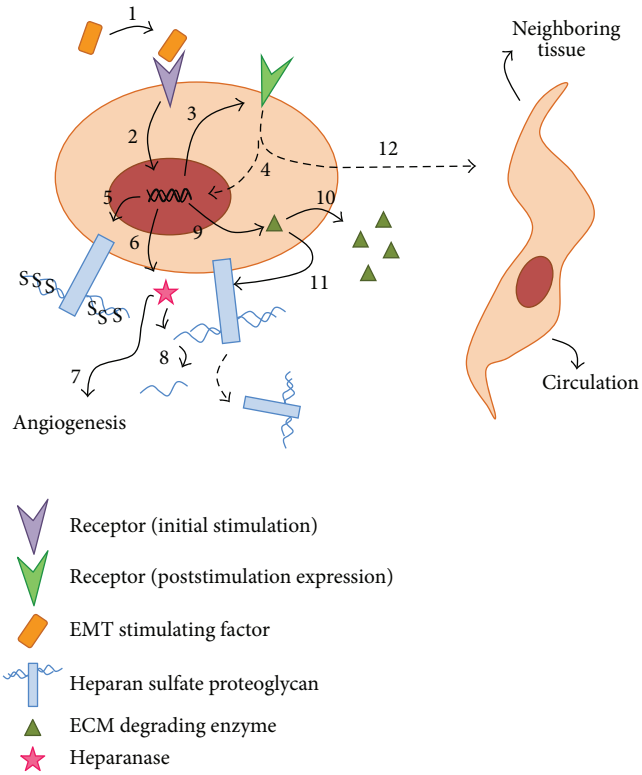


Figure 2: Model of how Heparan sulfate proteoglycans and heparanase participate in the epithelial-to-mesenchymal transition of a breast cancer cell. When a specific factor, such as TGF-$\beta$, stimulates its receptor on the tumor cell surface (1), the signaling cascade triggers transcriptional changes (2) that lead to a differential expression of specific receptors (3), which will allow the tumor cell to become responsive to other available factors that will culminate in the transition from an epithelial to a mesenchymal state (4). During this process, these transcriptional changes also lead to higher degree of sulfation of heparan sulfate chains (5), enhancing the cell ability to bind more extracellular molecules. Also, heparanase expression takes place (6), enhancing tumor angiogenesis (7) and degrading heparan sulfate chains (8) that will no longer be internalized, staying in the extracellular matrix bound with factors that also cooperate in the epithelial-to-mesenchymal transition process. Expression of extracellular matrix-degrading enzymes (9), such as metalloproteinases, promote extracellular matrix degradation (10) and heparan sulfate proteoglycans shedding (11). These processes altogether culminate in a complete transformation of an epithelial tumor cell into a mesenchymal phenotype (12) able to invade the neighboring tissue and circulation.

*3.2. Modulation of Heparanase Expression.* Several factors regulate heparanase gene expression in cancer cells. Early growth response gene 1 (EGR-1) is a zinc-finger transcription factor that plays dual role in tumor biology [85]. In breast tumor cells, EGR-1 binds to heparanase promoter and induces its activity in a dose-dependent manner [87]. Heparanase gene regulation is also modulated by estrogen, which is an important risk factor for breast cancer. Estrogen exposure enhances heparanase promoter activity in MCF-7 cells, and this can be associated with four estrogen response elements in the heparanase promoter [8]. The importance of these data was confirmed by breast cancer tissue array, which demonstrates a correlation between estrogen receptor (ER) and heparanase expression [88]. Moreover, this work reveals that Tamoxifen, a common drug used to treat breast cancer (ER+) patients, induces heparanase expression in MCF-7 and T47D cells.

Other transcriptional factors can modulate heparanase expression in breast carcinoma, such as the tumor suppressor p53. Mutation in tumor suppressor p53 gene is associated with several human tumors, altering cell behavior to favor tumor progression. Indeed, tumor suppressor p53 mutations are more common in high-grade ductal carcinoma in situ (DCIS) than in low-grade DCIS [89]. Additionally, p53 binding to heparanase promoter exerts inhibitory actions, whereas mutant p53 is not able to induce the same effect [90]. Also, ETS transcription factors, ETS1 and ETS2, regulate heparanase expression by binding to four different sites in the regulatory sequence of the heparanase gene [91].

*3.3. Heparanase as an Inducer of Angiogenesis.* Angiogenesis is a crucial step of cancer progression in a number of tumor types, including breast cancer. Heparanase seems to contribute to angiogenic responses by promoting cleavage of HS chains, releasing growth factors, such as bFGF and VEGF [81, 92, 93], to bind to endothelial cells. In addition, heparanase can promote VEGF gene regulation in a variety of cells. It was demonstrated that VEGF is upregulated in MDA-MB-435 human breast carcinoma cells overexpressing heparanase. Increase in VEGF expression is mediated via Src activation, and, although p38 phosphorylation is involved, it is not

essential [93]. It was also shown that conditioned medium from heparanase-overexpressing cells sustain endothelial cell proliferation. This effect is inhibited by the presence of soluble VEGF receptor, suggesting that VEGF secreted by heparanase-overexpressing cells is involved [93]. Moreover, VEGF upregulation seems to be independent of heparanase enzymatic activity. The enzyme cyclooxygenase-2 (COX-2) triggers heparanase-dependent angiogenic response. COX-2 localization in breast cancer specimens was similar to heparanase and there is a correlation between heparanase and COX-2 expression, which is evident in invasive breast cancer [94]. This work also shows that heparanase expression is related to higher incidence of metastasis and the size of the primary tumor.

*3.4. Heparanase and miRNA-1258 in Breast Cancer.* It has been recently reported that heparanase expression and activity in brain metastatic breast cancer cell (MDA-MB-231) can be inhibited by the microRNA miR-1258 [91]. According to this idea, it was shown that miR-1258 and heparanase levels are inversely correlated in breast carcinoma [95]. Thus, invasive ductal carcinoma and malignant cells showed attenuated expression of miR-1258. Moreover, western blot analysis indicates that miR-1258 inhibits phosphorylation and expression of heparanase-related proteins AKT, EGFR, MMP-9, and COX-2, resulting in decrease of breast cancer brain metastasis [95].

*3.5. Heparanase Inhibitors.* Taking into account the involvement of heparanase in breast cancer, several heparanase inhibitors have been developed in order to affect tumor growth and bulk angiogenesis [96, 97]. Progen Pharmaceuticals Company (Brisbane, QLD, Australia) designed a compound called PG545. This drug is a synthetic HS mimetic, a sulfated tetrasaccharide that decreases tumor growth in MDA-MB-231 xenograft and inhibits angiogenesis *in vivo* [96]. Recently, it was shown that PG545 blunts orthotopic tumor growth and inhibits lung spontaneous metastasis, contributing to overall survival. Importantly, PG545 treatment reduced heparanase expression in the primary tumor and at metastatic foci [97].

PI-88 (Phosphomannopentaose sulfate) is a heparanase inhibitor that is in clinical trials (phase II). Prostate cancer treatment with PI-88 in association with docetaxel decreases prostate specific antigen (PSA) response in 70% of patients (Clinical Trials, ID: NCT00268593). PI-88 was already employed in animal models to treat breast cancer [98]. In this context, it was revealed that PI-88 decreases tumor growth rate of highly invasive rat mammary adenocarcinoma, 13762 MAT cells, inhibits metastasis as well as tumoral vascularity. Laminarin sulfate, a linear polymer ($\beta$-1,3 glucan) sulfated at 2 and 6 position, known as heparanase inhibitor, inhibited lung colonization of 13672 MAT mammary-adenocarcinoma cells [99].

Due to its multiple roles in cancer progression, heparanase is seen as a potential target in cancer treatment. Besides inhibitors and antibodies against heparanase, vaccines have been developed. Two reasons explain why heparanase is being seen as a universal tumor-associated antigen: heparanase is expressed in several types of advanced tumors, and dendritic cell expressing heparanase are able to elicit heparanase-specific cytotoxicity T lymphocytes (CTL) against tumor cells [100, 101]. It was shown that vaccines made of heparanase multiple antigenic peptides induce CTL response in heparanase overexpressing MCF-7 cells [102]. Altogether, these data point to heparanase as a good target molecule to break breast cancer progression.

## 4. Future Directions

In addition to cancer genomics, host immunology, cell biology to develop less toxic treatments, and narrowing cancer therapy to individual patients, cancer research, including breast cancer, requires a multidisciplinary approach to study different aspects of the tumor biology. This includes tumor microenvironment, extracellular matrix components, and extracellular matrix-associated proteases, such as MMPs, sulfatases, and glycosidases. The knowledge generated in these basic studies should work in parallel with that generated in clinical studies to allow the development of new protocols. For example, nonanticoagulant heparin and heparan sulfate mimetics, as well as heparanase and sulfatase inhibitors, could be used as adjuvant therapies along with other chemotherapeutic protocols. Additionally, the concept of a premetastatic niche, created by evolutionary mechanisms during cancer progression, should be added to the overall picture (for review, see [103]) (Figure 3). This premetastatic microenvironment is constructed by signaling molecules (cytokines, chemokines, and exosomes), secreted by recruited bone marrow derived cells, and as activated resident cells such as fibroblast. As the premetastatic niche matures, as a result of an intense tissue remodeling, clone expansion and tumor promotion occur, leading to the establishment of metastatic focus. Therefore, the extracellular matrix composition of the premetastatic niche, with the probable occurrence of proteoglycans, hyaluronic acid, proteases, and glycosidases, should be an area of intense investigation in the future.

## Authors' Contribution

Angélica M. Gomes and Mariana P. Stelling contributed equally to the work.

## Acknowledgments

This work was supported by Programa de Oncobiologia-Fundação do Câncer, CNPq, CAPES-PROBRAL, FAPERJ, and "INFLAMA", IRSES program of FP7 no. 247516. AMG is a PhD student from CNPq. MPS is a graduate (postdoctorate) fellow from CNPq, and MSGP is a research fellow from CNPq and FAPERJ.

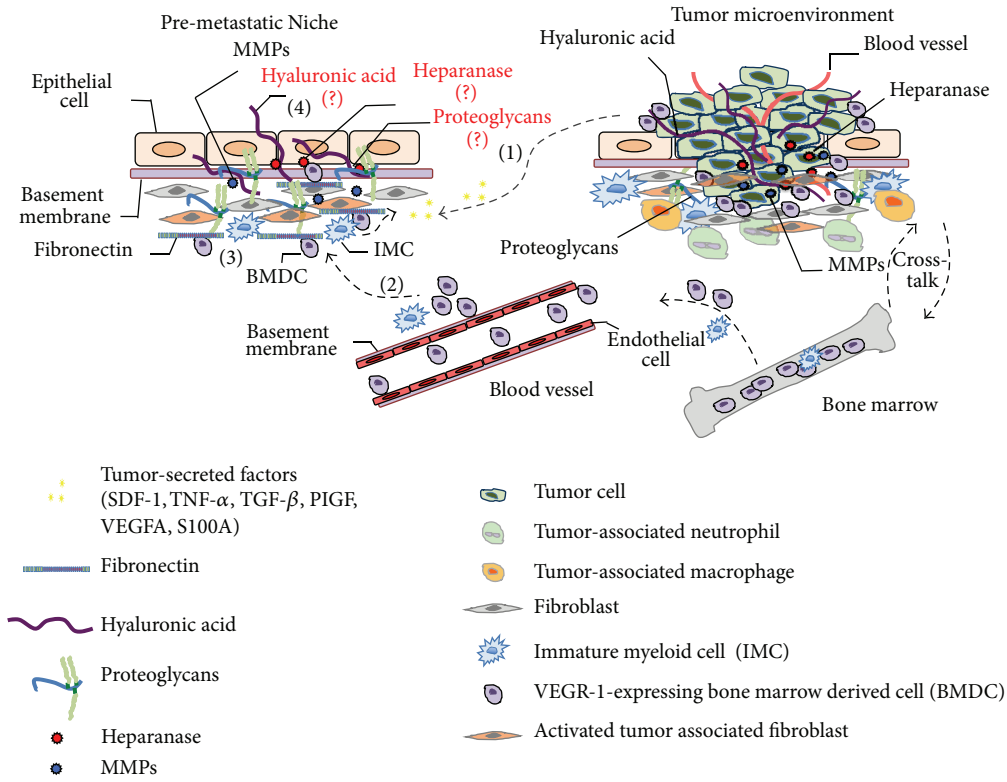


Figure 3: Cellular and molecular composition of premetastatic niche and metastatic microenvironment. premetastatic niche formation initiates by release of soluble factors, such as, VEGFA, TGF-beta, placental growth factor (PLGF), inflammatory chemokines S100, and Serum Amiloyd A3 (SAA3), as well as stromal-derived growth factor 1 (SDF1) by the primary tumor (1). As a result, bone marrow-derived hematopoietic progenitor cells (HPC) and immature myeloid cells are recruited to the premetastatic niche (2). Then, these bone marrow-derived cells start to populate the premetastatic niche with potent modified factors, such as tumor necrosis factor-$\alpha$ (TNF$\alpha$), matrix metalloproteinase 9 (MMP9), and TGF$\beta$, leading to stimulation of stromal cells that in turn modulate the extracellular matrix of the premetastatic microenvironment (3). For example, modified factors-mediated fibroblast activation initiates secretion of fibronectin, which constitutes an important adhesion protein in the niche. Additionally, other important extracellular matrix components such as hyaluronic acid, proteoglycans, glycosaminoglycan-modified enzymes, like heparanase and sulfatases are likely to be present (4), but it is yet to be confirmed and constitutes a new interesting area of research involving the premetastatic niche. Mature tumor microenvironment is composed by tumor cells, blood vessels, bone marrow-derived cells, proteoglycans, MMPs, hyaluronic acid, stromal cells, such as fibroblasts and several recruited cells like neutrophils, and macrophages. These cells, secrete several growth factors and cytokines that can drive epithelial to mesenchymal transition-mediated migration and invasion of tumor cells.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 152163, 7 pages
http://dx.doi.org/10.1155/2013/152163

*Review Article*

# LRP-1: A Checkpoint for the Extracellular Matrix Proteolysis

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Received 15 May 2013; Accepted 20 June 2013

Academic Editor: Davide Vigetti



Low-density lipoprotein receptor-related protein-(LRP-1) is a large endocytic receptor that binds more than 35 ligands and exhibits signaling properties. Proteinases capable of degrading extracellular matrix (ECM), called matrix proteinases in this paper, are mainly serine proteinases: the activators of plasminogen into plasmin, tissue-type (tPA) and urokinase-type (uPA) plasminogen activators, and the members of the matrix metalloproteinase (MMP) family. LRP-1 is responsible for clearing matrix proteinases, complexed or not with inhibitors. This paper attempts to summarize some aspects on the cellular and molecular bases of endocytic and signaling functions of LRP-1 that modulate extra- and pericellular levels of matrix proteinases.

## 1. Introduction

Extracellular matrix (ECM) remodeling occurs in both physiological and pathological situations [1]. Tissue homeostasis depends on a strict equilibrium between synthesis and degradation of ECM macromolecules. In contrast, fibrotic pathologies are classically related to a defect or an increased ECM breakdown, while an excessive proteolytic degradation is the hallmark of inflammatory processes or tumor invasion. Numerous proteolytic enzymes are able to degrade ECM macromolecules, including the serine proteinases tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) [2] and the members of the matrix metalloproteinase (MMP) family [3].

A series of specific or nonspecific inhibitors controls the activities of these powerful catalytic enzymes. Thus, the pan-protease inhibitor $\alpha$2-macroglobulin ($\alpha$2M) binds to and inhibits active members of the four classes of proteolytic enzymes [4]. More specifically, the serine proteinase inhibitors (serpins) and the plasminogen activator inhibitors (PAI) 1 and 2 block the activity of tPA and uPA [5]. Tissue inhibitors of metalloproteinases (TIMPs) inhibit the activity of MMPs [6] and also of adamalysins (a disintegrin and metalloproteinases, ADAMs) [7]. Besides this level of control, receptor-mediated endocytosis is an emergent and efficient biological mechanism to regulate extra- or pericellular levels of proteolytic enzymes by internalizing them for catabolism in lysosomes [8]. This paper briefly describes the main molecules involved in these events and reviews the different roles of low-density lipoprotein (LDL) receptor-related protein-(LRP-1) in controlling extracellular matrix remodeling.

## 2. Plasminogen Activators and Their Inhibitors

Urokinase-type plasminogen activator (uPA) and tissue-type PA (tPA) are serine proteinases that catalyze the conversion of the zymogen plasminogen to the active serine proteinase plasmin [9]. Plasmin degrades numerous ECM macromolecules including laminin, fibronectin, and proteoglycans, triggers the activation of pro-MMPs, and activates or releases growth factors from ECM including latent-transforming growth factor $\beta$ and vascular endothelial growth factor. Both $\alpha$2M and the serpin $\alpha$2-antiplasmin inhibit its activities [10]. Pro-uPA is synthesized as a one-chain molecule that is cleaved at a single peptide bond (K158-I159 in human uPA) by various proteases including plasmin to give active two-chain uPA of 55 kDa. Human tPA was first purified as

a single-chain form of approximately 70 kDa. A limited attack of the R275-I276 bond by plasmin generates a two-chain tPA. The plasminogen activation activity of single-chain tPA is 10- to 50-fold lower than that of the two-chain form [2]. The PA inhibitors PAI-1 and PAI-2 efficiently inhibited tPA and uPA catalytic activities [11].

The binding of uPA to its cell-surface receptor (uPAR) increases the affinity of uPAR for vitronectin and integrins, thus promoting cell adhesion, [12]. Interestingly enough by disrupting these interactions, PAI-1 detaches cells not only from vitronectin but also from fibronectin and collagen matrices [13]. This deadhesive property exhibited by PAI-1 could explain, at least partly, why paradoxically PAI-1 appears to be essential for cancer cell invasion and angiogenesis [14].

## 3. Matrix Metalloproteinases and Their Inhibitors

MMPs are the major matrix-degrading proteases due to the wide variety of their substrates and their role in numerous physiopathological processes [15, 16]. They belong to a large family of zinc-dependent endopeptidases. In humans, MMPs are represented by 23 members divided into two groups based on their localization (secreted or membrane-bound) or in five groups based on their domain organization and their substrate preference (collagenases, gelatinases, stromelysins, matrilysins, and membrane-type) [3, 17]. The general structure of MMPs consists in three domains that are common to almost all MMPs: the prodomain of about 80 amino acids, the catalytic metalloproteinase domain of about 170 amino acids, and the hemopexin domain of about 200 amino acids (except for MMP-7, -26, and -23). MMPs are secreted as a proenzyme, an enzymatically inactive state that results from the interaction between the "cysteine switch" motif in the prodomain and the zinc ion of the catalytic site [18]. The activation of these zymogens is an important regulatory step of MMP activity and occurs after the disruption of this interaction [15]. This process requires the proteolytic removal of the pro-domain by intracellular convertases such as furin or by extracellular proteinases (MMPs, plasmin, ..., etc.). A chemical perturbation of the cysteine-zinc interaction by SH reagents, by chaotropic agents (*in vitro*), or by antioxidant has been shown as sufficient to activate proMMPs [18].

After their activation, MMPs are regulated by two major types of endogenous inhibitors: $\alpha$2M and TIMPs [18]. $\alpha$2M is a plasma glycoprotein produced in the liver. Four nearly identical, disulfide-bonded domains of 180 kDa compose this 772 kDa protein. Inhibition mechanism involves the presentation of a cleavable region that, once proteolytically cleaved, induces a conformational change entrapping the proteinase that becomes covalently anchored by transacylation. Such a molecular complex is rapidly cleared by LRP-1-mediated endocytosis [19].

TIMPs are 184–194 amino acid proteins that have been described to form 1 : 1 stoichiometric complexes with active MMPs leading to the inhibition of their proteolytic activity. Four TIMPs (TIMP-1, -2, -3, and -4) have been identified in humans, inhibiting all MMPs tested so far, except TIMP-1 that was reported as being a poor inhibitor for MT1-MMP, MT3-MMP, MT5-MMP, and MMP-19 [6, 18, 19]. All structurally characterized inhibitory TIMP-metalloproteinase complexes are closely similar. Within the metalloproteinase active site, the catalytic zinc atom is chelated by the N-terminal amino group and the carbonyl group of cysteine 1 [20]. TIMPs are also able to interact with proMMPs: TIMP-2, TIMP-3 or TIMP-4 with proMMP-2 and TIMP-1 or TIMP-3, with proMMP-9 [20]. These complexes are stabilized by interaction between the TIMP C-terminal domain and hemopexin domain of the zymogen. Since these interactions do not involve the N-terminal domain of the TIMP, such molecular complexes are capable of interacting with a second MMP molecule. Except for the role of proMMP-2-TIMP-2 in the MT1-MMP-mediated activation of proMMP-2 [21], their functional significance remains unclear [20].

More recently, TIMPs have been reported to induce various biological processes (cell survival, differentiation, epithelial-mesenchymal transition, ..., etc.) independently from their MMP-inhibitory activity [22, 23]. These effects involved an interaction with specific cell-surface receptors leading to signaling pathway activation. For example, TIMP-1 promotes cell survival in erythroleukemic cells after binding with a CD44/proMMP-9 complex receptor [24, 25] and in breast and lung epithelial cells after interacting with a CD63/integrin-$\beta$1 complex receptor [26, 27].

## 4. Low-Density Lipoprotein Receptor-Related Protein-1

*4.1. General Features.* LRP-1 is the first member of a receptor family related to the LDL receptor [28]. The receptor for $\alpha$2M-proteinase complexes [29] and CD91, which interacts with heat-shock proteins at the surface of antigen-presenting cells [30], corresponds to LRP-1. It is synthesized as a single-chain molecule processed by furin in the trans-Golgi compartment into a 515 kDa $\alpha$-chain and an 85 kDa $\beta$-chain which remain non-covalently associated at the cell surface [8]. The extracellular $\alpha$-chain contains four basic amino acid residue-rich domains that interact with a number of ligands including proteins involved in lipoprotein metabolism, ECM proteins, growth factors, proteinases, and proteinase-inhibitor complexes. The transmembrane $\beta$-chain contains a cytoplasmic tail of 100 amino-acid residues including two NPxY motifs, necessary to trigger endocytosis and capable of interacting with many adaptors and signaling proteins.

The endocytic clearance of various ligands and signaling properties confer a main role to LRP-1 in a variety of pathophysiological processes including lipid metabolism, neurodegenerative diseases, blood-brain-barrier integrity, atherosclerosis, and cancer [8]. The importance of LRP-1 is confirmed by the lethality of mice carrying LRP-1 gene deletion at an early stage of embryonic development [31].

*4.2. Endocytic Function.* The LRP-1-mediated endocytic internalization of active proteinases linked to the pan-proteinase inhibitor $\alpha$2M represents a general process to eliminate the excess of active proteinases from cellular

Table 1: Main matrix proteinases and specific inhibitors known to bind to LRP-1.

| Serine proteinases, serpins, and serine proteinase/serpin complexes | | |
|---|---|---|
| tPA | PAI-1 | tPA, uPA/PAI-1 |
| (pro)uPA | | uPA/PAI-2 |
| MMPs, TIMPs, and MMP/TIMP complexes | | |
| (pro)MMP-2/TSP-1, -2 | TIMP-1 | (pro)MMP-2/TIMP-2<br>(pro)MMP-9/TIMP-1 |
| (pro)MMP-9 | TIMP-2 | |
| (pro)MMP-13 | TIMP-3 | |
| Other matrix proteinases | | |
| Heparanase precursor | | |
| Procathepsin-D | | |
| ADAMTS-5 | | |

environment [19, 32]. Here, we review additional LRP-1-mediated endocytosis that occurs independently from $\alpha$2M to regulate extracellular proteinase activities (Table 1).

*4.2.1. Serine Proteinases and Inhibitors.* The binding of tPA to cell surface has first been described through PAI-1-dependent [33] and PAI-1-independent [34] receptors. These receptors have been rapidly identified as being LRP-1 [35, 36]. Orth and colleagues [37] confirmed that tPA, under its free form or complexed to PAI-1, binds to LRP-1 to be intracellularly degraded. Also, LRP-1 was shown to mediate the internalization of uPA associated to PAI-1 [31, 38] and PAI-2 [39]. Pro-uPA binds to purified LRP-1 with affinity 15 to 20 fold, weaker than that of the uPA/PAI-1 complex [40]. In contrast, PAI-1 was described to interact with LRP-1 with high affinity when associated with proteinases [41]. These data strongly suggest that the binding of proteinase to PAI-1 could modify PAI-1 conformation, revealing a cryptic high-affinity binding site for LRP-1.

Besides its ability to link to LRP-1 to be internalized [40], pro-uPA is activated upon binding to uPAR [42]. Interestingly, both uPAR endocytosis and uPA catabolism are dependent on PAI-1 [43]. These important data support the role of LRP-1 in promoting the cell-surface PA activity by facilitating the clearance of uPA/PAI-1-occupied uPAR and the regeneration of unoccupied uPAR at the cell surface [44, 45]. Such a process requires a direct binding between uPAR and LRP-1 [46]. This cycle of binding uPA/PAI-1 to uPAR followed by association with LRP-1, internalization, and intracellular dissociation and recycling of unoccupied uPAR and free LRP-1 to the cell surface can explain, at least in part, the promigratory effect of PAI-1 observed in invasive cells [47].

*4.2.2. Matrix Metalloproteases and Inhibitors.* In addition to its effect on uPA and tPA activities, LRP-1 also regulates extracellular levels of MMP-2, -9, and -13 [48]. As for uPA and tPA, the endocytosis of MMP-2 and MMP-13 involves preliminary binding to adjacent receptors. Indeed, when bound to thrombospondin-2 (TSP-2), proMMP-2 first associates with an unknown cell-surface heparin-sulfate proteoglycan before interacting with LRP-1 [49]. When complexed with its specific inhibitor TIMP-2, proMMP-2 first binds to an unidentified coreceptor before being internalized by LRP-1 [50]. Likewise, the endocytic clearance of MMP-13 by LRP-1 requires a two-step process, involving a first binding to a 170 kDa co-receptor [51]. The internalization of MMP-9 by LRP-1 requires a more simple mechanism. Thus, proMMP-9/TIMP-1 directly interacts with LRP-1, leading to its endocytic uptake and degradation by lysosomal proteases [52]. The analysis of this interaction reveals that the hemopexin domain of MMP-9 contains a binding site for LRP-1 [53].

Although Hahn-Dantona et al. failed to demonstrate a direct interaction between TIMP-1 and LRP-1 in their *in vitro* study [52], our unpublished data reveal that LRP-1 could bind and endocytose TIMP-1 in neurons, in an MMP-independent way. Furthermore, noncomplexed TIMP-2 [50] and TIMP-3 [54, 55] also bind directly to LRP-1 to be internalized.

*4.2.3. Other Matrix Proteinases.* Heparanase-1 is secreted as an inactive heparanase precursor. Once activated, this endoglycosidase degrades heparan sulfate and consequently alters the stability of ECM [56]. The group of Guido David [57] has clearly identified LRP-1 as one of the receptors able to mediate the uptake of secreted heparanase precursor and its intracellular trafficking to the site of activation process. Recently, ADAM with thrombospondin motifs 5 (ADAMTS-5), a major aggrecan-degrading enzyme in cartilage, has been shown to be endocytosed by LRP-1 [58].

The aspartic proteinase cathepsin-D (cath-D) is capable of degrading ECM in an acidic microenvironment [59]. Recently, Liaudet-Coopman and colleagues [60, 61] identified pro-cath-D as the first ligand of the extracellular domain of LRP-1 $\beta$-chain.

*4.3. Signaling Function.* Additionally, LRP-1 acts in signaling pathways [8, 28, 62]. We recently demonstrated that the abrogation of LRP-1 expression inhibited migration and invasive capacities of thyroid carcinoma cells despite a strong stimulation of pericellular MMP-2 and uPA proteolytic activities [63]. We identified ERK and JNK as the main molecular relays by which LRP-1 regulates focal adhesion disassembly of malignant cells to support invasion [64].

A stimulating study reveals that LRP-1-mediated endocytosis of tPA and tPA/PAI-1 complex is accompanied by a decrease in tPA mRNA transcription [65], suggesting that a secreted protein could regulate its own biosynthesis. Furthermore, the binding of tPA to LRP-1 triggers intracellular signal transduction to induce the expression of another matrix proteinase, MMP-9, both in microvascular endothelial cells [66] and fibroblasts [67]. Probably more surprising, the binding of proteinase inhibitors to LRP-1 also induces MMP-9 expression, as demonstrated for the serpin nexin-1 in a mammary tumor model [68], and activate $\alpha$2M in macrophage-derived cell lines [69]. Recently, the knockdown of LRP-1 expression in human glioblastoma U87 cells revealed that LRP-1 promoted cell migration and invasion by inducing the expression of MMP-2 and MMP-9 [70].

Altogether, these data indicate a close link between MMP-9 and LRP-1: from one of its ligands to a product of LRP-1-induced expression. This suggests important functions for MMP-9 in normal and pathophysiological conditions.

*4.4. Regulation of LRP-1 Cell-Surface Level and Endocytic Activity by Shedding.* Most membrane proteins, including type I and type II transmembrane proteins, are subjected to a shedding process, that is, the proteolytic cleavage of their extracellular part or ectodomain [71]. LRP-1 also constitutes a membrane target for numerous proteinases. The LRP-1 ectodomain consists in the entire extracellular $\alpha$-chain (515 kDa) noncovalently associated to the extracellular part (55 kDa) of the transmembrane $\beta$-chain [72].

The product of LRP-1 shedding, the soluble LRP-1 (sLRP-1) $\alpha$-chain, was first detected in human plasma and serum [73]. A metalloproteinase, cleaving LRP-1 at the membrane-proximal region of the $\beta$-chain, was described in human BeWo choriocarcinoma cells [72]. Since this work was completed, different metalloproteinases have been identified, mainly among the ADAM family. Thus, ADAM-10 and ADAM-17 are associated to LRP-1 shedding in human brain [74]. We recently showed that ADAM-12 exhibited sheddase activity towards LRP-1 in human HT1080 fibrosarcoma cells [75]. Additionally, we reported that MT-MMP, first described to degrade LRP-1 in small fragments [76], was able to generate sLRP-1 in medium conditioned by HT1080 cells in culture [75]. Besides these metalloproteinases, tPA and BACE-1 were described to mediate shedding of LRP-1 [77, 78]. It has been reported that, during cerebral ischemia, tPA induces the shedding of LRP-1 from perivascular astrocytes followed by the development of cerebral edema [79]. These authors demonstrated that the interaction between tPA and LRP-1 in perivascular astrocytes induced Akt phosphorylation, leading to an increase of permeability in the blood-brain barrier.

Soluble LRP-1, which is composed of the entire extracellular $\alpha$-chain and noncovalently associated extracellular part of the transmembrane $\beta$-chain [72], retains ligand-binding capacity and acts as a decoy receptor [80]. Thus, Quinn and colleagues first reported that the addition of sLRP-1 to cultured rat hepatocytes resulted in an inhibition of tPA clearance [73]. Immunoprecipitation assays confirmed that tPA interacted with LRP-1 [72]. Also, LRP-1 shedding from human lung fibroblasts impairs endocytosis of MMP-2 and -9 [81]. We similarly reported that the inhibition of LRP-1 shedding increased MMP-2 and -9 activities, in cultures of human endometrial explants [82] and fibrosarcoma cells [75]. Finally, we recently demonstrated that TIMP-3 bound to sLRP-1, which is resistant to endocytosis, retained its inhibitory activity against metalloproteinases [55].

## 5. Conclusion

Understanding the precise role of LRP-1 in the regulation of ECM breakdown remains an exciting challenge, as it appears to be a multifunctional "Swiss knife." Thus, besides the endocytosis of proteinases, LRP-1 mediates the clearance of their own inhibitors [8]. Moreover, LRP-1 acts as a membrane receptor that transduces intracellular signals to induce the MMP-9 expression [66–69]. Finally, the proteolytic cleavage of LRP-1 at the cell surface solubilizes the LRP-1 ectodomain, which conserves ligand-binding capacities. Such a property could allow matrix proteinases—but also inhibitors—to increase their extracellular half-life time by escaping from endocytic clearance mediated by membrane-LRP 1.

Despite a strong stimulation of pericellular MMP-2 and uPA proteolytic activities, carcinoma cell invasion decreased by LRP-1 silencing [63]. This result clearly indicates that, depending on parameters yet to be elucidated, the signaling function of LRP-1 can counteract or override its endocytic function.

Another paradox is represented by a proteinase, tPA, for instance, which can either be endocytosed by LRP-1 or solubilize the ectodomain of LRP-1. At the molecular level, interactions between tPA and LRP-1 will be different according to the event: binding to domains 2 and 4 of the LRP-1 $\alpha$-chain for endocytic pathway or cleaving at a single site of both $\alpha$- and $\beta$-chains at the vicinity of the cell surface for shedding LRP-1 ectodomain. Which is determinant the enzyme or the cell?

On the whole, these data strongly suggest that LRP-1 does not act alone but with membrane partners, which vary according to numerous parameters including cell origin, ECM composition, pathological conditions, and so forth. In this way, we recently demonstrated [83] that LRP-1 forms complex with the hyaluronan receptor CD44, which may bind proMMP-9 [24]. The identification of these partners could represent a key to the understanding of the LRP-1 roles in ECM remodeling.

## Acknowledgments

The authors thank Claude-Annie Turlier for editorial assistance. They are supported by the Université de Reims Champagne-Ardenne and the Centre National de la Recherche Scientifique (CNRS), France. They acknowledge support from the Agence Nationale pour la Recherche (ANR-08-MNPS-042-02, TIMPAD project). Laurie Verzeaux and Stéphane Dedieu were recipients of grants from the Région Champagne-Ardenne.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 107954, 10 pages
http://dx.doi.org/10.1155/2013/107954

*Review Article*

# Prostate Stem Cells in the Development of Benign Prostate Hyperplasia and Prostate Cancer: Emerging Role and Concepts

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Received 23 April 2013; Revised 14 June 2013; Accepted 14 June 2013

Academic Editor: Mauro S. G. Pavao



Benign Prostate hyperplasia (BPH) and prostate cancer (PCa) are the most common prostatic disorders affecting elderly men. Multiple factors including hormonal imbalance, disruption of cell proliferation, apoptosis, chronic inflammation, and aging are thought to be responsible for the pathophysiology of these diseases. Both BPH and PCa are considered to be arisen from aberrant proliferation of prostate stem cells. Recent studies on BPH and PCa have provided significant evidence for the origin of these diseases from stem cells that share characteristics with normal prostate stem cells. Aberrant changes in prostate stem cell regulatory factors may contribute to the development of BPH or PCa. Understanding these regulatory factors may provide insight into the mechanisms that convert quiescent adult prostate cells into proliferating compartments and lead to BPH or carcinoma. Ultimately, the knowledge of the unique prostate stem or stem-like cells in the pathogenesis and development of hyperplasia will facilitate the development of new therapeutic targets for BPH and PCa. In this review, we address recent progress towards understanding the putative role and complexities of stem cells in the development of BPH and PCa.

## 1. Introduction

Prostate gland is a male accessory reproductive endocrine organ, which expels proteolytic solution in the urethra during ejaculation. In humans, the prostate is located immediately below the base of the bladder surrounding the neck region of the urethra. It is mainly associated with three types of disorders, namely, benign prostate hyperplasia (BPH), prostate cancer (PCa), and prostatitis. BPH and PCa are the most common pathophysiological conditions of prostate gland in elderly men. These diseases already represent significant challenges for health-care systems in most parts of the world. Epidemiologically, BPH is more prevalent in Asian population [1, 2]. Whereas, PCa is more common in the western world [3, 4]. Both the diseases are complex and multifactorial. Factors predisposing to the development of BPH or PCa include hormonal imbalance, oxidative stress, environmental pollutants, inflammation, hereditary, aging, and, more particularly, stromal to epithelial cells crosstalk [5–7]. So far, variety of growth factors and hormonal factors, including androgens and estrogens, has been described in the hyperplastic development of the prostate gland [8–10]. However, the cellular and molecular processes underlying the pathogenesis and development of BPH or PCa are poorly understood.

Stem cells have an extensive capacity to propagate themselves by self-renewal and to differentiate into tissue-specific progeny. It is well know that stem cells are required to maintain and repair tissues throughout the lifetime. The requirement to understand the biology of stem cells derived from the prostate is increasing, as new evidence suggests that BPH and PCa may arise from the stem or stem-like cell compartments [11–13]. This review summarises the biology of prostate stem or stem-like cells and their contribution in pathogenesis and development of BPH and PCa.

## 2. Prostatic Cellular Compartments

The prostate is a hormonally regulated glandular organ whose growth accelerates at sexual maturity due to androgen action on both stromal and epithelial cells [14, 15]. The human

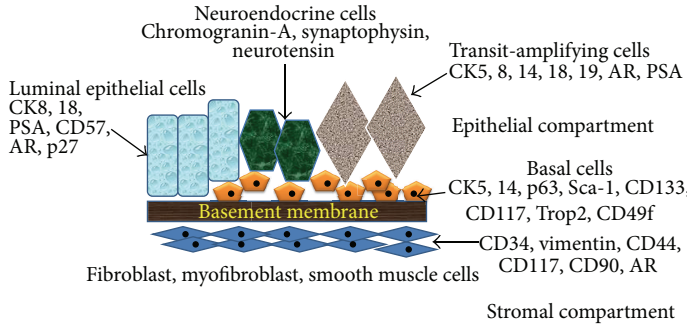


Figure 1: Prostatic cellular compartments and stem cell identity markers. Pictorial representation of different prostatic cells and their respective cellular markers.

prostate is a complex ductal-acinar gland that is divided into three anatomically distinct zones: peripheral, transitional, and central zones, which are surrounded by a dense and continuous fibromuscular stroma [16–18]. BPH, a nonmalignant overgrowth found in older men, mainly, develops in the transitional zone, while PCa arises primarily in the peripheral zone [19].

At histological level, human prostate contains mainly two types of cells that are called epithelial and stromal cells. The stromal to epithelial ratio in normal prostate of human is 2 : 1 [18, 20]. The epithelial cell layer is composed of four differentiated cell types known as basal, secretory luminal, neuroendocrine (NE), and transit-amplifying (TA) cells that are identified by their morphology, location, and distinct marker expression (Figure 1). The basal cells form a layer of flattened to cuboidal shaped cells above the basement membrane and express p63 (a homolog of the tumor suppressor gene *p53*), Bc1-2 (an anti-apoptotic factor), Cluster designation (CD) 44, hepatocyte growth factor (HGF), and the high molecular weight cytokeratins (CK) 5 and 14. The expression of androgen receptor (AR) is low or undetectable in the basal cells, which makes the basal cells independent of androgens for their survival [21–23]. The luminal cells are the major cell type of the prostate that form a layer of columnar-shaped cells above the basal layer and constitute the exocrine compartment of the prostate, secreting prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) into the lumen. They are terminally differentiated, androgen dependent, and nonproliferating cells, expressing low molecular weight CK8 and 18, CD57 and $p27^{Kip1}$ (a cell cycle inhibitor) [22–24] along with high levels of AR. NE cells are rare cells scattered in the basal and luminal layers of the prostate. They are terminally differentiated and androgen-insensitive cells, expressing chromogranin A, synaptophysin, and neuron-specific enolase (NSF) [23, 25, 26]. The NE cells also produce and secrete neuropeptides such as bombesin, calcitonin, and neurotensin that are believed to support epithelial cell growth and differentiation [19, 27, 28]. Additionally, there is a small group of intermediate cells referred to as TA cells that express both basal as well as luminal cell markers (CK5, CK8, CK14, CK18, AR, and PSA) [29–32]. The epithelial layer is surrounded by a stromal layer, which forms a peripheral boundary of the prostate gland. The stromal cell layer consists of several types of cells that include smooth muscle cells (the most abundant cell type in stroma), fibroblasts, and myofibroblasts. Stromal cells express mesenchymal markers like CD34, vimentin, CD44, CD117, and CD90 [33].

## 3. Stem Cell in Normal Prostate

Prostatic epithelium is, structurally and functionally, a highly complex tissue composed of multiple differentiated cell types, including basal, luminal, and neuroendocrine cells, along with small population of relatively undifferentiated cells generally known as "stem cells" that are endowed with self-renewal and differentiation capacities [26]. If the stem cells are key target for mutagenic changes and tumourigenesis in human prostate, we need to understand more about stem cell status in normal prostate tissue.

As the adult prostate is relatively slow-growing organ with limited cycles of cell proliferation and apoptosis, the possible existence of adult prostate stem cells (PSCs) was controversial for many years. Several investigations based on stem cell models have elegantly defined role of stem cells in cellular turnover and morphogenesis of normal prostate [30, 34]. Evidence for the existence of the stem cells in normal prostate came from the studies which demonstrated that adult rodent prostate can undergo multiple rounds of castration-induced regression and testosterone-induced regrowth [35–37]. Adult PSCs were believed to reside within the basal cell layer because of the ability of the basal cells to survive and undergo regression and regeneration following repeated castration and androgen replacement [38–40]. Adult mouse prostate epithelial cells, when transplanted along with the urogenital sinus mesenchymal cells under the renal capsule, generated normal murine prostate like structures [41]. Prostate glands were also regenerated when dissociated cells were implanted in Matrigel subcutaneously into immunodeficient mice [42]. Studies, including 5-bromo-2-deoxyuridine (BrdU) retention analysis, showed that the enriched population of BrdU-labelled cells possessing stem cell features (quiescent, high proliferation potential) are localized at the proximal region of mouse prostate duct [43] and are programmed to regenerate proximal-distal ductal axis [44]. The proximal region of the prostatic duct is surrounded by a thick band of smooth muscle cells [45] that are known to produce high level of transforming growth factor-beta (TGF-$\beta$) [46], which is known to play a critical role in maintaining the relative

dormancy of the PSCs [47]. Independent study by Burger et al. also identified a candidate population of PSCs in the proximal region of mouse prostatic ducts, using stem cell surface marker known as stem cell antigen 1 (Sca-1, also known as Ly6a) [48]. In addition to high expression of Sca-1, these cells were shown to coexpress integrin $\alpha$6 (CD49f) and Bcl-2. The cells with these properties showed a higher efficiency to generate prostatic tissue in an *in vivo* reconstitution assay [48]. Lawson et al. showed that sorting prostatic cells for CD45(−)CD31(−)Ter119(−)Sca-1(+)CD49f(+) antigenic profile results in a 60-fold enrichment for colony and sphere-forming cells that can self-renew and expand to form spheres for many generations [49]. Leong and colleagues identified CD117 (c-Kit, stem cell factor receptor) as a new marker of a rare adult mouse PSC population that showed all the functional characteristics of stem cells including self-renewal and full differentiation potential. The CD117(+) single stem cell defined by the phenotype Lin(−)Sca-1(+)CD133(+)CD44(+)CD117(+) regenerated functional, secretion-producing prostate after transplantation *in vivo*. Moreover, CD117(+) PSCs showed long-term self renewal capacity after serial isolation and transplantation *in vivo*. CD117 expression was predominantly localized to the proximal region of the mouse prostate and was upregulated after castration-induced prostate involution, consistent with prostate stem cell identity and function [50].

Stem cells in the human prostate have been identified and isolated using the cell surface markers such as integrin $\alpha2\beta1$ [51], CD133 (Prominin-1) [52], and CK6a (cytokeratin 6a) [53]. Based on high expression of $\alpha2\beta1$ integrin, Collins and colleagues identified PSCs in the basal layer and showed that the $\alpha2\beta1^{high}$ integrin cells represent ~1% of basal cell population in the human prostate [51]. This selected PSC population was enriched through rapid adherence to the type I collagen and showed higher colony-forming efficiency *in vitro*. Furthermore, when the $\alpha2\beta1^{high}$ integrin cells were grafted subcutaneously together with stromal cells in Matrigel into nude mice, they formed prostatic gland structures *in vivo*. Nevertheless, these glandular-like structures, although containing basal cytokeratin positive as well as AR, PAP, and PSA positive cells, lack well-defined basal and luminal organizations [51]. However, recent studies by Missol-Kolka et al. have reported that the overall expression of CD133 in human prostate is not strictly limited to the rare basal stem and progenitor cells, but it is also expressed in some of the secretory luminal cells [54]. Furthermore, it has been shown that CD133 is downregulated in prostate cancer tissues and upregulated in the luminal cells in the vicinity of cancer area. In contrast to the human CD133, the mouse CD133 has been shown to express widely in prostate [54]. Several other surface markers, such as aldehyde dehydrogenase (ALDH), tumor-associated calcium signal transducer 2 (Trop-2), ATP-binding cassette transporter family membrane efflux pump (ABCG2), p63, and CD44, have also been reported for identification and isolation of the PSCs from the prostate tissues of human and mouse [49, 55–60]. Moreover, Trop2(+)CD44(+)CD49f(+) were used as the markers to identify basal stem cells with enhanced prostasphere-forming and tissue-regenerating abilities [61]. Unlike the murine PSCs, the human PSCs are randomly distributed within the basal epithelial layer throughout the acini and ductal regions of the prostate [51, 52]. In addition to the expression of stem-cell-specific markers, different studies have also shown that PSCs express both basal and luminal cell-specific markers in fetal and adult stages of prostate development [13, 22, 31, 62, 63]. Several studies have proposed the existence of different cell compartments based on stem-cell-driven differentiation hierarchical arrangements within the prostate epithelium [24, 29, 30, 64].

In addition to prostate epithelial stem cells, stromal stem cells (SSCs) have also been reported to exist in the prostate, where they are postulated to carry out function of replacing and regenerating local cells that are lost to normal tissue turnover, injury, or aging [65–67]. These subpopulation of SSCs expressed mesenchymal stem cell (MSC) markers such as CD34 and Sca-1, showed a high proliferative activity and ability to differentiate into fibroblastic, myogenic, adipogenic, and osteogenic lineages [68]. Of all these potential lineages, the most characteristic cell type derived from prostate stromal stem cell is fibroblast or smooth muscle cells [68, 69]. Growth factors that have regulatory effects on SSCs include members of TGF-$\beta$ superfamily, the insulin-like growth factors, the fibroblast growth factors, the platelet-derived growth factor, and Wnts [70]. It is believed that the differentiation of stromal stem cells to smooth muscle cells is due to paracrine effects of prostrate epithelial cells, which permanently commit the stromal stem cells to mature into androgen receptor (AR) expressing smooth muscle cells [68].

## 4. Stem Cell in Benign Prostate Hyperplasia (BPH)

BPH is a slow progressive enlargement of the prostate gland which can lead to lower urinary tract symptoms (LUTS) in elderly men. It is characterized by hyperproliferation of epithelial and stromal cells in the transition zone of the prostate gland, which can be observed histopathologically [71]. Despite of its obvious importance as a major health problem, little is known in terms of biological processes that contribute to the development of BPH. To explain the etiology behind the pathogenesis of BPH, several theories, including stem cell, hormonal imbalance, apoptosis, epithelial-mesenchymal transition, embryonic awakening, and inflammation, have been proposed in recent years, and all of them seem to contribute together to some extent in the pathogenesis of BPH [12, 72]. According to stem cell theory, the stem cell population residing in the prostate gland is increased due to abnormal proliferation and apoptosis of stem cells, which may eventually contribute to BPH pathogenesis. Earlier, it was reported by Berry et al. that stem cell population is responsible for prostate gland maintenance [73]. Changes in tissue consistency and cellular hyperplasia are accompanied by downregulation of apoptotic factors and increased level of antiapoptotic factors that decrease the rate of prostatic cell death and, thus, contributing to hyperproliferation of prostatic tissue [74]. It has been reported that stromal to epithelial

ratio is altered in BPH, where the ratio increases from 2 : 1 in normal glands to 5 : 1 in BPH [75]. Because stromal hyperproliferative activity is thought to promote the development of BPH, the existence of adult stem cells in the prostate stromal compartment is speculated to expand the stroma in response to stimuli during the pathogenesis of BPH [68]. Lin et al. showed that primary culture of prostate cells from BPH patients possessed many common stem cell markers, including CD30, CD44, CD54, neuronspecific enolase (NSE), CD34, vascular endothelial growth factor receptor-1 (Flt-1), and stem cell factor (SCF, also known as KIT ligand or steel factor) [68]. Compared to CD30, CD44, CD54, and NSE, the CD34, Flt-1, and SCF markers were expressed at low level. These stem cells were negative for CD11b, stem cell antigen-1 (SCA-1), SH2, AA4.1, and c-Kit. Furthermore, among this stem cell population only a fraction (5%) of the stem cells was positive for CD133 [68]. Although the origin of these stem cells is not known, the CD49(+)CD54(+)NSE(+)SCF(+) cell marker profile of these cells suggests that they are in a lineage closely related to MSCs. The stem cell population with the above profile possessed ability to differentiate or transdifferentiate into myogenic, adipogenic, and osteogenic lineages [68, 76]. Ceder et al. reported the possible existence of prostate stromal stem/progenitor cells in the adult human prostate [76]. This stromal population expressed vimentin (a mesenchymal marker), CD133, c-Kit, and SCF, with expression profiles similar to those observed in the Cajal cells of gastrointestinal tract, which represent a subset of stem cell-like cells. Several studies have identified c-Kit-expressing interstitial cells in the stromal compartment of human prostate [77–79]. Altered patterns of c-Kit expression have been reported in benign lesions of prostate and breast tissues [80, 81]. It has been shown that the c-Kit expression and number of c-Kit(+) interstitial cells were significantly higher in BPH than those of the normal prostate. Furthermore, it has been suggested that c-Kit regulates cell proliferation in prostate and plays a crucial role in the pathophysiology of BPH via altering the expression of JAK2 and STAT1 [77].

Stem cells from the BPH samples expressing CD49f, CD44, or CD133 markers have been shown to possess monolayer- and spheroid-colony-forming ability, where the highest (98%) recovery of colony-forming cells (CFCs) was achieved by CD49f(+) cells as compared to CD44(+) (17%) or CD133(+) (3%) cells [82]. These CFCs showed the capacity to undergo clonal proliferation, generates branching ductal structures, and they expressed both basal and luminal lineage markers. Further characterization of CD49f(+) cells revealed that they are comprised of two cell types: CK5(+) basal epithelial cells and CD31(+) endothelial cells [82]. Sca-1- and CD34-expressing cells isolated from BPH tissue showed a high proliferative capacity and increased plasticity, as these cells were able to differentiate into fibroblastic, myogenic, adipogenic and osteogenic lineages, similar to that of MSCs [68, 83]. Furthermore, Burger and colleagues found that cells with high Sca-1 expression had considerably more growth potential, and proliferative capabilities than cells expressing low or no Sca-1 antigen [48]. Expression of pluripotency markers such as *Oct4A*, *Sox2*, *c-Myc*, and *Klf4* might represent a stemness-specific gene signature. A very recent study has demonstrated a relatively high expression of stemness-associated genes, including *Oct4A*, *Sox2*, *c-Myc*, *Nanog*, and *Klf4*, in BPH as compared to normal prostate tissue [84]. Thus, several studies have revealed the presence of stem cells that express pluripotency-associated markers and are hyperproliferative and capable of differentiation into different cell lineages within the hyperplastic prostate tissue. The presence of these high proliferative and plastic stem cells in the BPH tissue samples suggests that BPH could occur as a result of changes in the stem cell properties that could ultimately give rise to a clonal expansion of cell populations.

## 5. Stem Cell in Prostate Cancer (PCa)

PCa is the most prevalent and is the second most frequently diagnosed cancer and sixth leading cause of cancer-related deaths among men in the world [85]. Its etiology, although not clear, is partly attributed to multigenic and epigenetic mechanisms and the heterogeneous nature of this disease [4, 86–88]. Gleason and others described that when the transition of normal gland into adenocarcinoma of prostate takes place, its normal histological structure is disrupted and results in abnormal proliferation of the glandular structure, destruction of basement membrane, and progressive loss of basal cells (<1%) [87, 89]. In addition, AR(+) luminal cells increase and contribute in bulk of prostate mass (>99%) in PCa [90]. It is hypothesised that prostate cancer arises from AR(+) luminal cells and dramatic loss of basal cells. To support this hypothesis several investigations have been conducted [4, 91–93]. In addition, mouse basal population expressing Lin(−)Sca-1(+)CD49f$^{high}$ cells can differentiate into luminal cells in xenograft [49]. Lin(−)Sca-1(+)CD49f$^{high}$ cells from a Pten−/− mouse model display cancer stem cells phenotypes, which gave rise to adenocarcinoma after transplantation [94]. It has been reported that basal cells are the possible cells of prostate cancer origin [95]. When Goldstein et al., especially injected the mixture of urogenital sinus mesenchyme (UGSM) with human prostate basal (expressing CD49f$^{high}$ and Trop2$^{high}$) or luminal cells (expressing CD49f$^{low}$ and Trop2$^{high}$) into the subcutaneous space of immunodeficient NOD(−)SCID(−)IL(−)2Rg−/− mice, only basal cells formed prostatic duct after 16 week, whereas no prostatic duct or adenocarcinoma developed when using luminal cells [91, 95]. Luminal derived grafts lack epithelial structures and mimicked transplantation of UGSM cell alone [95]. Collins et al. reported basal cancer stem cells isolated from human prostate cancer biopsies expressing Cd44(+), $\alpha 2\beta 1^{high}$, and Cd133(+) and cell surface markers were of self renewal *in vitro* [96]. ALDH$^{high}$ is another marker used for cancer stem cells in human prostate cancer cell lines. Cells expressing ALDH$^{high}\alpha 2(+)/\alpha 6(+)/\alpha v(+)$-integrin CD44(+) showed increased tumourigenicity and metastasis *in vivo* and enhanced invasiveness *in vitro* [97]. Prostate cancer stem cells isolated from LNCaP and DU145 cell lines also showed expression of CD44(+), $\alpha 2\beta 1^{high}$, and CD133(+) markers [98, 99]. In addition, CD44(+) population isolated from xenograft human tumour and cell lines displayed high tumour initiating

ability and metastasis *in vitro* [100]. Recently, Rajasekhar and his group isolated a small cell population expressing TRA-1-60(+)CD151(+)CD166(+) markers that displayed stem cell like features with increased NF-kB signalling along with basal cell markers, and this recapitulates the cellular hierarchy of the tumour origin from basal cells [101].

Over all data from several investigators indicated that origin of prostate cancer can be from basal stem cell population, which expresses CD44(+), $\alpha 2\beta 1^{high}$, CD133(+), $ALDH^{high}$, and other normal basal stem cell markers.

## 6. Stem Cell Niche and Plasticity

Stem cells are localized in a defined microenvironment, which is known as their "niche." The main function of a niche probably is to provide specific factors necessary for the maintenance of the stem cell properties via a combination of intracellular and intercellular signalling. These factors include a complex array of growth factors, cytokines, chemokines, and adhesive molecules known to be capable of altering the balance between proliferation, differentiation, and quiescence in stem cell populations [102, 103]. One can probably assume that this is equally true for prostatic stem cells as it is for other stem cell populations.

PSCs reside in niche areas within the basal layer of the epithelial compartment at a low percentage of approximately 0.5–1% [34]. PSCs population in the prostate undergoes a series of phenotype changes. Specifically, the basal SCs do not express the AR or the p63 protein. They have extended proliferative potential by slow cycling. According to these studies, it is postulated that, in addition to the reserve stem-cell population, there is a "TA" cell type, which is characterized by the expression of p63, as well as other basal markers such as CK5 and 14, Jagged-1, and Notch-1 [64, 104, 105]. A TA cell does not express AR protein and it is dependent, for proliferation, but not for survival, on andromedans secreted by stromal cells [105]. Under normal conditions a PSC is slow cycling in that it divides occasionally, undergoing asymmetric division to give rise to a new PSC along with a more differentiated TA daughter cell. TA cell undergoes a limited number of rapidly amplifying cell division cycles to increase the cell population derived from a single PSC before leaving the proliferative compartment to produce intermediate cell [106]. This intermediate cell expresses both epithelial specific (CK5 and 14) and luminal specific (CK8 and 18) cytokines, AR mRNA (but not protein), and prostate stem cell antigen (PSCA) [105, 107]. As an intermediate cell migrates through the basal layer, it differentiates into various terminally differentiated cell lineages of prostate epithelium.

## 7. Is BPH/PCa a Stem Cells Disease?

Numerous investigators demonstrated presence of stem cell in prostate tissue by using various high-end techniques that may contribute to local invasive to metastatic disease in human and research animals. In normal tissue-development, homeostasis is maintained by differentiation of stem cells and

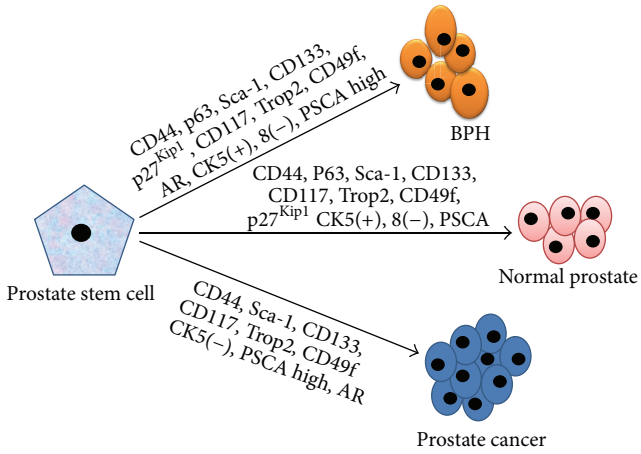


Figure 2: Cellular identity of stem cells in prostate. Stem cell model of normal tissue renewal, BPH and PCa.

programmed cells death in regular cell cycle. This mechanism is established through interactions with tissue specific environmental factors such as growth factors and steroid hormones. Many signalling molecules and factors involvement have been reported in stem cell self-renewal and implication in cancer stem cells (CSCs) regulation (Figure 2).

Although the precise role of stem cells in tumourigenesis is still in debate, it is widely accepted that cancers can arise from normal stem cells which may accumulate mutation, genetic changes, and molecular pathway alterations that disrupt self-renewal control capacity (Table 1). It has been reported that, in prostate, putative stem/progenitor cells can reside in CK5(+) 8(−) basal cells. A diagnostic feature of human prostate cancer is the loss of basal cells [108], indicating cancer origin cells as basal cells. In BPH, CD133(+) cells expressed genes related to undifferentiated cells such as TDGF1 (teratocarcinoma-derived growth factor 1) and targets of the Wnt and Hedgehog developmental pathways, whereas CD133(−) cells showed upregulation of genes related to proliferation and metabolism. In cancer, CD133(+) cells specifically displayed more TA population phenotype with increased metabolic activity and proliferation, possibly explaining the transition from a relatively quiescent state to an active growing tumour phenotype. This reflects that CD133 isolates from benign and malignant tissues show biologically distinct characteristics [109]. CSCs exploit many of the signal pathways such as notch, hedgehog- and TGF-$\beta$, which play, important role in proliferation and differentiation in prostate stem cell [110, 111]. The sonic hedgehog signalling element receptor PTCH1 and glioma-associated oncogene homolog-1 (GL1) transcription factor were especially reported to be colocalized with p63 basal marker in BPH and PCa cells, expressing CD44/CK8/14. This suggests that hedgehog pathway may induce differentiation of prostate stem/progenitor cells into CD44(+)/P63(+/−) hyperplasia basal cells [112]. Other studies on DNA damage and proliferation markers $p27^{Kip1}$, cyclin D3, and Ki-67, revealed interesting findings. It has been shown that $p27^{Kip1}$ is significantly upregulated in BPH,

Table 1: Molecular alterations in BPH and PCa.

| Factors | Normal prostate | BPH | PCa |
|---|---|---|---|
| Prostate-specific factors | | | |
| 5 $\alpha$ reductase | Normal | Upregulated | Upregulated |
| Androgen receptor (AR) | Normal | Upregulated | Upregulated |
| AR coactivator | Normal | Upregulated | Upregulated |
| Androgen corepressor | Normal | Upregulated | Upregulated |
| PSA level in serum | (0–4 ng/mL) | (2–8 ng/mL) | (4–10 ng/mL) |
| Growth factors | FGF-2,7,9<br>IGF 1,2<br>IGFBP-2 | FGF 1,2,9<br>IGF-2 high<br>IGFBP-3 | FGF-1,2,6,8<br>IGF-1 high<br>IGFBP-2 high<br>IGFBP-3 high |
| NE cells | Normal | Number decrease | Number increase |
| Luminal cell factors | Vimentin<br>Intracellular space normal<br>PMSA normal | Vimentin increase<br>Intracellular space increase<br>PMSA decrease | Vimentin over exp<br>Intracellular space decrease<br>PMSA increase |
| Basal cells | Present | Present | Absent |
| Stromal cell factor | Fibroblast content normal<br>NMMHC<br>Elastin<br>SMMHC | Fibroblast content increase<br>NMMHC increase<br>Elastin decrease<br>SMMHC decrease | Fibroblast content increase<br>NMMHC<br>Elastin increase<br>SMMHC decrease |
| Stem cell markers | CD44, P63, Sca-1, CD133, CD117, Trop2, CD49f, $p27^{Kip1}$, CK5(+), 8(−), PSCA | CD44, p63, Sca-1, CD133, $p27^{Kip1}$, CD117, Trop2, CD49f, AR, CK5(+), 8(−), PSCA high | CD44, Sca-1, CD133, CD117, Trop2, CD49f, CK5(−), PSCA high, AR |

whereas it is downregulated in PCa. In addition to downregulation of $p27^{Kip1}$, there is also up regulation of Ki-67 and cyclin D3 in PCa [113].

Several lines of evidence have been indicated that CSCs exhibit both stem cells and cancer cells characteristics. CSCs have the ability to form tumors when transplanted into an animal host. CSCs can be distinguished from other cells within the tumor by cell division and alterations in their gene expression profile [114].

Advanced prostate cancer is androgen independent and basal cells can be phenotypically identified in the majority of metastases [115]. Studies from several investigators revealed that tumor-initiating cells are negative for AR and p63 and expressed the stem cell markers Oct-4, Nanog, Sox-2, Nestin, CD44, CD133, and CD117. Moreover, Sca-1-positive cells having the ability with prostate-regeneration activity, showed evidence of a basal and luminal lineage [96, 100, 116, 117]. Gu et al. demonstrated human telomerase reverse transcriptase-(hTERT-) positive epithelial cells could regenerate tumor in mice that resembled the original tumor in patients [118]. These finding may be indicative of CSC role in prostate cancer.

The growing understanding of the prostate stem cell biology provides the rationale for acute approaches. But without a clear definition of stem cells in normal prostate and BPH/PCa, it is difficult to determine whether the cancer cell of origin in prostate is a stem cell, multipotent progenitor/TA cells, or a more differentiated progeny. Nonetheless, evidence exists that the cellular origin can include both basal and luminal cells.

## 8. Conclusion

The prostate stem cells are a key role player in prostate tumourigenesis and enlargement disorders. But their precise role in disease pathogenesis remains unknown. The prostate stromal and epithelial compartments and their reciprocal paracrine and autocrine interactions are crucial regulators of prostatic tissue homeostasis. The combination of the prostatic cell surface markers, such as Sca-1, CD133, p63, and CD49f, can aid in the identification of prostate stem cell populations. However, a prostate-specific stem cell marker has yet to be identified. The study of CSCs is still in its early stages. No standard treatments have yet been developed as a result of research on CSCs. The isolation and characterization of epithelial, stromal stem cells and cancer stem cells in the prostate will lead to understanding normal stem cells and CSCs activity to identify new strategies for the control of prostate diseases without harming normal cells milieu.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 147514, 14 pages
http://dx.doi.org/10.1155/2013/147514

*Research Article*

# The Exposure of Breast Cancer Cells to Fulvestrant and Tamoxifen Modulates Cell Migration Differently

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Received 30 April 2013; Accepted 10 June 2013

Academic Editor: Davide Vigetti



There is no doubt that there are increased benefits of hormonal therapy to breast cancer patients; however, current evidence suggests that estrogen receptor (ER) blockage using antiestrogens is associated with a small induction of invasiveness *in vitro*. The mechanism by which epithelial tumor cells escape from the primary tumor and colonize to a distant site is not entirely understood. This study investigates the effect of two selective antagonists of the ER, Fulvestrant (Fulv) and Tamoxifen (Tam), on the invasive ability of breast cancer cells. We found that 17$\beta$-estradiol ($E_2$) demonstrated a protective role regarding cell migration and invasion. Fulv did not alter this effect while Tam stimulated active cell migration according to an increase in Snail and a decrease in E-cadherin protein expression. Furthermore, both tested agents increased expression of matrix metalloproteinases (MMPs) and enhanced invasive potential of breast cancer cells. These changes were in line with focal adhesion kinase (FAK) rearrangement. Our data indicate that the anti-estrogens counteracted the protective role of $E_2$ concerning migration and invasion since their effect was not limited to antiproliferative events. Although Fulv caused a less aggressive result compared to Tam, the benefits of hormonal therapy concerning invasion and metastasis yet remain to be investigated.

## 1. Background

Breast cancer is the most frequent malignancy cancer in women. It is estimated that approximately 75% of breast tumors are estrogen receptor (ER) positive, and their growth is stimulated by estrogens [1]. Estrogen-based therapies represent the mainstay in the treatment of hormone-dependent breast cancer with the ER modulator Tamoxifen (Tam) improving significantly the clinical outcome of patients with both early and advanced breast cancer [2]. Furthermore, Fulvestrant (Fulv) that belongs to a recently developed group of antiestrogens (selective estrogen receptor downregulators—SERDs) has extended the therapeutic options in the management of breast cancer patients [2, 3].

Invasion is considered as the hallmark of malignancy and is the first in the cascade of events leading to tumor development and metastasis. During invasion, the tumor cells penetrate into tissues breaking the basement membrane and allowing tumor growth. The invading tumor cells are able to enter the circulation so as distant metastasis occurs [4, 5]. Both invasion and metastasis require cell migration. The cell type and tissue microenvironment define the way of cell movement that is generally categorized as single and collective cell migration. During single cell migration, cells disseminate from the primary tumor as individual using either amoeboid or mesenchymal type movement, while in collective migration cells move as cell sheets or clusters [6, 7].

Degradation of the extracellular matrix (ECM) is one of the most important events in the spread of malignant cells, and it is well documented that it plays an essential role in tumor prognosis [8]. Matrix metalloproteinases (MMPs), zinc finger dependent enzymes, promote invasion, metastasis, and angiogenesis through the digestion of ECM components as well as surface factors' receptor and junctional proteins involved in cell-cell and cell-ECM interactions. MMPs consist of 23 members, which are classified into

different groups, including gelatinases. MMP-2 and MMP-9 are gelatinases that are related to tumor invasion and metastasis by their capacity for tissue remodeling via ECM, as well as their involvement in epithelial mesenchymal transition (EMT) [8, 9]. EMT is the key mechanism by which tumor cells gain invasive and metastatic ability, as EMT enables separation of individual cells from the primary tumor mass and promotes cell migration. During EMT, epithelial cells lose polarity and cell-cell contacts and undergo a complete remodeling of the cytoskeleton that leads to the acquisition of the mesenchymal features such as motility, invasiveness, and resistance to apoptosis [10–12]. One of the most pivotal steps in this process is the loss of E-cadherin, a cell-adhesion protein that maintains the cell-cell contacts [13]. However, the expression of E-cadherin is regulated by several transcription factors including Snail, Slug, and Twist. Furthermore, the nonreceptor tyrosine kinase focal adhesion kinase (FAK) is associated with highly invasive breast cancers, and it mediates several pathways leading to proliferation, migration, and adhesion [14]. Phosphorylation is required for FAK activation, and it has been shown that estrogens are able to promote rapid phosphorylation of FAK at tyrosines residues [15].

Despite the undoubted benefits that estrogen-based therapies offer to $ER^+$ breast cancer patients, *de novo* and acquired resistance to such therapies presents a major clinical problem [16]. The aim of the current study is to evaluate the effect of antiestrogens Fulv and Tam as well as the active metabolites of Tam, Endoxifen (End), and 4-OH-Tamoxifen (4-OH-T) on migration of 17$\beta$-estradiol- ($E_2$-) stimulated breast cancer cells. We focused on single and collective cell migration since these are the main ways for cells to migrate. To understand the effect of estrogen receptors' inhibition on cell migration, we assessed the effect of the antiestrogens on MMPs levels, on protein levels as well as on localization of E-cadherin and Snail and colocalization of FAK phosphorylated form with actin fibers.

## 2. Methods

*2.1. Cell Culture and Reagents.* In the current study, the human hormone-dependent breast cancer cell lines MCF-7 and T47D were purchased from the American Type Culture Collection (ATCC, USA). The adenocarcinoma cell line MCF-7 was cultured in EMEM supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% fetal bovine serum (FBS). The ductal carcinoma cell line T47D was cultured in RPMI 1640 supplemented with 4.5 g/L glucose (Sigma-Aldrich, Inc., USA) and 10% FBS. Both mediums were supplemented with 0.01 mg/mL insulin (Sigma-Aldrich, Inc., USA), 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 100 $\mu$g/mL penicillin G/streptomycin, 2.5 $\mu$g/mL amphotericin B, and 50 $\mu$g/mL gentamycin. All mediums and supplements were purchased from Biochrom (Berlin, Germany) unless otherwise indicated. Cells were cultured at 37°C, 5% $CO_2$, and 100% humidity.

$E_2$, Fulv, Tam, End, and 4-OH-T were purchased from Sigma-Aldrich (Sigma-Aldrich, Inc., USA). All experiments were performed according to the following conditions: after reaching 70% confluence, cells were washed with phosphate buffer saline (PBS) and incubated with phenol red-free RPMI (rf-RPMI) (Biochrom, Berlin, Germany) with 1% charcoal-stripped serum (CSS) for 24 h to deplete estrogen [17]. Thereafter, cells were treated with $E_2$ and the tested agents at the indicated time points and doses according to appropriate assay.

*2.2. Cell Proliferation Assay.* The effect of $E_2$ and the tested agents on proliferation of cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium-bromide (MTT) assay, as previously described [18]. Briefly, both MCF-7 and T47D cells, were seeded at a density of $2 \times 10^4$ cells/well in 24-well plates with rf-RPMI supplemented with 1% CSS. Cells were treated with $E_2$ 10 nM alone or in combination with the tested agents: Fulv + $E_2$, Tam + $E_2$, End + $E_2$, and 4-OH-T + $E_2$ for 48 h. The tested agents were added at two different concentrations: 100 nM and 1 $\mu$M. MTT solution (5 mg/mL in PBS) was prepared and a volume equal to 1/10 was added to each well and incubated for 2 h, at 37°C. Medium was removed and 100 $\mu$L acidified isopropanol (0.33 mL HCl in 100 mL isopropanol) was added in each well in order solubilise the dark blue formazan crystals. The solution was transferred to 96-well plates and immediately read in a microplate reader (Tecan, Sunrise, Magellan 2) at a wavelength of 570 nm using reference wavelength 620 nm.

*2.3. Migration Assay.* Migration assay was performed using boyden chambers (Costar, Avon, France) containing uncoated polycarbonate membranes with 8 $\mu$m pores. Briefly, cells were treated with $E_2$ and the tested agents for 24 h with rf-RPMI supplemented with 1% CSS. Cells were trypsinized and resuspended at $2 \times 10^4$ cells/0.1 mL in the same medium in presence of $E_2$ and the tested agents. The bottom chamber was filled with 0.6 mL of rf-RPMI with 10% CSS. The upper chamber was loaded with the solution of $2 \times 10^4$ cells and incubated for 36 h. After incubation, the membrane was fixed with saline-buffered formalin and stained in 1% toluidine blue solution. Images of cells that have migrated through the filter were captured using an inverted microscope of Nikon (Eclipse TE 2000-U) at magnification of 10X.

*2.4. Invasion Assay.* To evaluate the effect of $E_2$ and tested agents on capacity of cell to invade, a Boyden chamber containing matrigel-coated polycarbonate membranes with 8 $\mu$m (Invasion Chambers, BD Biosciences, Oxford, UK) was used. Briefly, cells were treated with $E_2$ and the tested agents for 24 h with rf-RPMI supplemented with 1% CSS. Cells were trypsinized and resuspended at $1.25 \times 10^5$/mL in the same medium in presence of $E_2$ and the tested agents. The bottom chamber was filled with 0.7 mL of rf-RPMI with 10% CSS. The upper chamber was loaded with the solution of $1.25 \times 10^5$cells and incubated for 72 h at 37°C. After the incubation, the noninvading cells were removed from the upper compartment using a cotton swab. Transwell filters were fixed with saline-buffered formalin for 10 min and then in 100% methanol for 20 min. Cells were stained

in toluidine blue solution for 10 min and washed twice in 1% PBS. Images of cells that have migrated through the matrigel-coated filter were captured using an inverted microscope of Nikon (Eclipse TE 2000-U) at magnification of 10X.

*2.5. Scratch-Wound Assay.* The effect of $E_2$ and the tested agents on collective cell migration was evaluated using 2D scratch-wound assay. Briefly, cells were seeded in 6-well plates at a density of $10^5$ cells/well. After reaching 100% of confluence, cells were treated with $E_2$ and the tested agents in the appropriate medium rf-RPMI with 10% CSS for 24 h. In the confluent cells' monolayer an artificial gap was created with a yellow pipette tip. Then cells were rinsed several times with the appropriate medium to remove dislodged cells. Images of living cells were captured at the indicated time points of 0, 24, and 48 h at magnification of 4X using an inverted microscope (Nikon Eclipse TE 2000-U).

*2.6. Zymography.* Zymography was used to evaluate the expression both of pro- and active forms of MMP-2 and MMP-9. Supernatants from both cell lines were collected in 48 h, concentrated 80-fold to 50 $\mu$L, and analyzed as previously described [18].

*2.7. Immunoblotting.* E-cadherin and Snail were studied using western blot analysis. Briefly, MCF-7 and T47D cells were treated with $E_2$ and the tested agents for 24 and 48 h, and then cells were lysed in buffer containing 0.5% NP-40, 0.5% NaDOC, 0.1% SDS, 50 mM Tris (pH 7.0), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM NaF, and a protease inhibitor cocktail (Sigma-Aldrich, Inc., USA), as previously described [19]. Cell extracts were incubated on ice for 30 min, with vortexing every 10 min and centrifuged at 13000 rpm for 30 min. Supernatants were collected and protein concentration was determined with Bradford (Sigma-Aldrich, Inc, USA) assay. Specific protein amount was analyzed using the standard procedure of western blot analysis. A mouse anti-E-cadherin (1 : 1000, Invitrogen Corporation, Camarillo, CA, USA), a rat anti-Snail (1 : 1000, Cell Signaling Technology, Inc., Boston, USA), and a mouse antiactin (1 : 1000, Chemicon, Millipore, Temecula, CA, USA) were used. Detection of the immunoreactive proteins was performed by chemiluminescence using horseradish peroxidase substrate SuperSignal (Pierce, Rockford, IL, USA), according to the manufacturer's instructions.

*2.8. Immunofluorescence.* Cells were grown in 4-well coverslips ($15 \times 10^3$ cells/well) in the presence or absence of $E_2$ and the tested agents for 48 h. Cells were fixed with saline-buffered formalin for 15 min and permeabilized with 0.1% Triton for 5 min. Blocking was performed with 3% bovine serum albumin (BSA) in phosphate buffer saline (PBS) containing 10% FBS for 1 h at 37°C. After the incubation, cells were rinsed once with PBS for 5 min and then incubated with a mouse anti-E-cadherin (1 : 1000, Invitrogen Corporation, Camarillo, CA, USA), a rat anti-Snail (1 : 500, Cell Signaling Technology, Inc., Boston, MA, USA), a rabbit anti-$Tyr^{397}$-FAK antibody (dilution 1 : 200, R&D Systems, Deutschland, Germany), a mouse anti-ER-$\alpha$ antibody (dilution 1 : 500, Chemicon International Inc., Temecula, CA, USA), and phalloidin-fluorescein isothiocyanate labeled (Sigma-Aldrich, Inc., USA) for 1 h at 37°C. Cells were rinsed $3 \times 5$ min with PBS and then a chicken anti-mouse Alexa Fluor 488, a chicken anti-rat Alexa Fluor 568, or a donkey anti-rabbit antibody Alexa Fluor 594, (1 : 1000, molecular probe, Invitrogen Corporation, Camarillo, CA, USA) diluted in blocking solution and an incubation for 30 min at 37°C was followed. Cells were rinsed $2 \times 5$ min with PBS; then incubation for 5 min with 5 $\mu$M Draq 5 (Biostatus Limited, Shepshed, UK) or DAPI (Vectashield, Vector Laboratories, Inc., US) diluted in PBS was followed for nucleus staining and cells mounted on glass sides. Fluorescence was visualized using a Leica microscope at 63X magnification.

*2.9. Statistical Analysis.* Differences between groups and controls were tested by one-way ANOVA. Each experiment included at least triplicate measurements. All results are expressed as mean ± SEM from at least three independent experiments.

## 3. Results

*3.1. Fulv, Tam, and the Metabolites End and 4-OH-T Partially Decrease $E_2$-Induced Cell Proliferation.* In the current study, MCF-7 and T47D breast cancer cells were treated with Fulv, Tam, and its metabolites End and 4-OH-T, so as to determine the optimum concentration regarding their effect on cell proliferation. $E_2$ was used at a concentration of 0.01 $\mu$M as previously described [20]. Fulv, Tam, End, and 4-OH-T were tested at the concentrations of 0.1 and 1 $\mu$M, as previously described [21–24]. Both cell lines were treated with Fulv and Tam as well as the metabolites of the latter with simultaneous addition of $E_2$. We showed that $E_2$ induced cell proliferation in both cell lines 48 h after its addition (Figure 1(a)), as previously described [25]. All the tested agents demonstrated an antiproliferative effect in both concentrations in a dose-dependent manner compared to untreated cells in both cell lines 48 h after their addition, as was expected (Figures 1(b) and 1(c)). Thereafter, all the experiments were performed using 0.01 $\mu$M $E_2$ and 0.1 $\mu$M of the tested agent.

*3.2. Tam but Not Fulv Stimulates Single Cell Migration.* Migration is a pivotal process for both invasion and metastasis allowing cells to change position into tissues or metastasize to distant organs [5, 26]. Cancer cells utilize different ways to migrate, either individual or multicellular [4]. To assess the effect of the tested agents on single cell migration, we used the boyden chamber assay in both cell lines. Cells were pretreated with $E_2$ and the tested agents for 24 h, and then we observed their ability to migrate through the membrane after 36 h incubation. MCF-7 cells showed greater ability to pass through the membrane compared to T47D cells (Figure 2). $E_2$ alone or in combination with Fulv did not affect MCF-7 cell migration compared to untreated cells. In contrast the treatment of MCF-7 cells with the combination of $E_2$ with Tam and its metabolites significantly promotes the motility of cells to migrate through the pores of the membrane

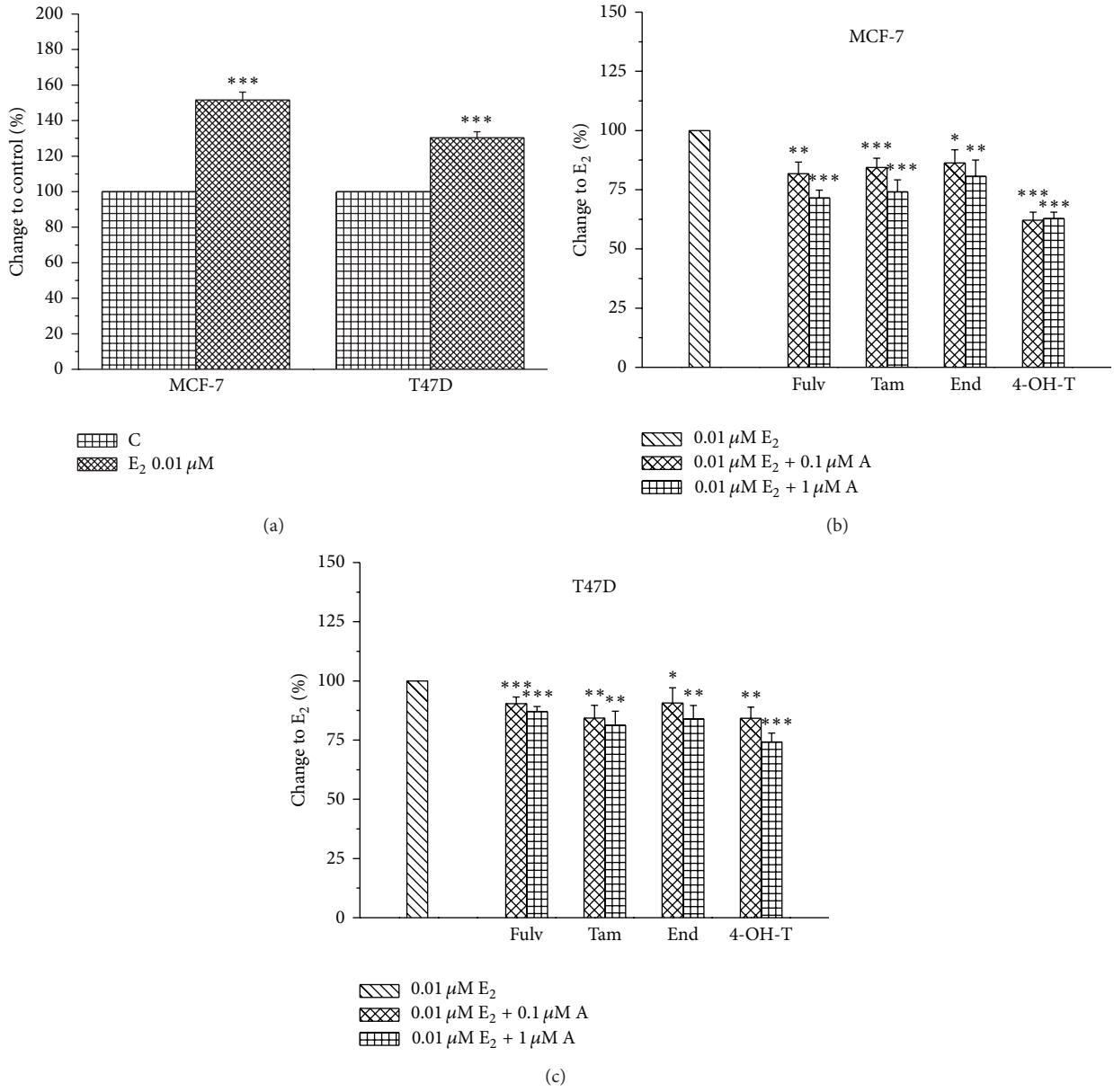


Figure 1: The effect of $E_2$ and the tested agents on cell proliferation. $E_2$ alone induces cell proliferation of MCF-7 and T47D (a). Cells were pretreated with $E_2$ (0.01 $\mu$M), and the tested agents (A) were added at the concentrations of 0.1 and 1 $\mu$M at MCF-7 (b) and T47D (c). Results are expressed as mean ± SEM of the % change compared to the untreated cells and/or $E_2$. Asterisks denote a statistically significant difference compared to control (untreated) cells. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

(Figure 2). In T47D cells the effect of $E_2$ and the tested agents on cell migration is not reliable since very low number of cells passed through the membrane. The difference in the ratio of ER$\alpha$/ER$\beta$ might contribute to low metastatic ability of T47D cells. MCF-7 cells express very low levels of ER$\beta$ compared to T47D cells [27]. According to recent data, ER$\beta$ exerts a protective role for the cell by inhibiting the invasiveness and promoting the adhesion [28]. Further, a previous study demonstrated that treatment of MCF-7 cells with $E_2$ caused a degradation of ER$\alpha$ and an increase of ER$\beta$ [29]. This might explain the absence of any effect on MCF-7 cell migration after their treatment with $E_2$ alone or in combination with Fulv since Fulv exerts its effect through ER$\alpha$ degradation.

*3.3. Collective Cell Migration Is Not Affected by Fulv but It Is Reduced by Tam.* Since $E_2$ alone or in combination with Fulv did not affect single cell migration, we studied the effect of tested agents on collective cell migration using the scratch wound assay [30]. Both cell lines were treated with $E_2$ and the tested agents for 24 and 48 h. In MCF-7 cells we found that $E_2$ alone increased cell migration compared to untreated cells up

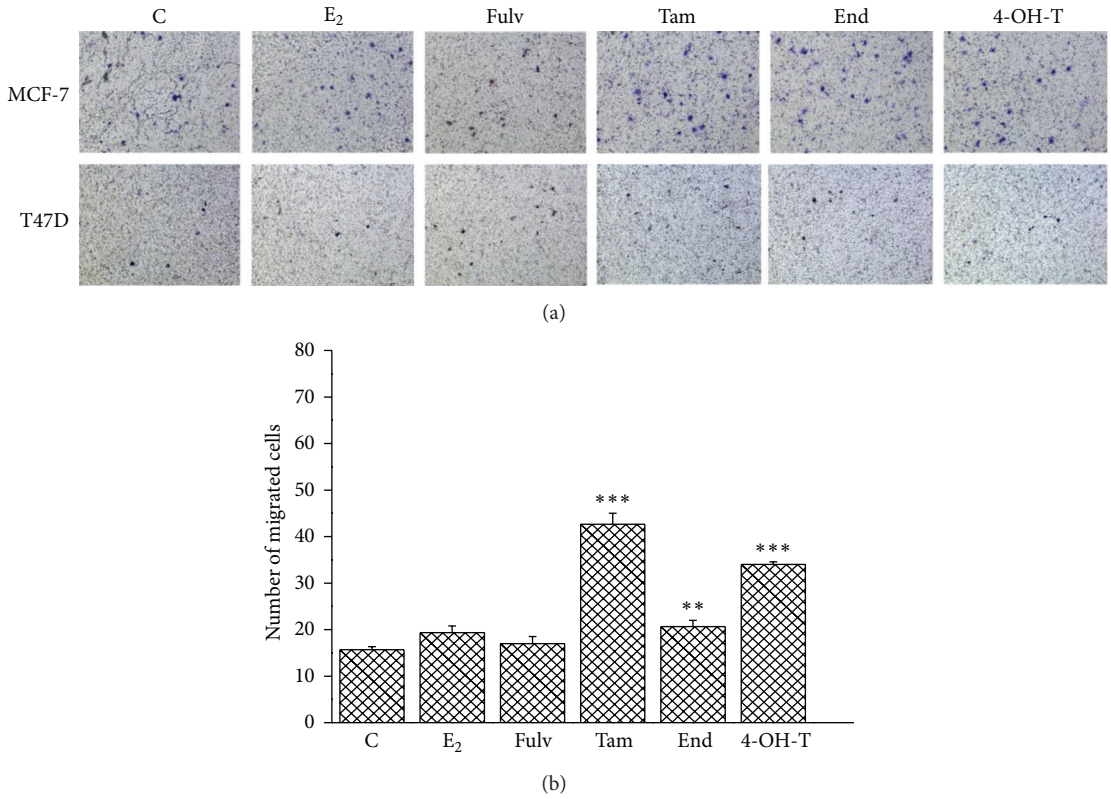


Figure 2: Single cell migration in MCF-7 and T47D cells after their treatment with $E_2$ and antiestrogens. C: control (untreated cells); $E_2$: cells treated with 17$\beta$-estradiol; Fulv: cells treated with $E_2$ + 100 nM Fulv; Tam: cells treated with $E_2$ + 100 nM Tam; End: cells treated with $E_2$ + 100 nM End; and 4-OH-T: cells treated with $E_2$ + 100 nM 4-OH-T. The image is representative of three independent experiments using a magnification of 10X (a). Quantification of images from boyden chamber assay in MCF-7 cells (b). Results are expressed as mean ± SEM of the % change compared to the untreated cells. Asterisks denote a statistically significant difference compared to $E_2$ treated cells. $^{**}P < 0.01$ and $^{***}P < 0.001$.

to 48 h (Figure 3). The combination of $E_2$ with Fulv reversed slightly the effect of $E_2$ alone. This reversal was more potent when $E_2$ combined with Tam, End, and 4-OT-T as shown in Figure 3. The same effect of $E_2$ and tested agents was observed in T47D (data not shown).

*3.4. Fulv and Tam Totally Reverse the Protective Effect of $E_2$ in Cell Invasion.* Because migration plays a crucial role during tumor invasion, we evaluated the influence of Fulv, Tam, and its active metabolites on the invasive capacity of breast cancer cells lines. Cell invasion was studied using a modified boyden chamber assay with a membrane coated with matrigel. Cells were treated with $E_2$ and tested agents, and the invasion was observed 72 h later. In MCF-7 cells, we found that $E_2$ alone reduced cell ability to invade and this effect was partially reversed by the combination of $E_2$ and the tested agents (Figure 4). Fulv and 4-OH-T exerted a better inhibitory effect than Tam and End (Figure 4). T47D cells were not used in this set of experiments, because of the low capacity to migrate the membrane in typical boyden chamber assay. Although, MCF-7 cells are also characterized by low invasive capacity compared to other breast cancer lines, we showed that the treatment with $E_2$ and the tested agents altered their motility and this prompted us to investigate it further.

*3.5. Fulv and Tam Facilitate Invasion through MMPs' Modulation.* MMPs are key players in invasion and metastasis since they promote the invasive potential through digestion of the ECM components [5, 31, 32]. In $ER^+$ breast tumors $E_2$ exerts a protective role since it regulates the expression both of MMP-2 and MMP-9 as well as syndecan-4 [29] and, therefore, limits the ability of cells to invade the adjacent tissues. By contrast, antiestrogens seem to reverse this effect increasing the level of MMPs [33]. We evaluated the influence of $E_2$ alone and/or in combination with the tested agents on MMP-2 and MMP-9 levels 24 and 48 h after treatment of cells. Zymography analysis in MCF-7 cells demonstrated a slight decrease on the expression of both MMP-2 and MMP-9 followed the treatment with $E_2$ up to 48 h. In addition, the combination of cells with $E_2$ and tested agents reversed the effect of $E_2$ inducing MMPs levels 24 h after treatment of cells (Figure 5). This phenomenon was preserved for Fulv and End up to 48 h after cells treatment. At the same time point, when $E_2$ combined with Tam, MMPs levels were not changed compared to $E_2$ alone while the combination of $E_2$ with 4-OH-T reduced the levels of MMPs and particularly MMP-9 (Figure 5). In T47D cells any change in MMPs levels was not found after cells treatment with $E_2$ and the tested agents at any time point tested (data not shown).

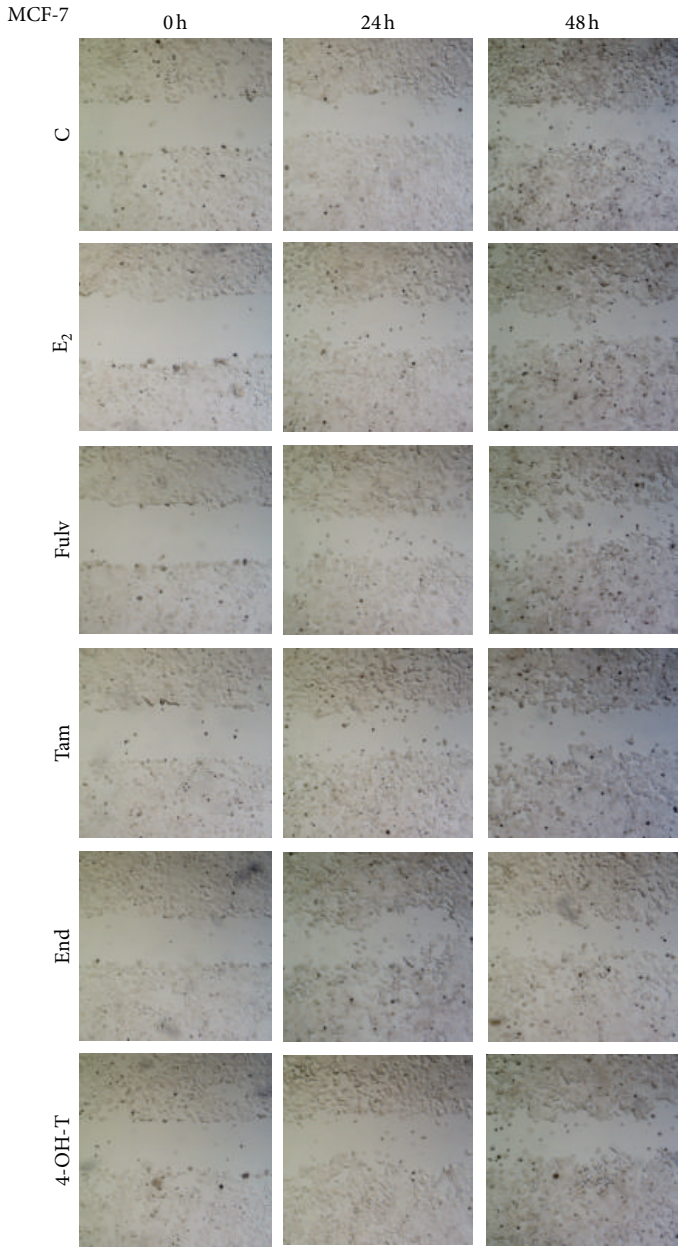


Figure 3: Collective cell migration in MCF-7 cells treated with $E_2$ and antiestrogens. C: control (untreated cells); $E_2$: cells treated with 17$\beta$-estradiol; Fulv: cells treated with $E_2$ + 100 nM Fulv; Tam: cells treated with $E_2$ + 100 nM Tam; End: cells treated with $E_2$ + 100 nM End; and 4-OH-T: cells treated with $E_2$ + 100 nM 4-OH-T. The image is representative of three independent experiments using a magnification of 4X.

*3.6. Tam and End Stimulate EMT-A Different Role for Snail.* At the leading edge of invasiveness and metastasis, epithelial cells undergo EMT. Two major partners of EMT are E-cadherin and Snail. E-cadherin is reversibly downregulated in EMT, and this reduction is associated with increased levels of Snail, a repressor of E-cadherin [28, 34, 35]. Regarding E-cadherin protein levels, we found that $E_2$ alone and/or in combination with 4-OH-T did not alter protein status 48 h after their addition to MCF-7 cells. The combinations of $E_2$ with Fulv, Tam, and End caused a decrease in E-cadherin protein levels (Figure 6(a)). Further, regarding Snail protein levels, we found that $E_2$ alone increased Snail protein status at the same time point. The combination of $E_2$ with Fulv and/or with 4-OH-T decreased Snail protein. This phenomenon was more potent in the case of $E_2$ with 4-OH-T. The combination of $E_2$ with Tam and End increased Snail levels (Figure 6(a)). In T47D cells, $E_2$ alone as well as its combinations with Tam, End and 4-OH-T did not alter E-cadherin protein levels. The treatment of cells with $E_2$ and Fulv caused a slight decrease in protein levels (Figure 6(b)). Furthermore Snail protein was decreased only when $E_2$ combined with Fulv and 4-OH-T (Figure 6(b)).

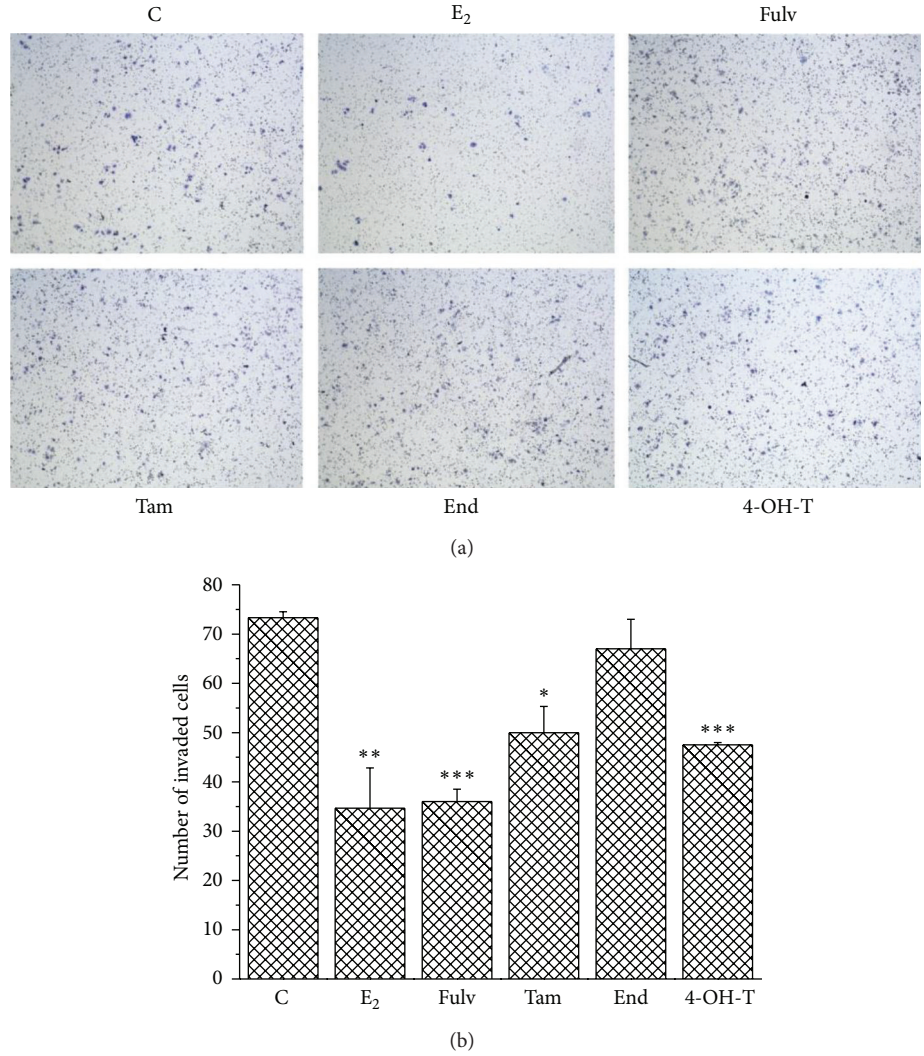


Figure 4: The effect of $E_2$ and the tested agents on MCF-7 cell invasion. C: control (untreated cells); $E_2$: cells treated with 17$\beta$-estradiol; Fulv: cells treated with $E_2$ + 100 nM Fulv; Tam: cells treated with $E_2$ + 100 nM Tam; End: cells treated with $E_2$ + 100 nM End; and 4-OH-T: cells treated with $E_2$ + 100 nM 4-OH-T. The image is representative of three independent experiments using a magnification of 10X (a). Quantification of images from boyden chamber assay in MCF-7 cells (b). Results are expressed as mean ± SEM of the % change compared to the untreated cells. Asterisks denote a statistically significant difference compared to untreated cells. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

The complicated results from western blot analysis revealed that, in both cell lines, the protein changes of E-cadherin did not follow the changes of Snail protein levels in order for an EMT phenomenon to be observed. Only in the case that $E_2$ combined with Tam or End, a decrease of E-cadherin levels followed an increase of Snail levels. In addition, the most important changes were observed at Snail protein after cell treatment with the combinations of $E_2$ with Fulv and 4-OH-T where Snail levels were decreased (Figure 6). Besides in EMT, the role of Snail is also very important for cell survival. Previous studies have shown that a decrease in Snail protein sensitizes cell to death [34, 36].

*3.7. The Antiestrogens on Localization of E-Cadherin and Snail.* In order for the transcription factor Snail to act as repressor of E-cadherin, its nuclear translocation is required. Since western blot analysis did not reveal any significant connection between EMT proteins' expression and treatment of cells with the $E_2$ and the tested agents, we studied the effect of antiestrogens on these proteins' localization, 48 h after cell treatment. We found that E-cadherin is located in cell membrane and cell-cell junctions in untreated MCF-7 cells as well as in cells treated with $E_2$ and the tested agents (Figure 7). Snail was localized at both nucleus and cytoplasm in untreated cells or cells treated with $E_2$ (Figure 7). The combinations of $E_2$ with Fulv and 4-OH-T retained the cytoplasmic localization and enhanced the nuclear localization. The combinations of $E_2$ with Tam and End retained the cytoplasmic localization of Snail. Similar effects of $E_2$ and the tested agents were observed at T47D cells (data not shown).

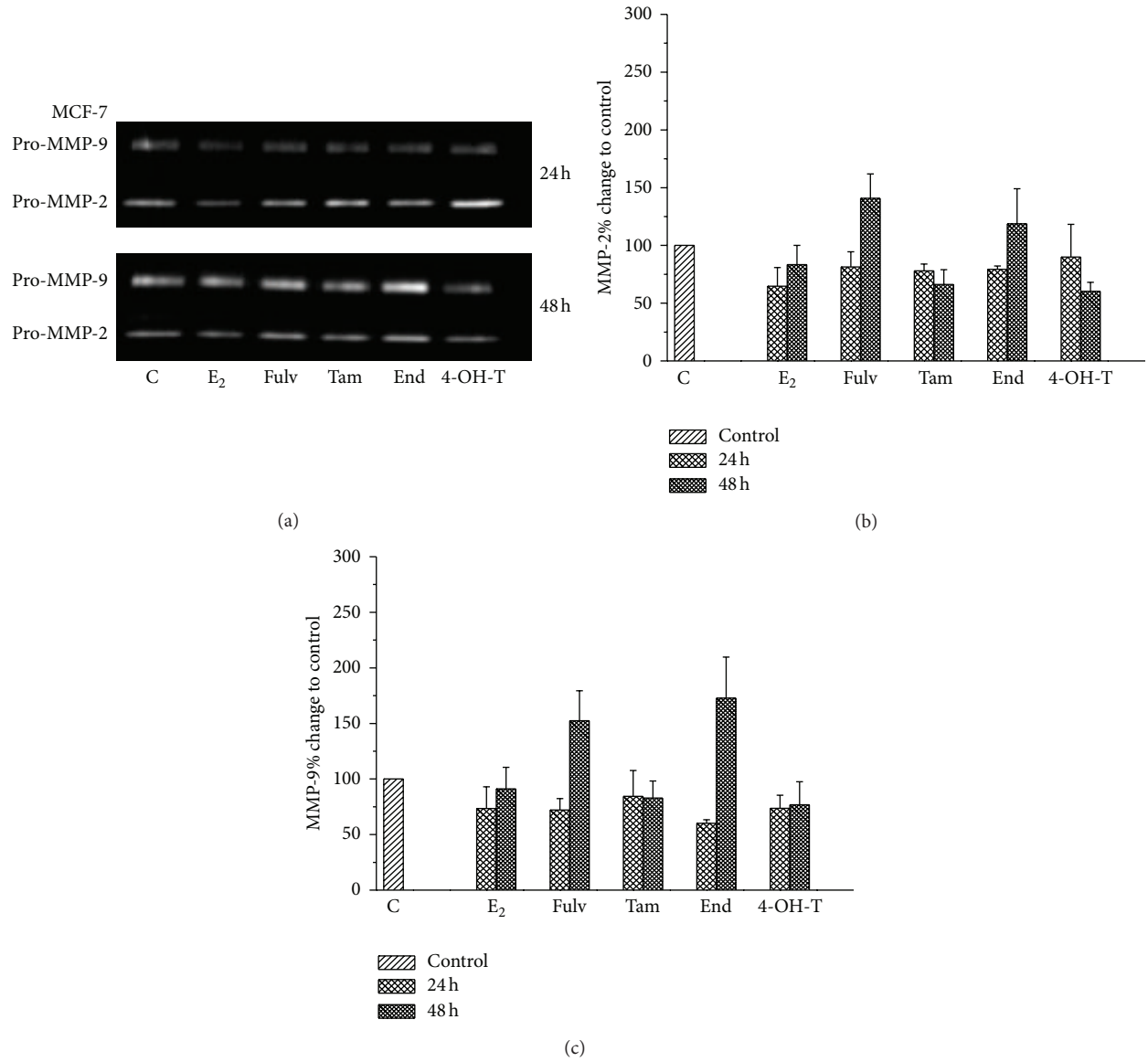


Figure 5: MMP-9 and MMP-2 enzyme expression after treatment of MCF-7 cells with $E_2$ and the tested agents. (a) A representative image of three independent experiments. A quantitative analysis of images for (b) MMP-2 and (c) MMP-9 expression using appropriate software. Results are expressed as mean ± SEM of the % change compared to the untreated cells.

*3.8. Fulv and Tam Affect Migration through FAK Phosphorylation and F-Actin Rearrangement.* FAK exerts a central role on cell migration and invasion, and its activation is correlated with malignant transformation [37, 38]. In addition, a specific phosphorylation at $Tyr^{397}$ residue is correlated with Tam-resistance [22]. In MCF-7 cells, $E_2$ exposure resulted in autophosphorylation of FAK in $Tyr^{397}$ residue, which entails activation of FAK. This phenomenon was time dependent, and the highest phosphorylation was observed in 10 min (Figure 8). Thereafter, the phosphorylated signal was down-regulated.

At the time point of 10 min, when the maximum FAK phosphorylation was found, we investigated the impact of Fulv, Tam, and its metabolites in spatial organization of actin fibers. The main finding to emerge was that the treatment of cells with $E_2$ combined with Fulv either Tam or End resulted in a less round-like morphology with more leading edges than the other groups (Figure 8). The colocalisation of F-actin with $Tyr^{397}$ FAK appeared mainly at the leading edges. In untreated cells as well as in cells treated with $E_2$ alone or in combination with 4-OH-T, the spots of $Tyr^{397}$ FAK are scattered all around the cell membrane which is attributed to increased stability (Figure 8). Similar effects of $E_2$ and the tested agents were observed at T47D cells (data not shown).

## 4. Discussion

Hormonal therapy has been established for the treatment of $ER^+$ breast cancer patients. Several clinical trials [39–41] have demonstrated the benefits of this type of treatment,

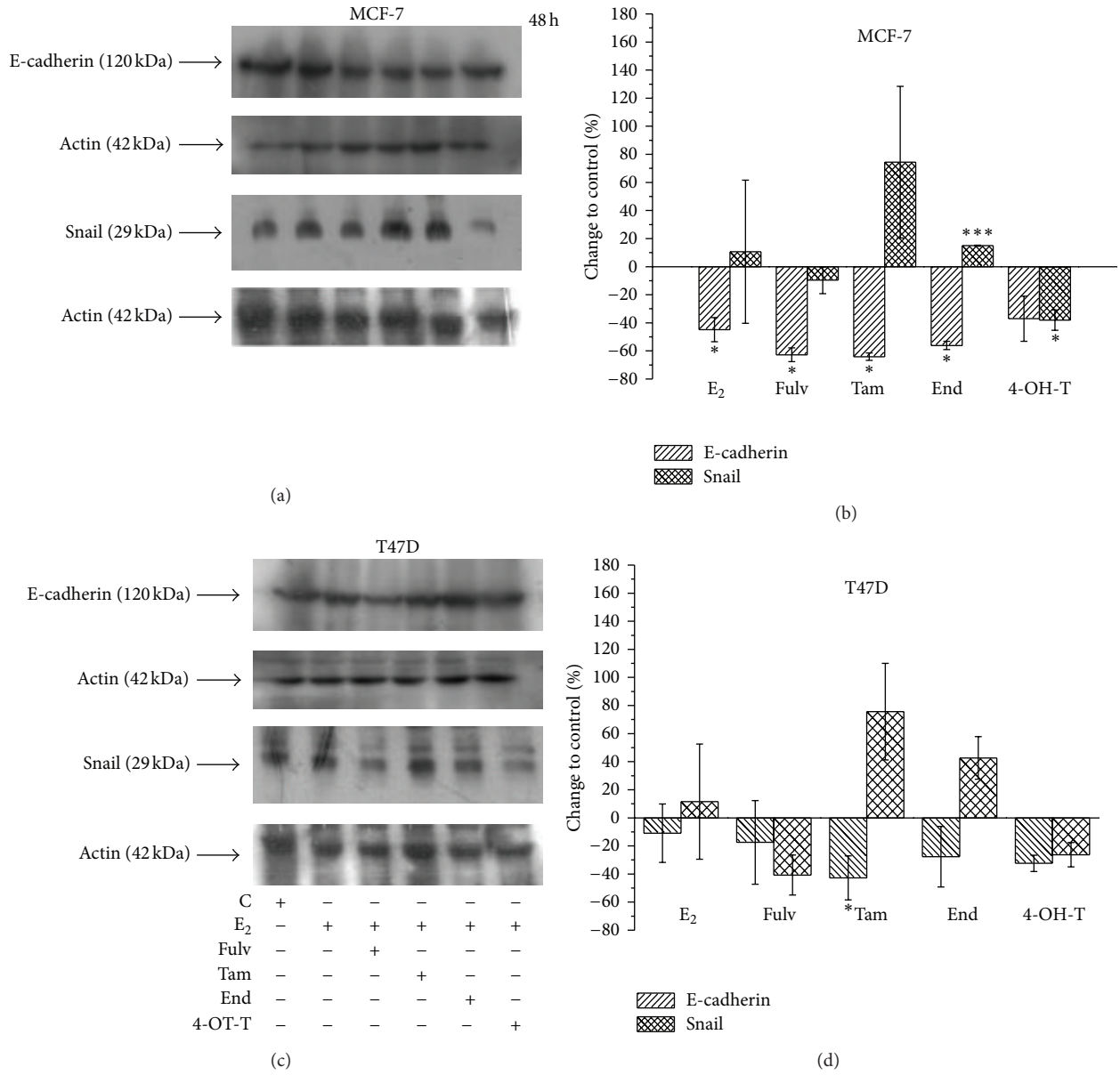


Figure 6: E-cadherin and Snail protein expression in MCF-7 and T47D cells 48 h after treatment of cells with $E_2$ and the tested agents. A representative image of three independent experiments for both cell lines using western blot analysis, (a) and (c). Quantification of images from western blot analysis in both cell lines, (b) and (d). Results are expressed as % change compared to the untreated cells ± SEM. Asterisks denote a statistically significant difference compared to untreated cells. $^{*}P < 0.05$ and $^{***}P < 0.001$.

and it is generally acceptable that it has contributed to the decrease in breast cancer mortality. Despite the benefits of hormonal therapy, the disease often relapses and secondary tumors develop due to their metastatic potential [42, 43]. *In vitro* studies have assessed the impact of antiestrogens on breast cancer cell invasiveness and MMPs expression [16, 33, 44, 45]. In the present study we evaluated the effect of the antiestrogens Fulv and Tam from a different standpoint, namely, migration that leads to tumor growth, invasion, and metastasis.

There are many types of cell movement that lead to cell migration and invasion according to cell type and microenvironment [4]. Epithelial cells undergoing EMT can migrate individually. On the other hand, basal- and squamous-originated epithelial cells following EMT or moderately differentiated epithelial cells lacking EMT can migrate collectively [4]. In order to evaluate the effect of $E_2$ on single and collective cell migration, we applied 2 typical assays: boyden chamber and wound healing, respectively. We found that in MCF-7 cells, $E_2$ alone failed to stimulate single cell migration while promoting collective cell migration in both cell lines. The failure of $E_2$ to stimulate single cell migration is in line with the unclear results of western blot analysis for the interaction of EMT proteins, E-cadherin, and Snail as well

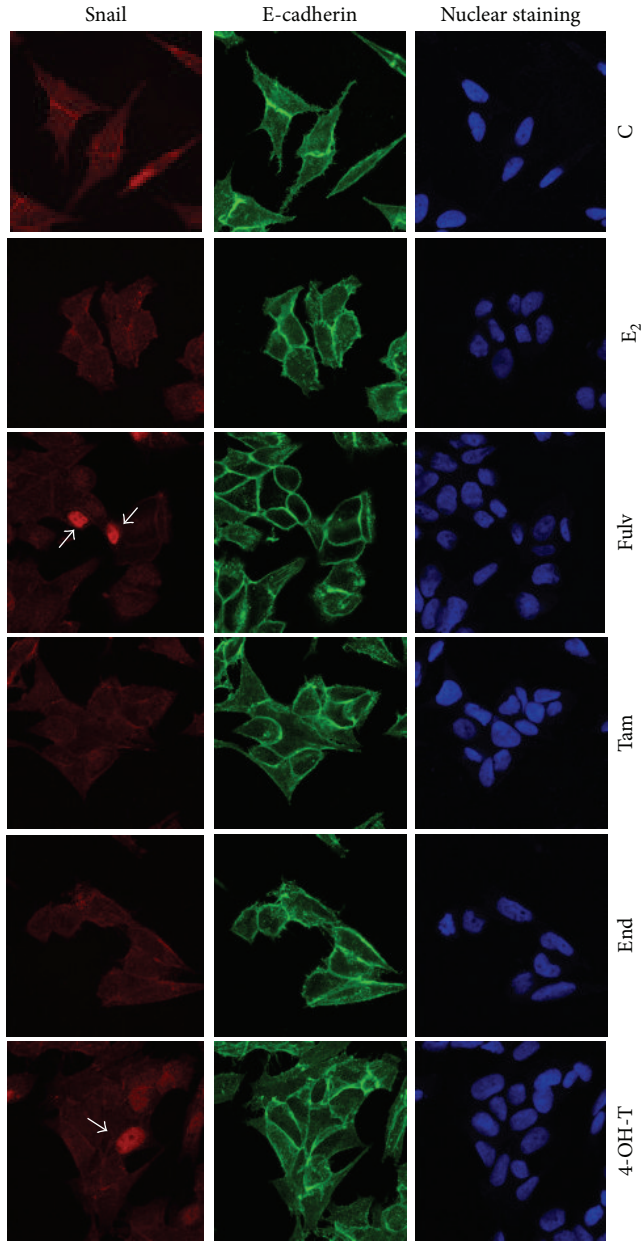


Figure 7: E-cadherin and Snail protein localization in MCF-7 cells 48 h after treatment of cells with $E_2$ and the tested agents. C: control (untreated cells); $E_2$: cells treated with 17$\beta$-estradiol; Fulv: cells treated with $E_2$ + 100 nM Fulv; Tam: cells treated with $E_2$ + 100 nM Tam; End: cells treated with $E_2$ + 100 nM End; and 4-OH-T: cells treated with $E_2$ + 100 nM 4-OH-T. The image is representative of three independent experiments using a magnification of 63X.

as with the absence of Snail import to the nucleus. Snail is a highly unstable protein and is dually regulated by protein stability and cellular localization. In order for Snail to exert its effect, a nuclear translocation is required [34]. The increase in collective cell migration after treatment of cells with $E_2$ is in line with the increase in cell proliferation of both cell lines since these are indications of expansive growth with the absence of active migration [46]. In contrast to the increase in cell proliferation and collective cell migration, we found that $E_2$ decreased the capacity of cells to invade. The decrease in invasiveness was associated with decrease in MMPs. This is not the first time that a protective role of $E_2$ is described. Previous studies have shown that $E_2$ may inhibit breast cancer cell invasion by affecting proteins that modulate cell-cell interactions or increasing the number of desmosomes [47]. The reduced invasiveness of $E_2$-stimulated cells is also supported by the findings from immunofluorescence assay, where cells demonstrated a more spherical morphology with focal adhesions all a round the cell membrane, which is associated with increased stability.

Using Fulv, the mitogenic effect of $E_2$ was partially reversed with a decrease in Snail protein levels associated

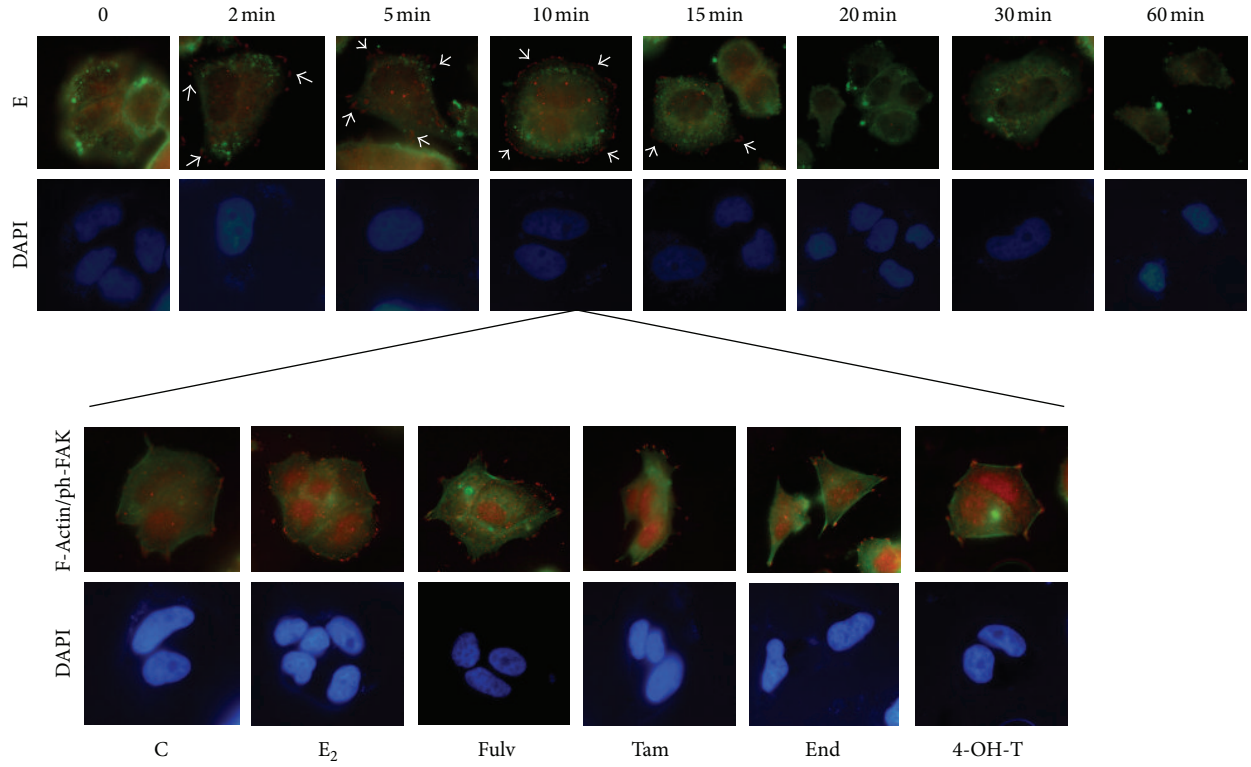


FIGURE 8: The impact of $E_2$ and the tested agents on $Tyr^{397}$ FAK phosphorylation and F-actin rearrangement. MCF-7 cells exposed to $E_2$ in a time course manner up to 60 min for detection of the maximum FAK phosphorylation. ER$\alpha$ and $Tyr^{397}$ FAK localisation is indicated with green and red fluorescence, respectively. At the time point of 10 min, F-actin and $Tyr^{397}$ FAK colocalisation (green and red fluorescence, resp.) was observed after the exposure of MCF-7 cells to the tested agents. C: control (untreated cells); $E_2$: cells treated with 17$\beta$-estradiol; Fulv: cells treated with $E_2$ + 100 nM Fulv; Tam: cells treated with $E_2$ + 100 nM Tam; End: cells treated with $E_2$ + 100 nM End; and 4-OH-T: cells treated with $E_2$ + 100 nM 4-OH-T. The image is representative of three independent experiments using a magnification of 60X.

with its import to nucleus. However, the effect of $E_2$ either on single or collective cell migration was not altered. Fulv is a selective estrogen downregulator that binds to ER forming an unstable ER-Fulv complex, which is rapidly degraded resulting in ER reduction. Fulv may exert genomic as well as non genomic effects on target cells [16, 48]. A recent publication by Song et al. [48] shows that Fulv at the concentration of 0, 1 $\mu$M shuttles ER$\alpha$ from the nucleus to the cytosol and plasma membrane. When Fulv is extranuclear acts as an estrogen agonist but after its entrance to the nucleus blocks the genomic effects of estrogens in transcription and cell proliferation. This might explain the effect on cell proliferation but not on cell migration. Previous data have shown that functional ER$\alpha$ is associated with E-cadherin expression, and this expression as well as cell-cell adhesion may be modulated by antiestrogens resulting in an invasive phenotype [16]. Indeed, we found that Fulv decreased E-cadherin protein expression and increased cell invasion and MMPs expression versus $E_2$. These data were confirmed by immunofluorescence assay where cells exhibited a less round-like morphology, indication of increased invasiveness.

Tam is a prodrug that is metabolized to End and 4-OH-T so as to exert its therapeutic effect. Although both metabolites are equivalent regarding ER$\alpha$ binding and inhibition of $E_2$-induced cell proliferation, it is proposed that End is the principal antiestrogenic metabolite for the antitumour activity observed in breast cancer patients [49]. In the current study we used both metabolites to verify that they act in the same way. Tam and its metabolites stimulated single cell migration and reduced collective cell migration. Regarding Tam and End, the stimulation of single cell migration is in concordance with the E-cadherin protein decrease and Snail protein increase. This might be an indication that an active migration through EMT induction occurs after Tam and End treatment. Although Snail was not detected to the nucleus at the same time point we cannot exclude a positive role of cytosolic Snail in cell migration [50]. These data are also in agreement with the less round-like shape of cells as well as with the scattering of focal adhesions at the leading edges of F-actin revealing a more invasive and potent phenotype. An increase in both MMPs expression and cell invasiveness might facilitate EMT induction. In the case of 4-OH-T, it seems that an EMT phenomenon did not occur because no decrease in E-cadherin or increase in Snail protein levels was detected. In contrast, a reduction in Snail protein in association with a nuclear localization was detected. So far, our data indicate that 4-OH-T promoted single cell migration without EMT. A detailed review of Friedl and Alexander [4], related to the types of cancer cell movement, referred

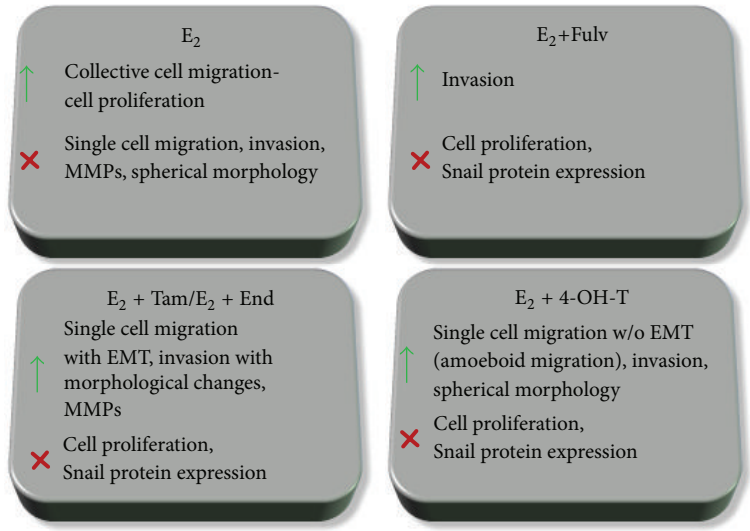


Figure 9: An overview of the effect of Fulv, Tam, and metabolites of Tam in migration and invasion of MCF-7 and T47D cells.

to a single cell movement currently known as amoeboid migration. In this type of migration, cells adopt a more spherical shape and migrate without ECM proteolysis. The decrease in MMPs levels and the spherical shape of cells found in our study after treatment with 4-OH-T using zymography and immunofluorescence, respectively, support this type of migration. The decrease of Snail protein and its nuclear location after 4-OH-T treatment seem to correlate with the inhibition of cell proliferation rather than migration. This is compatible with the decrease of cell proliferation that we found after 4-OH-T treatment. This decrease was more potent for 4-OH-T compared to the other agents which did not reduce Snail protein. Regarding invasion, it seems that the active single cell migration with or without EMT was associated with increased invasiveness.

## 5. Conclusions

Our working hypothesis was that different approaches of estrogen inhibition affected differently breast cancer cell migration and invasion. Summarizing our data, we may conclude that in breast cancer cells after serum $E_2$ withdrawal (i) $E_2$ stimulated expansive growth of cells with the absence of EMT but exerted a protective effect by reducing invasiveness, MMPs expression and preserving a more stable phenotype with focal adhesions all around the cell membrane; (ii) the antiestrogens partially counteracted the $E_2$-induced effect; (iii) Fulv did not affect the expansive growth stimulated by $E_2$ and promoted cell invasion; (iv) Tam and its metabolites stimulated active single cell migration and increased cell invasiveness. An overview of Fulv and Tam effect is observed in Figure 9.

Although Fulv might result in a less aggressive behaviour of cells compared to Tam, the benefits of hormonal therapy concerning invasion and metastasis yet remain under question.

## Abbreviations

ER: Estrogen receptor
Fulv: Fulvestrant
Tam: Tamoxifen
E2: 17$\beta$-Estradiol
End: Endoxifen
4OHT: 4-OH-Tamoxifen
MMPs: Matrix metalloproteinases
SERDs: Selective estrogen receptor downregulators
ECM: Extracellular matrix
EMT: Epithelial mesenchymal transition
FAK: Focal adhesion kinase
FBS: Fetal bovine serum
rf-RPMI: Phenol red-free RPMI
CSS: Charcoal-stripped serum
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2, 5-dephenyltetrazolium-bromide.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Authors' Contribution

Dionysia Lymperatou participated in acquisition of data as well as data analysis and was involved in drafting the paper; Efstathia Giannopoulou has made substantial contribution to conception and design of the project, participated in acquisition and analysis of data, and was involved in drafting the paper, Angelos K. Koutras has made substantial contribution to conception of the project, was involved in drafting the paper, and revised it critically for important intellectual content; Haralabos P. Kalofonos has made substantial contribution to conception and design of the project, was involved in drafting the paper, and revised it critically for important

intellectual content. All authors have given final approval of the version to be published.

## Acknowledgments

The authors would like to thank the Medical School, University of Patras, Greece, for providing them with the Advanced Light Microscopy facility and EOGE Oncological Research Fund for financial support.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 230805, 18 pages
http://dx.doi.org/10.1155/2013/230805

*Review Article*

# Extracellular Matrix Degradation and Tissue Remodeling in Periprosthetic Loosening and Osteolysis: Focus on Matrix Metalloproteinases, Their Endogenous Tissue Inhibitors, and the Proteasome

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Received 30 April 2013; Accepted 31 May 2013

Academic Editor: Achilleas D. Theocharis



The leading complication of total joint replacement is periprosthetic osteolysis, which often results in aseptic loosening of the implant, leading to revision surgery. Extracellular matrix degradation and connective tissue remodeling around implants have been considered as major biological events in the periprosthetic loosening. Critical mediators of wear particle-induced inflammatory osteolysis released by periprosthetic synovial cells (mainly macrophages) are inflammatory cytokines, chemokines, and proteolytic enzymes, mainly matrix metalloproteinases (MMPs). Numerous studies reveal a strong interdependence of MMP expression and activity with the molecular mechanisms that control the composition and turnover of periprosthetic matrices. MMPs can either actively modulate or be modulated by the molecular mechanisms that determine the debris-induced remodeling of the periprosthetic microenvironment. In the present study, the molecular mechanisms that control the composition, turnover, and activity of matrix macromolecules within the periprosthetic microenvironment exposed to wear debris are summarized and presented. Special emphasis is given to MMPs and their endogenous tissue inhibitors (TIMPs), as well as to the proteasome pathway, which appears to be an elegant molecular regulator of specific matrix macromolecules (including specific MMPs and TIMPs). Furthermore, strong rationale for potential clinical applications of the described molecular mechanisms to the treatment of periprosthetic loosening and osteolysis is provided.

## 1. Pathobiology of Periprosthetic Loosening Process

The total hip or knee replacement is an operation whereby the damaged cartilage and the subchondral sclerotic bone of the hip or knee joint are surgically replaced with artificial materials. The continuous improvement of the materials and the surgical techniques have given comfort to patients suffering from painful diseases of the joints, such as primary osteoarthritis and secondary ones caused by rheumatoid arthritis, posttraumatic conditions, congenital dysplasia or dislocation, and aseptic necrosis of the femoral head. After the improvement in prophylaxis against infection, aseptic loosening of endoprostheses represents the predominant complication of this operation, which usually occurs during the second decade, after the primary arthroplasty. Although many reports have been published on the pathogenesis of periprosthetic loosening, the precise biological mechanisms responsible for this process have not yet been completely elucidated.

Wear-generated particular debris at the interface between implant components is associated with chronic inflammation and osteolysis, limits the lifespan of the implants, and is the main cause of initiating this destructive process. However, many other factors, such as cyclic loading or micromotion of the implants and hydrostatic fluid pressure, have also been implicated revealing the high heterogeneity in the histology of the tissue around the prosthesis [1]. Evidence in support

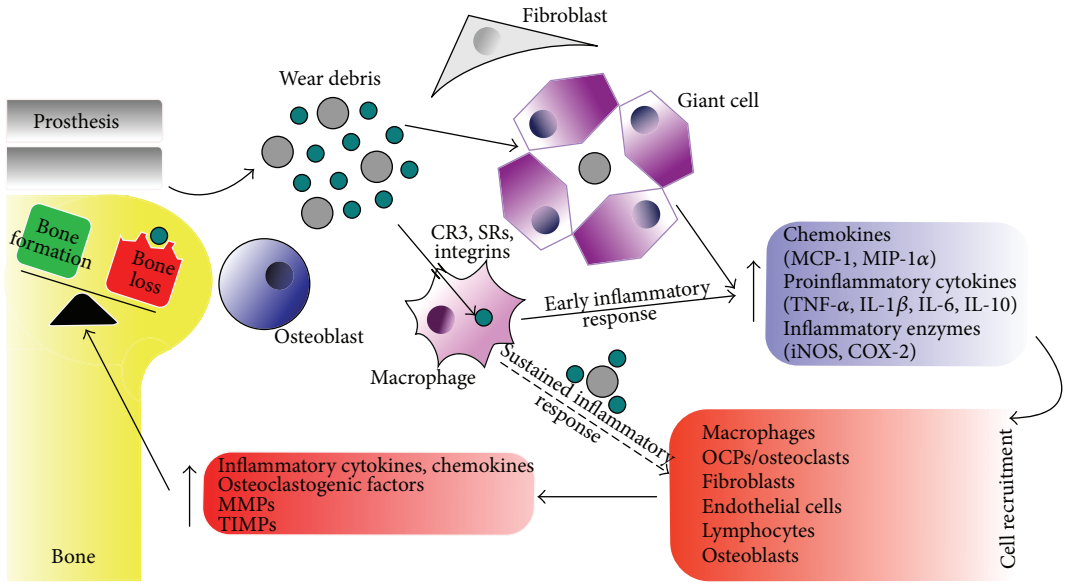


Figure 1: Schematic representation of periprosthetic loosening and osteolysis. Implant-derived wear debris induces an early inflammatory response from the resident or infiltrating macrophages in the periprosthetic tissue. Small particles are phagocytosed, whereas the larger induce fusion of macrophages and giant cell formation. Activated macrophages release proinflammatory cytokines, chemokines, and enzymes that recruit multiple cell types within periprosthetic tissue, which are further activated by the particles resulting in sustained inflammation, increased secretion of cytokines/chemokines/osteoclastogenic factors/MMPs/TIMPs, and osteolysis.

of the central role of wear debris in periprosthetic loosening and osteolysis includes the observations that osteolysis is correlated with higher wear rates [2] and that vast numbers of wear particles are found associated with the periprosthetic interfacial membrane removed during revision surgery [3–5]. Furthermore, experimental systems have demonstrated that particulate debris can induce osteolysis in a variety of animal models [6–12] and inflammatory responses in cultured macrophages [8, 13–17]. Wear debris may include particles from all the various components of the prosthesis (such as polyethylene, metal, and ceramic) as well as bone cement [18]. Since cellular responses are highly dependent upon the composition, size, and shape of particles, the type of prosthesis and bearing surface used may have a significant impact on the potential for development of osteolysis [19].

The release of implant-derived particles induces a cellular host response, which initially is taking place in the pseudocapsular tissue (PCT). This membranous tissue is formed postoperatively around the artificial joint and practically replaces the normal joint capsular tissue, which is usually removed during the primary joint replacement procedure. The most important and active cells in this tissue are macrophages and fibroblasts, which after their interaction with the wear debris produce most of the soluble chemical factors and mediators, which are going to be analyzed below. Additionally these soluble factors migrate through the joint fluid (pseudosynovial fluid, PSSF) in the layer between the implant and the bone (interface), where they continue their action, mainly affecting the bony tissue. Finally the fibrous interface tissue (IFT), between the prosthesis and the bone, is formed and this leads to failure of the implant, which becomes loose. The communication of the interface layer with the space of the initial foreign body reaction is described as effective joint space, may result an early micromotion of the implant, and could be related to the surgical technique [20]. The interface tissue is heavily infiltrated with several different cell types, mainly macrophages, lymphocytes, fibroblasts, endothelial cells, and osteoclast precursors (OCPs)/osteoclasts. Beside enhanced and chronic inflammatory reactions in the periprosthetic region, the cellular recruitment to this region is promoted by induced chemokine expression [21–25]. Macrophages activation by phagocytosis of the wear debris particles, which are impervious to enzymatic degradation, has been shown to be the principle pathophysiologic mechanism in particle-induced periprosthetic osteolysis. Activated macrophages secrete proinflammatory and osteoclastogenic cytokines as well as proteolytic enzymes exacerbating the inflammatory response leading to activation of a periprosthetic osteolytic cascade (Figure 1). It is known that particles smaller than 8–10 $\mu$m are phagocytosed by macrophages, while bigger particles induce giant cell reaction and are associated with such cells [26]. However, it has been reported that contact between wear particles and macrophages without phagocytosis is also important for the signal transduction of cytokines and activation of macrophages [14].

The identity of the macrophage surface receptors responsible for recognition of the particles and the full repertoire of signaling cascades initiated or modified by particle binding remain poorly understood although macrophages are the best-characterized cellular target for particle action. One important consideration in determining the involvement of specific cell surface receptors is the extent to which different particles become opsonized with host serum proteins prior

to phagocytosis. There is evidence that polyethylene activates complement [27], and this would argue in favor of a role for complement receptors, such as complement receptor 3 (CR3), in particle uptake. Indeed, CR3 expressing phagocytes have been detected in granulomatous lesions associated with hip replacement [28]. An involvement of CR3 in particle action is also supported by observations that antibodies against CR3 reduce particle uptake [14] and that activation of this receptor mimics several aspects of downstream signaling by particles in that MAP kinases [29] and the transcription factors nuclear factor $\kappa$B (NF-$\kappa$B) and activator protein-1 (AP-1) are activated [14, 30], and production of proinflammatory cytokines [14] and chemokines [30] is elevated. By contrast, research on alveolar macrophage response to environmental particulate matter has implicated scavenger receptors (SRs), such as scavenger receptor A (macrophage receptor with collagenous structure; MARCO), in opsonin-independent uptake of titanium particles [31], suggesting that different particles may use different surface receptors. Accordingly, Rakshit and coworkers suggested the involvement of opsonization, complement, and integrin receptors, including CR3 and fibronectin receptors, in polymethylmethacrylate action, and an involvement of scavenger receptors (scavenger receptor A) in macrophages responses to titanium [32]. This would provide an intriguing explanation of the abilities of different types of wear debris to elicit particle type-specific responses in cultured macrophages. The concept that opsonization may differentially regulate uptake of different compositions of wear debris is also supported by observations that the spectra of adherent human serum proteins demonstrate a level of particle specificity [33].

Other cell types that are abundant within the periprosthetic tissue are fibroblasts and osteoclasts. Frequently, a proliferation of periprosthetic fibroblasts, which constitute a major source of proinflammatory and osteoclastogenic mediators [34–37], is accompanied by tissue hypervascularization. Periprosthetic fibroblasts exposed to wear and/or proinflammatory mediators are a major source of the receptor activator for nuclear factor $\kappa$B ligand (RANKL) required to drive osteoclastogenesis in patients with osteolysis (discussed below). Particles can also induce production in cultured fibroblasts of proinflammatory mediators, collagenases, and stromelysins [36, 37], which contribute to the development of osteolysis and chemokines, which promote the recruitment of increased numbers of osteoclast precursors to periprosthetic tissues. The final cellular consequence of particle action is an excess of osteoclast activity, which results in uncontrolled bone erosion. Osteoclasts, which are the unique cell type capable to resorb bone, are derived from circulating hematopoietic cells of the monocyte/macrophage lineage. Therefore, wear particles might increase osteoclast activity either by generation of functional osteoclasts from osteoclast precursor cells within the periprosthetic space or recruitment of osteoclast precursor cells from the blood or both [19]. However, it is not only an increased osteoclastic bone resorption due to particle exposure that can disrupt the balance in the bone remodeling process, but also a reduced bone formation caused by a direct negative impact of particles on osteoblasts [38]. As shown by Lochner and coworkers, wear particles can alter the metabolism of human primary osteoblasts [39]. In particular, metallic particles in the wear debris of cemented hip endoprostheses can compromise the vitality and activity of bone cells and bone matrix. In consequence, this may lead to a reduction of implant integration strength. Osteoblasts are rather responsible for bone formation but can indirectly participate in bone degeneration by changing cell viability and expression of specific chemokines as well as directly by the secretion of preosteolytic mediators and specific proteinases.

Collectively, the extensive body of research on *in vitro* cellular responses to wear debris suggests that while an inflammatory response by macrophages is central to the development of periprosthetic osteolysis, the detailed nature of this response will vary based upon several parameters, including prosthetic type, patterns of wear, cellular crosstalk, host factors, and cell-associated/extracellular molecular effectors.

## 2. Matrix Metalloproteinases, Their Endogenous Tissue Inhibitors, and Cytokines/Chemokines in the Periprosthetic Extracellular Matrix

Extracellular matrix (ECM) degradation and connective tissue remodeling around implants have been considered as major biological events in the periprosthetic loosening. Critical mediators of wear particle-induced inflammatory osteolysis released by periprosthetic synovial cells (mainly macrophages) are inflammatory cytokines (such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interleukin- (IL-) 1$\beta$, IL-6, and IL-10), chemokines (monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1alpha (MIP-1$\alpha$)), inflammatory enzymes (inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2)), and proteolytic enzymes, mainly matrix metalloproteinases (MMPs).

TNF-$\alpha$, IL-1$\beta$, and IL-6 are known to be important molecules involved in the foreign body reaction process, and their upregulation is considered to be a marker of inflammation. They are well recognized as key proinflammatory cytokines that provoke cellular proliferation, stimulate osteoclast formation, and increase bone resorption around prostheses [40–44]. In particular, TNF-$\alpha$ has a catabolic effect on bone. It can upregulate bone resorption in cultured mouse calvaria by a prostaglandin-independent mechanism and stimulates osteoblasts to produce osteoresorptive factors such as IL-6 and prostaglandin $E_2$ ($PGE_2$) [45, 46]. High levels of TNF-$\alpha$ have been detected in periprosthetic tissues of loose endoprostheses with focal osteolysis [47]. It has also been shown to exhibit a synergistic effect with titanium particles, when added in osteoblast culture [48]. IL-1$\beta$ induces differentiation and proliferation of osteoclasts as well as the production of MMPs and $PGE_2$ from fibroblasts and synovial cells [49, 50]. It also reduces the osteocalcin production by the osteoblasts [51]. According to Jiranek and coworkers, IL-1$\beta$ might play a significant role in the formation of IFT, because of its stimulatory activity on fibroblasts [52]. Kusano and coworkers have shown that IL-1$\beta$ augments bone

resorption in mouse calvaria culture *in vitro*, by inducing MMP-2, MMP-3, MMP-9, and MMP-13 production [53]. IL-6 is strongly implicated in bone catabolism. It is produced by the osteoblasts and induces bone resorption [54]. It also stimulates the formation of osteoclast-like cells in long-term human marrow cultures [55]. In periprosthetic tissues from loose orthopaedic implants with osteolysis, IL-6 levels are much higher than in tissues from loose implants without bone loss [47]. The role of prostaglandins in mediating pseudomembrane-associated bone resorption remains questionable. It is proposed, from *in vitro* studies, that prostaglandins play an important role in bone resorption [56]. Periprosthetic tissue, cultured in the presence of indomethacin, showed less bone resorptive capacity. Other investigators have shown that conditioned media from predialysed periprosthetic tissue cultures maintained their ability to cause bone resorption, indicating that the prostaglandins, removed by dialysis, had no effect whatsoever upon bone resorption [57]. Therefore, prostaglandins may be implicated in the loosening process through complex mechanisms involving interactions with MMPs and cytokines. On the other hand, IL-10 is synthesized by activated immune cells, in particular monocytes/macrophages, and has profound anti-inflammatory and immunoregulatory effects. This anti-inflammatory cytokine diminishes the expression of inflammatory mediators, inhibits antigen presentation, and induces expression of endogenous TNF-$\alpha$ inhibitors (soluble TNF receptors) to suppress the effects of proinflammatory cytokines in periprosthetic tissues [47, 58].

Chemokines play pivotal roles in the recruitment of inflammatory and immune cells subsequent to the development of periprosthetic inflammation following wear particle generation. MCP-1 and MIP-1$\alpha$ are two chemokines involved in this adverse process by recruiting monocytes/macrophages and lymphocytes to the site around prostheses and play important roles in periprosthetic osteolysis [59–61]. Previous studies suggest that high levels of inflammatory enzymes, such as iNOS and COX-2, are also present in the tissues around prostheses and therefore may account for periprosthetic bone resorption [62]. Macrophages are the major inflammatory cells accounting for this response. iNOS is closely involved in regulating inflammatory responses and COX-2 is induced by many cytokines, such as TNF-$\alpha$ and IL-1$\beta$, and the overexpression of these two enzymes plays a key role in chronic inflammatory diseases [63]. Furthermore, iNOS and COX-2, as well as TNF-$\alpha$ and IL-6, are inductive regulators of osteoclastogenesis [64].

A key role in periprosthetic ECM remodeling and destruction belongs to MMPs because of their ability to degrade in concert most extracellular matrix components, such as collagens, gelatin, elastin, laminin, fibronectin, or proteoglycan core proteins. MMPs contain four well-defined domains: a signal peptide, a propeptide with a conserved cysteine residue, a catalytic domain with a Zn-binding site, and a hemopexin-like domain at the COOH-terminal region, and they are frequently subgrouped based on substrate specificities and sequence characteristics. There are six main families of MMPs: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), matrilysins (MMP-7 and MMP-26), membrane-type MMPs (MT-MMPs: MMP-14, -15, -16, -17, -24, and -25), and other MMPs, which are not categorized in any of the previous groups (MMP-12, -19, -20, -21, -23, -27, and -28). The expression of MMPs is under tight control at the transcription level and their proteolytic activity is regulated posttranslationally in several ways [65]. MMPs are synthesized as zymogens, which are then activated extracellularly, with the exception of MMP-11 (stromelysin 3), MT-MMPs, MMP-21, MMP-23, and MMP-28. Although pro-MMPs can be activated *in vitro* by various proteolytic and nonproteolytic means, the *in vivo* activation mechanisms have not yet been completely clarified. Further, the proteolytic activity of MMPs is regulated by specific tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs have been identified (named TIMP-1 to -4), which form high-affinity 1 : 1 noncovalent complexes with all active MMPs, thereby inhibiting their action. TIMPs inhibit all MMPs tested so far, but TIMP-1 is a poor inhibitor for MT3-MMP, MT5-MMP, and MMP-19. TIMP-3 has been shown to inhibit members of the ADAM (a disintegrin and metalloproteinase) family (ADAM-10, -12, and -17) and ADAMTSs (ADAM with thrombospondin motifs) (ADAMTS-1, -4, and -5). TIMP-1 inhibits ADAM-10. While TIMP-1-null mice and TIMP-2-null mice do not exhibit obvious abnormalities, TIMP-3 ablation in mice causes lung emphysema-like alveolar damage [66] and faster apoptosis of mammary epithelial cells after weaning [67], indicating that TIMP-3 is a major regulator of metalloproteinase activities *in vivo*. However, the functions of TIMPs go beyond the inhibition of MMPs and are also partakers in the activation and coactivation of others [68]. The balance between the levels of activated MMPs and free TIMPs determines in part the net MMP activity. In addition to regulating the MMPs, TIMPs have also been shown to have angiogenic and growth factor-like activities [69].

Numerous studies have demonstrated that specific MMPs and TIMPs are expressed in periprosthetic tissues and are critically involved in the bone resorption and subsequent implant failure (Tables 1 and 2). In a study conducted by Takei and coworkers, the mRNA expression patterns of 16 different types of MMPs in synovium-like interface tissues between bone and prosthesis of loose artificial hip joints were analyzed to evaluate which MMPs were present at the mRNA level and possibly contributed to periprosthetic loosening [70]. It was shown that periprosthetic tissues were characterized by highly elevated expression of MMP-1, MMP-9, MMP-10, MMP-12, and MMP-13; moderate expression of MMP-2, MMP-7, MMP-8, MMP-11, MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17), and MMP-19; lower expression of MMP-3; and little significance of MMP-20. Quantitative analysis of mRNA expression of their endogenous inhibitors (TIMPs) in periprosthetic tissues showed a significant upregulation of TIMP-1, -2, and -3 mRNA expressions in contrast to the decreased levels of TIMP-4 [71]. On protein level, strong immunoreactivity was observed for the extracellular matrix metalloproteinase inducer (EMMPRIN/CD147) in the lining-like layers, sublining area, and vascular endothelium of synovium-like interface tissue around loosened prostheses.

Table 1: Matrix metalloproteinases (MMPs) in periprosthetic microenvironment (expression and/or activity: ↑ with bold data: high; ↑ without bold data: moderate).

| MMPs | | Substrates | Expression and/or activity in periprosthetic microenvironment [References] |
|---|---|---|---|
| *Collagenases* | | | |
| Contain hemopexin domain and peptide linking with catalytic domain | **MMP-1** (interstitial collagenase; collagenase 1)<br>**MMP-8** (neutrophil collagenase; collagenase 2)<br>**MMP-13** (collagenase 3) | Collagen type I, III, V, VII, VIII, X, gelatin, IL-1$\beta$, MMP-2, -9, fibronectin | ↑**MMP-1** [39, 58, 70, 72, 80, 82, 90]<br>↑MMP-8 [70]<br>↑**MMP-13** [70, 82, 89] |
| *Gelatinases* | | | |
| High substrate specificity to native collagen and gelatin | **MMP-2** (gelatinase A; 72 kDa metalloproteinase)<br>**MMP-9** (gelatinase B; 92 kDa metalloproteinase) | Collagen type IV, V, VII, X, proteoglycans, gelatin, elastin, laminin | ↑**MMP-2** [58, 70, 73, 74, 76, 77, 80, 90]<br>↑**MMP-9** [58, 70, 76, 77, 80, 90, 91] |
| *Stromelysins* | | | |
| Metalloproteinases of stroma | **MMP-3** (stromelysin 1)<br>**MMP-10** (stromelysin 2)<br>**MMP-11** (stromelysin 3) | Proteoglycans, fibronectin, laminin, elastin, gelatin, plasminogen, vitronectin, fibrinogen, fibrin, collagen type III, IV, V, antithrombin III, MMP-1, -2, -8, -9, -13 | ↑MMP-3 [58, 70, 74, 80]<br>↑**MMP-10** [70]<br>↑MMP-11 [70] |
| *Matrilysins* | | | |
| The smallest among MMPs, lack of hemopexin domain | **MMP-7** (matrilysin, metalloendopeptidase)<br>**MMP-26** (matrilysin-2, endometase) | Collagen type IV, proteoglycans, glycoproteins, gelatin | ↑MMP-7 [70] |
| *Membrane-type MMPs* | | | |
| (A) Transmembrane-type MMPs | **MMP-14** (MT1-MMP)<br>**MMP-15** (MT2-MMP)<br>**MMP-16** (MT3-MMP)<br>**MMP-24** (MT5-MMP) | Collagen type I, II, III, gelatin, elastin, laminin, fibronectin, fibrin, proteoglycans, proMMP-2, proMMP-13 | ↑**MMP-14** [70, 73]<br>↑MMP-15 [70]<br>↑MMP-16 [70] |
| (B) GPI-anchored MMPs | **MMP-17** (MT4-MMP)<br>**MMP-25** (MT6-MMP) | | ↑MMP-17 [70] |
| *Other MMPs* | | | |
| MMPs that are not categorized in any of the previous groups | **MMP-12** (macrophage metalloelastase)<br>**MMP-19**<br>**MMP-20** (enamelysin)<br>**MMP-21, MMP-23**<br>**MMP-27, MMP-28** | | ↑**MMP-12** [70]<br>↑MMP-19 [70] |

Moreover, double immunofluorescence labeling revealed EMMPRIN/MMP-1 double-positive cells in lining-like areas and the sublining area of interface tissue. These data indicated that EMMPRIN expression was upregulated in interface tissues, and that locally accumulated EMMPRIN may modulate MMP-1 expression [72].

In another study, Nawrocki and coworkers used immunohistochemistry (IHC) to identify the cells responsible for the synthesis of MMPs in the periprosthetic microenvironment [73]. MMP-2 (gelatinase A) and its activator MT1-MMP were strongly detected in macrophages and multinucleated giant cells in contact with polyethylene wear debris. Similar results have been also obtained by other IHC studies on MMP-2 in this pathological process [58, 74, 75]. Indeed, these studies reported the expression of MMP-2, as well as those of other MMPs, such as MMP-9 and MMP-1 and, in a more restricted pattern, MMP-3, in macrophages, fibroblasts, and endothelial cells. The strong expression of MMP-2 and its activator MT1-MMP in phagocytic cells of periprosthetic samples suggests their contribution to aseptic loosening of prosthetic components. These data are supported by the observation that high levels of gelatinolytic activities were also previously detected in the same type of lesion [76–79]. Of particular interest was the colocalization of MMP-2, MT1-MMP, and TIMP-2 in the same cells [73]. The strong expression of TIMP-2 in interface tissue around implants was also reported by Ishiguro and

Table 2: Tissue inhibitors of metalloproteinases (TIMPs) in periprosthetic microenvironment (expression and/or activity: ↑ with bold data: high; ↓ without bold data: low).

| TIMPs | Preferred MMP/ADAM/ADAMTS | Expression and/or activity in periprosthetic microenvironment [References] |
|---|---|---|
| TIMP-1 | Most MMPs, ADAM-10 (inhibition). MT3-MMP, MT5-MMP, MMP-19 (weak inhibition) | ↑**TIMP-1** [71, 74, 76, 78, 80, 82] |
| TIMP-2 | Most MMPs (inhibition). MMP-2 (activation) | ↑**TIMP-2** [71, 73, 76, 80, 82] |
| TIMP-3 | Most MMPs, ADAM-10, -12, -17, and ADAMTS-1, -4, -5 (inhibition). MMP-2, MT3-MMP (activation) | ↑**TIMP-3** [71] |
| TIMP-4 | Most MMPs (inhibition) | ↓TIMP-4 [71] |

coworkers [80]. These data support the concept of Strongin and coworkers, who postulated that proMMP-2 activation could be mediated by a trimolecular stoichiometric complex involving MMP-2, TIMP-2, and MT1-MMP [81]. More specifically, these authors demonstrated that the activated form of MT1-MMP acts as a cell surface TIMP-2 receptor. The MT1-MMP/TIMP-2 complex may in turn serve as a receptor for proMMP-2, leading to its processing into the active enzyme. Interestingly, the detection of a soluble type of MT1-MMP (~56 kDa) in synovial and pseudosynovial fluid of patients with rheumatoid arthritis, osteoarthritis, and loose arthroplasty endoprostheses has been previously reported, without clarifying the origin of this type or its activation state. It was proposed that this form was probably processed proteolytically from the transmembrane type of MT1-MMP [75]. A protein band of ~56 kDa was also detected in periprosthetic tissues extracts and pseudosynovial fluids from loose arthroplasty endoprostheses that was ascribed to a soluble form of MT1-MMP [82].

The contribution of different members of the MMP family in gelatinolytic and collagenolytic potential was evaluated by gelatin zymography, and the degradation of synthetic dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg (DNP-S) together with reverse phase high performance liquid chromatography, respectively [82]. Activated species of both MMP-1 and MMP-13 were identified in most periprosthetic tissues, which could be responsible for the detected DNP-S-degrading activity, while the gelatinases MMP-2 and MMP-9 did not contribute in this potential, since they mainly existed in complex with TIMP-2 and TIMP-1, respectively. These data indicated that MMP-1 and MMP-13 may play a key role in the degradation of periprosthetic ECM, since they degrade native type-I and type-III collagens. Moreover, they may directly contribute to bone resorption, by removing the osteoid layer from calcified bone, facilitating the osteoclastic bone resorption [83–85]. Accordingly, it has been previously reported that periprosthetic tissue extracts exhibited high TIMP-free collagenolytic activity although TIMP-1 and TIMP-2 have been detected in periprosthetic tissues [78, 79]. TIMPs produced by pseudosynoviocytes may be released into synovial fluid to limit MMP proteolysis, but their localization far from local degradation sites leads to the hypothesis of a disruption of the MMP-TIMP balance in favor of MMPs surrounding wear particles.

Immunohistochemical study of the plasminogen activation system, which is closely associated with MMP activities, disclosed localization in periprosthetic tissues of urokinase plasminogen activator (uPA), uPA-receptor (uPAR), and tissue type plasminogen activator (tPA) in macrophages with phagocytosed metal, polyethylene, cement particles, or accompanying pieces of necrotic bone [86]. Plasminogen activator inhibitor-1 (PAI-1) staining was present in the neighboring areas that stained for uPA or tPA, but PAI-1 staining was also found overlapping and outside these areas. These findings suggest a role for the uPA/uPAR and PAI-1 in activation and focalization of extracellular matrix degradation in periprosthetic tissues. The expression of the plasminogen activation system by macrophages containing phagocytosed material suggests undegradable microdebris as a possible initiating and perpetuating stimulus for a proteolytic activation cascade, which may contribute to loosening of the prosthesis. In contrast to most ECM-degrading proteases, uPA has restricted substrate specificity. Although uPA best-documented proteolytic action is the conversion of inactive plasminogen to active plasmin, it has been also reported that it is able to activate the cell surface MT1-MMP proenzyme [87]. Like uPA, plasmin is also a serine protease but, in contrast to uPA, has broad substrate specificity. Apart from native collagen, plasmin can degrade most proteins present in the ECM. It can also activate the precursor forms of a number of MMPs, such as MMP-3, MMP-9, MMP-12, and MMP-13 [88]. Elevated protein levels of MMP-13, together with uPA and PAI-1 in periprosthetic pseudocapsular and interface tissues were also reported by Diehl and coworkers [89]. However, no significant correlation between the protein expression of these factors and years from arthroplasty to revision or to type of fixation (cemented versus cementless) was observed.

It should be noted that the physical characteristics of wear particles (size, shape, and sintering temperature) as well as their amounts in the periprosthetic tissues can modify the toxicity of the biomaterials and the production of cytokines, MMPs, and TIMPs by various cell types. For example, macrophages seemed to release MMPs (MMP-1, -2, and -9) in proportion to the amount of particulate debris at the prosthetic interface [90]. Laquerriere and coworkers demonstrated that sintering temperature (that modify crystal size and surface area) had little effect on MMPs and TIMPs production. Nonphagocytable particles induced more MMP-9, although phagocytable particles induced more IL-1$\beta$ release. The shape of the particles was the most important factor since needle-shaped particles induced the most significant upregulated expression of MMPs (mostly MMP-9) and IL-1$\beta$ [91]. In another study, human osteoblasts were incubated with particles experimentally generated in the interface between hip stems with rough and smooth surface finishings as well as different material compositions [39].

The results revealed distinct effects on the cytokine release of human osteoblasts towards particulate debris. Thereby, human osteoblasts released increased levels of IL-6 and IL-8 after treatment with metallic wear particles. The expression of VEGF was slightly induced by all particle entities at lower concentrations. Apoptotic rates were enhanced for osteoblasts exposed to all the tested particles. Furthermore, the *de novo* synthesis of type 1 collagen was reduced and the expression of MMP-1 was considerably increased. Therefore, by the secretion of degrading effectors, osteoblasts may actively contribute to matrix weakening.

## 3. Molecular Mechanisms Controlling the Periprosthetic Microenvironment: Implication of MMPs/TIMPs and an Emerging Role for Proteasome

A large body of studies reveals a strong interdependence of MMP expression and activity with the molecular mechanisms that control the composition and turnover of periprosthetic ECMs. MMPs can either actively modulate or be modulated by the molecular mechanisms that determine the debris-induced remodeling of the periprosthetic microenvironment (summarized in Figure 2). One likely mechanism whereby particulate debris may induce osteoclast generation and activation is an indirect one, mediated through the actions of proinflammatory mediators that can act on osteoclast precursors and, most importantly, modulate the RANKL/osteoprotegerin (OPG) ratio through actions on cells within the periprosthetic tissue. RANKL is a type II homotrimeric transmembrane protein, which is normally expressed on osteoblastic cell membrane but is also expressed by fibroblasts and activated T cells [92]. Binding of RANKL to RANK on preosteoclasts (OCPs) activates NF-$\kappa$B and Jun N-terminal kinases (JNKs) pathways to induce cell differentiation [93]. NF-$\kappa$B is likely the most notable transcription factor implicated in wear debris action. This protein complex, long known as a key regulator of inflammatory gene expression, is also emerging as an important player during osteoclastogenesis. Supporting evidence for a role of NF-$\kappa$B in periprosthetic osteolysis comes from observations that deficiency of NF-$\kappa$B in mice protects against titanium-induced calvarial osteolysis [94], and that inhibition of NF-$\kappa$B blocks wear debris induction of osteoclastogenesis *in vitro* [95, 96]. Osteoblasts also secrete OPG, a soluble decoy receptor for RANKL, which strongly binds to RANKL and effectively inhibits its activity on preosteoclasts differentiation and maturation [97]. OPG is a glycoprotein possessing 4 cysteine-rich domains at its Nterminus by which it binds to RANKL, whereas its C-terminus contains 22 homologous death domains of unknown function and a heparin binding domain by which the glycoprotein interacts with matrix macromolecules, such as glycosaminoglycans and proteoglycans. Importantly, any imbalance in the RANKL/OPG ratio impairs normal bone remodeling and evidence suggests a role of RANKL/OPG ratio in wear debris-induced osteolysis. In particular, it has been shown that RANKL blockade with OPG [98, 99] or RANK:Fc (RANKL antagonist consisting of the extracellular region of RANK fused to the Fc portion of human IgG1), or by using mice genetically deficient in RANK prevents wear debris-induced osteolysis in the murine calvarial model [100]. Moreover, wear debris can increase the RANKL/OPG ratio in murine calvarial tissues [101], and several reports have identified elevated RANKL expression in IFTs [102–105]. However, the fact that several different cell types within the periprosthetic tissue are capable of RANKL expression, including osteoblasts, fibroblasts, T lymphocytes, and also macrophages and giant cells [102–106], makes the cellular basis for elevated RANKL expression very complicated.

Several MMPs are overexpressed and correlated with osteoclast differentiation, maturation and activation by interfering with the RANK/RANKL/OPG system in inflammation and cancer [107]. MMP-9 is likely to play an important role in the recruitment of osteoclasts at inflammatory and metastatic sites since the use of chemical inhibitors or antisense oligonucleotides against MMP-9 abrogated the recruitment of osteoclasts [108]. Franco and coworkers showed that doxycycline (Dox), which can suppress the enzyme activity of MMP-9 [109], as well as MMP-9 inhibitor (MMP-9 inhibitor I), downregulated the expression of RANKL-induced osteoclast maturation genes in conjunction with the suppression of RANKL-induced osteoclastogenesis [110]. These findings indicated that MMP-9 induced by RANKL plays a role as an upstream effector of osteoclast gene expression, and, as such, it may also be a regulator of osteoclastogenesis. Previous studies reported that MMP inhibitor (RP59794) [111] or MMP-9 gene knockout [112] reduced osteoclast migration, which results in reduction of the resorption process in the growth plate and, as a consequence, attenuated development of bone marrow cavity. However, since the latter studies treated the aspect of osteoclast migration, but not differentiation, the study by Franco and coworkers is the first to report the involvement of MMP-9 activity in RANKL-induced osteoclastogenesis. In another report, MMP-7 could solubilize RANKL in mouse models of prostate and breast cancer promoting osteoclast activation and osteolysis [113]. The limiting step in RANKL-dependent osteoclastogenesis is the contact of RANKL-expressing osteoblasts with RANK on the cell surface of osteoclasts. This limitation is prohibited by proteolytic cleavage of RANKL from the cell surface through the action of MMP-7 and cathepsin G. Importantly, it has been shown in tumor-induced bone disease that soluble RANKL retains its activity and is liberated at the tumor-bone interface promoting osteoclastogenesis without the necessity of direct interaction of osteoblast with osteoclasts [113–115].

Possible accumulation of cell membrane and matrix proteoglycans at the inflammatory periprosthetic ECM may also modulate the RANK/RANKL/OPG system through both MMP-independent and -dependent manners. For example, it has been shown that myeloma cells decrease OPG availability by internalizing it through binding to glycosaminoglycan side chains of surface syndecan-1 and degradation to lysosomes, thereby regulating its inhibitory effect on RANKL [116]. Moreover, shed syndecan-1 secreted by myeloma cells may also bind OPG [117] and block its inhibitory activity to RANKL triggering further osteoclast differentiation and activation. Syndecan ectodomain shedding is an important

regulatory mechanism, because it rapidly changes cell surface receptor dynamics and generates soluble ectodomains that can function as paracrine or autocrine effectors or competitive inhibitors. Strong evidence indicates the involvement of several MMPs in syndecan cleavage *in vitro* and *in vivo* [118]. Matrilysin (MMP-7) cleaves syndecan-1 [119], gelatinases MMP-2 and MMP-9 can cleave syndecans-1, -2, and -4 [120, 121], whereas the membrane-associated metalloproteinases MT1-MMP and MT3-MMP are known to cleave syndecan-1 [122]. Taken together, these data suggest a critical role of certain members of MMPs in interfering with the RANK/RANKL signaling axis by directly and/or indirectly regulating OPG and RANKL availability, thereby modulating osteoclast generation and activation within the periprosthetic tissue (Figure 2).

An important observation in several studies was that specific gene responses were induced in different cell types of the periprosthetic microenvironment by an initial and early particulate biomaterial-cell interaction. The differential gene expression indicated that particle-cell interactions activated specific signaling events and transcription factors. Vermes and coworkers have found that particles rapidly activated protein tyrosine phosphorylation and induced the nuclear transcription factor NF-$\kappa$B in osteoblasts [123]. The rapid kinetics of the activation suggested that the particles elicited signals before the phagocytosis process. Importantly, inhibition of NF-$\kappa$B function by either tyrosine kinase inhibitors or antioxidants reversed the suppressive effect of titanium particles on procollagen a1[I] gene expression suggesting a functional relationship in osteoblasts between tyrosine phosphorylation, NF-$\kappa$B activation, and collagen gene expression. Thus, particle-cell interactions before their phagocytosis appear to initiate an intracellular tyrosine phosphorylation cascade that targets the nuclear activation of the inducible transcription factor NF-$\kappa$B.

A role for protein tyrosine kinases (PTKs) in regulation of the activation of MMPs/TIMPs in ion-induced activation of macrophages was suggested by Luo and coworkers [124]. In particular, cobalt (Co) and chromium (Cr) ions, two corrosion products found in the periprosthetic environment of metal-on-metal prostheses, were shown to upregulate MMP-1, TIMP-1, and cytokines (such as TNF-$\alpha$) in cultures of human U937 macrophages. The inhibitory effect of genistein suggested the implication of PTKs in the induction of MMP-1 and TIMP-1 expressions by $Co^{2+}$ and $Cr^{3+}$ ions in macrophages, the most important cellular target of wear debris. Genistein, a soy isoflavonoid that is a natural broad spectrum PTK (such as EGFR, PDGFR, IGFR, Src) inhibitor, has been shown to regulate the transcription of several MMPs and their endogenous inhibitors (TIMPs) by breast cancer cells [125, 126]. Moreover, previous *in vitro* studies demonstrated that genistein downregulates the expression of vascular endothelial growth factor (VEGF), which is a major signaling protein that contributes to angiogenesis [127]. VEGF is produced by multiple cell types, including macrophages and osteoblasts [128, 129]. It exerts its biological activity by binding to two TK receptors, VEGF receptor-1 (VEGFR-1; Flt-1) and VEGFR-2 (Flk-1/KDR) [130]. VEGF is actively involved in the process of inflammation, osteoclastogenesis, and bone resorption [131–133] and probably plays an important role in wear debris-induced inflammatory osteolysis since the periprosthetic tissues at the bone-implant interface show a high degree of vascularization [134]. Notably, Luo and coworkers showed a more potent effect of herbimycin A, Src kinase-specific inhibitor, on the expression of MMP-1 and TIMP-1 compared to genistein [124], providing strong evidence for a critical role of Src kinases in modulating the expression levels of MMP-1 and TIMP-1 in macrophages in the presence of $Co^{2+}$ and $Cr^{3+}$ ions (Figure 2). Other ions released from hip prostheses, such as titanium [135] and nickel [136], have been shown to stimulate TNF-a in a manner similar to Co and Cr, suggesting that other ions may also modulate tyrosine kinase activity probably affecting the amounts and activities of MMPs/TIMPs in periprosthetic ECMs.

Moreover, the adhesion of macrophages to phosphorylcholine-polymer coated surfaces stimulated the expression of MMP-1 and TIMP-1, suggesting that cell adhesion induced a remodeling of the macrophage ECM. The inhibition of the expression of these genes by genistein and herbimycin A suggested that PTKs were also implicated in this remodeling [124]. Interestingly, this kind of materials-stimulated expression of genes implicated in ECM remodeling was also observed in fibroblasts induced by three-dimensional collagen [137, 138]. In these studies, alpha1beta1 and alpha2beta1 integrins mediated the signals inducing downregulation of collagen gene expression and upregulation of MMP-1, respectively. Therefore, the potential impact of macrophage surface integrins-evoked signals on the periprosthetic microenvironment should be further investigated to better understand the cellular effects of particles liberated from the articular surface of prostheses (Figure 2).

One major part of the organism's first line of defense against infection is a family of pattern recognition receptors (PRRs) called the Toll-like receptors (TLRs). TLRs are transmembrane proteins found in various cells and recognize infectious and endogenous threats, so-called danger signals, which evoke inflammation and assist adaptive immune reactions. It has been suggested that TLRs play a role in periprosthetic tissues and arthritic synovium. Tamaki and coworkers found that peri-implant tissues were well equipped with TLRs and, in aseptic loosening, monocytes/macrophages were the main TLR-expressing cells [139]. This could lead to production of inflammatory cytokines and MMPs after phagocytosis of wear debris derived from an implant. A major conclusion of the study was that inflammatory cells in both aseptic and septic tissues were equipped with TLRs, providing them with responsiveness to both endogenous and exogenous TLR ligands. In this line, the high expression of TLRs in the periprosthetic tissues could be potentially important, as they can reflect occurrence of subclinical biofilms on the prosthetic surfaces. Activation of TLRs has been suggested to modulate the expression levels of certain MMPs but not TIMPs. In a recent study, Lisboa and coworkers showed that activation of TLR-2 and TLR-4, two TLR members expressed by a variety of human cells that participate in the recognition

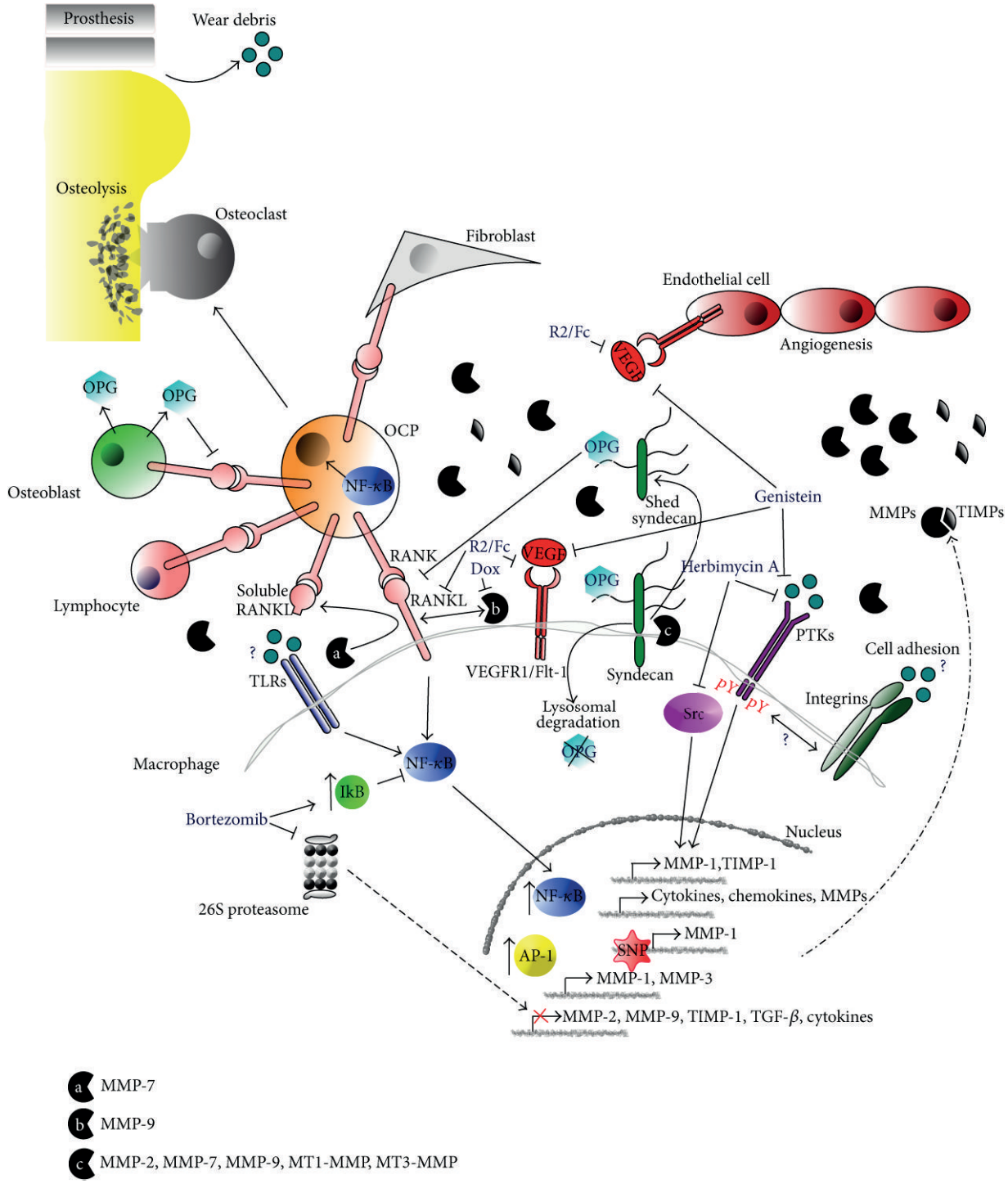


FIGURE 2: Hypothetical model of the molecular mechanisms that control periprosthetic microenvironment and potential molecular targeting with regard to the expression and activity of MMPs/TIMPs to prevent osteolysis *(see text for details)*.

of bacterial lipoproteins and lipopolysaccharides (LPS) [140], induced an increase in the secretion of MMPs-1, -3, and -10 by cultured periodontal fibroblasts, and this was mediated via the p38, JNK1/2, and NF-$\kappa$B pathways [141]. It is likely that there is a broad variation in the response of cells to TLR ligands that is dependent on the type of stimulus in the periprosthetic microenvironment. Therefore, the possibility of potentiation of MMPs activation concomitant with TLR activation in periprosthetic tissues needs to be further investigated (Figure 2).

Genetic variation may determine individual responses in terms of susceptibility to osteolysis and recovery. Expression

levels of the MMPs at both the mRNA and protein levels can be affected by the introduction or loss of transcription binding sites by single nucleotide polymorphisms (SNPs). SNPs are the most common sequence variation in the human genome and can affect coding sequences, splicing, or transcription regulation. In a case control, it was shown that a single-nucleotide polymorphism (SNP) of MMP-1 was highly associated with total hip replacement aseptic failure [142]. This SNP existed within a promoter region of the gene and as such may have a direct effect on the amount of gene expression (Figure 2). However, the mechanisms of MMP gene regulation are still not fully delineated, and it is likely that many more functionally important elements in their promoter regions are yet to be identified [143–145]. Moreover, investigation of SNPs in the TIMP genes would be a necessary complement for any study of MMP SNPs, given the evidence that the MMP-to-TIMP ratio plays a role in defining overall MMP activity.

In another line of research, Ortiz-Lazareno and coworkers found that the proteasome inhibitor MG-132 significantly diminished proinflammatory cytokines (TNF-$\alpha$, IL-1$\beta$, IL-6) release by U937 macrophages, whereas, induced a decrease in the membrane receptors TNF-R1 and IL-1R1 and an increase in the soluble receptors sTNF-R1 and sIL-1R1. However, MG-132 increased the IL-6R and decreased sIL-6R [146]. In another report, Mao and coworkers investigated the effects of Ti particles and the specific proteasome inhibitor bortezomib on the secretory profile of inflammatory cytokines, chemokines, and inflammatory enzymes in a murine macrophage cell line [147]. It was shown that Ti particles increased the production of TNF-$\alpha$, IL-1$\beta$, IL-6, IL-10, MCP-1, MIP-1$\alpha$, iNOS, and COX-2 in this cell line, while bortezomib inhibited the expression of all factors, except IL-10, in a time-dependent manner. Bortezomib, a potent, reversible and selective inhibitor of the chymotryptic activity of the proteasome, prevents the degradation of I$\kappa$B proteins, which mask the nuclear localization sequence of NF-$\kappa$B, therefore inhibiting the translocation of NF-$\kappa$B into the nucleus and further inhibiting the transcription and secretion of inflammatory mediators. It is known that NF-$\kappa$B regulates the transcription of a variety of genes of inflammatory cytokines (TNF-$\alpha$, IL-1$\beta$, IL-2, IL-6, and GM-CSF (granulocytemacrophage colony-stimulating factor)), chemokines (IL-8, MCP-1, and MIP-1$\alpha$), and inflammatory enzymes (iNOS and COX-2) [148]. Therefore, bortezomib may inhibit the proinflammatory factors mentioned earlier through inhibition of NF-$\kappa$B activity. Moreover, proteasome inhibition with bortezomib alters the binding of other transcription factors to the promoter region of several molecular effectors, thus modulating their expression levels [149]. The anti-inflammatory cytokine IL-10 induced by bortezomib inhibits TNF-$\alpha$ gene expression via inhibiting NF-$\kappa$B activity or by directly inhibiting TNF-$\alpha$ itself [150, 151]. It should be noted that bortezomib (as well as other proteasome inhibitors) exhibits beneficial effect on bone metabolism as it inhibits osteoclastic function and promotes osteoblastic activity by inhibiting NF-$\kappa$B activation induced by the RANK-RANKL signaling axis, which is the master regulator of differentiation and activation of osteoclasts [152–155].

The inhibitory effects of bortezomib and MG-132 on the secretion of inflammatory cytokines and their receptors by macrophages suggested the potential involvement of the proteasome pathway in periprosthetic loosening and osteolysis process. The proteasome is a major cellular protease complex that functions as the main driver of intracellular degradation of a wide variety of cellular proteins implicated in several physiological and pathological cellular functions [156]. Interestingly, the proteasome pathway controls via transcriptional and posttranslational mechanisms the concentration and turnover of several ECM macromolecules (including proteoglycans/glycosaminoglycans, MMPs/TIMPs, and collagens) [157]. Importantly, the proteasome provides a link between the regulation of extracellular proteolytic events to intracellular proteolysis by modulating MMP/TIMP expression and activity. In particular, it has been shown that proteasome blockade by proteasome inhibitors resulted in a marked modification of gene expression and activity of MMPs (upregulation of MMP-1, -3 and downregulation of MMP-2, -9) and TIMPs (downregulation of TIMP-1). Moreover, proteasome inhibition regulated also the synthesis and activity of other ECM constituents, such as TGF-$\beta$ (downregulation), decorin (upregulation), and collagen type-I and type-IV (downregulation) [157]. Therefore, since matrix remodeling and degradation can be tightly regulated by proteasome activities, its modulation may be considered as a novel strategy to control the properties of periprosthetic ECMs as has been recently suggested for tumor microenvironment (Figure 2).

## 4. Potential Therapeutic Perspectives

Much progress has recently been made in understanding the molecular and cellular mechanisms whereby prosthetic wear debris can ultimately cause aseptic loosening and osteolysis. However, the complex nature of the interactions between wear particles and periprosthetic cells as well as the multiple intracellular signaling pathways activated by such interactions results in the reality that development of therapeutic approaches to the treatment of periprosthetic osteolysis is long overdue. In the following lines, we will try to address strong rationale for potential clinical applications of the described molecular mechanisms for periprosthetic loosening and osteolysis treatment.

The finding that proteasome inhibitors (i.e., bortezomib, MG-132) altered the macrophage secretory profile of inflammatory cytokines, chemokines, and inflammatory enzymes [146, 147], which play a key-role in the inflammatory response of periprosthetic tissue to wear debris, reveals a critical role for proteasome in the development of periprosthetic loosening and osteolysis. In a recent review, we have highlighted the novel approach of targeting the proteasome as a mechanism to control the synthesis and bioactivity of ECM effectors in tumors, since the proteasome appears to be an elegant molecular regulator of specific matrix macromolecules [157]. From the data described in the present review, proteasome signaling pathway emerges as a promising target to selectively regulate the synthesis and activity of inflammatory factors

(such as TNF-$\alpha$, IL-1$\beta$, IL-6, IL-8), their membrane receptors, and matrix degrading effectors (such as specific MMPs) in the periprosthetic microenvironment. To this aim, a promising agent is bortezomib, which exhibits multiple functions by interfering also with other intracellular signaling pathways such as the RANK-RANKL system thereby regulating new bone formation by both inducing osteoblastic function and inhibiting osteoclastogenesis. However, the elevated production of reactive oxygen species (ROS) by activated macrophages and osteoclasts in the presence of wear particles [158] should be considered in this context, since proteasome inhibitors have been also shown to induce ROS [159], which further attenuate the proteasomal system activation [160]. This proteasomal inhibition would potentially result in the accumulation of phosphorylated c-Jun and activation of AP-1 that ultimately induce MMP-1 and MMP-3 expression levels [160]. Therefore, proteasome inhibitors may have a synergistic effect with wear particles on ROS production and strongly induce the expression of specific MMPs within the periprosthetic microenvironment although their inhibitory effect on inflammatory cytokines and their receptors as well as other MMPs has been documented. Moreover, MG-132 has been found to significantly downregulate TIMP-1 expression in organ interface tissue cultures and primary IFT fibroblast cultures (Aletras and coworkers, unpublished data), which is in line with the findings of Fineschi and coworkers in dermal fibroblasts [161], revealing the complex and questionable role of proteasome in regulating distinct molecular effectors that would potentially be beneficial for periprosthetic osteolysis treatment. Therefore, the efficacy of proteasome inhibitors (such as bortezomib) to prevent periprosthetic loosening and osteolysis caused by implant-derived particles is an emerging concept and needs to be further investigated.

Taking under consideration these data, it should be investigated whether an alternative strategy associated with proteasome activation would be more beneficial in the treatment of periprosthetic loosening. Several activators of the proteasome, such as isoflavonoids, should be tested in order to reverse the effects on the expression levels of specific MMPs and TIMPs described previously, as a result of the reduced proteasome activity in IFT. Proteasome activation might be further induced by combined treatment with activators of nuclear factor erythroid 2-related factor 2 (Nrf2), such as sulforaphane [162]. Notably, Nrf2 upregulates the transcription of multiple antioxidant enzymes providing an effective means of reducing elevated ROS levels in IFT.

The observation that genistein and herbimycin A strongly attenuated the expression of MMP-1 and TIMP-1 by macrophages implied that tyrosine kinases play also an essential role in the signaling pathways regulating the remodeling of macrophage ECM in the periprosthetic microenvironment [124]. Therefore, PTKs (e.g., Src kinases) may serve as an additional target for selective inhibition of periprosthetic osteolysis. Importantly, proteasome is implicated in this process since it has been reported that herbimycin A targets the degradation of tyrosine kinases by the 20S proteasome [163]. Moreover, apart from its inhibitory action on PTKs, genistein was found to downregulate the expression of VEGF, a major angiogenic factor in periprosthetic microenvironment. The interactive network of the VEGF/Flt-1 and RANKL/RANK pathways may play important roles in the initiation, progression, and resolution of aseptic loosening. In a study by Ren and coworkers, it was shown that VEGF may be actively involved in the regulation of RANK/RANKL gene expression, and that it exerted a regulatory effect on the development of particle-induced inflammatory osteoclastogenesis through its unique Flt-1, rather than Flk-1, receptor located on monocyte/macrophage cell lineages [164]. In particular, they found that treatment with R2/Fc (a VEGF neutralizing antibody) but not SU5416 (an Flk-1 receptor inhibitor) resulted in the inhibition of polyethylene particle-enhanced VEGF/Flt-1 signaling and inflammatory osteolysis by trapping VEGF in the periprosthetic milieu (Figure 2). Taken together, these findings provide the biological rationale for a combined VEGF/Flt-1- and RANKL/RANK-targeted treatment strategy, especially in the early stages of wear debris-induced inflammatory response. The fact that the RANK/RANKL/OPG system is of crucial importance for the development of periprosthetic osteolysis together with the finding that Dox inhibits RANKL-induced osteoclastogenesis by its inhibitory effect on MMP-9 enzyme activity [110] provides a reasonable rationale for a pharmaceutical advantage of tetracycline antibiotics against periprosthetic osteolysis. It should be noted that this class of antibiotics, including Dox, has been effectively utilized for the treatment of bone resorptive diseases because of their activity to suppress osteoclastogenesis induced by RANKL.

Given that excessive osteoclast activity represents the cellular endpoint of osteolysis, it is not surprising that the bisphosphonate class of osteoclast inhibitors have come in for much discussion as possible therapeutic agents for this disease. Again, however, despite promising results in animal models, there is no clinical evidence supporting the effectiveness of these drugs in the treatment of osteolysis patients. Alendronate inhibits wear debris-induced osteolysis in the rat loaded tibial implant model of osteolysis [165] and in a similar canine model [9] and is also effective in preventing osteolysis in the murine calvarial model [6]. A single dose of zoledronic acid administered directly after surgery also suppressed particle-induced osteolysis in mouse calvaria [166]. Bisphosphonates inhibit osteoclast formation by blocking the mevalonate pathway of isoprenoid biosynthesis. Their potential effect in periprosthetic osteolysis should be also considered with regard to their ability to inhibit the enzymatic activity of various MMPs. Certain bisphosphonates showed beneficial effects as a result of altering the expression pattern of MMPs/TIMPs by inhibiting and increasing the gene and protein expression of several MMPs and TIMPs, respectively, in breast cancer cells. In particular, it has been shown that zoledronic acid suppressed the expression of metalloproteinases MMP-2, -9, the membrane type MT1- and MT2-MMP, whereas it increased the expression of their endogenous tissue inhibitors [167].

Though not extensively studied, other mechanisms that should be further investigated with regard to their contribution to the remodeling of periprosthetic ECM include SNPs

of certain MMP/TIMP genes as well as the involvement of TLRs in periprosthetic inflammation. An SNP of MMP1 gene was highly associated with total hip replacement aseptic failure [142]. It should be noted that MMPs do not possess only degrading functions but they also play protective and anti-inflammatory roles. Therefore, the association that exists with a particular polymorphic form of MMP-1 does not necessarily show that particular form is associated with increased MMP-1 activity; in fact, the opposite may be true. The possibility that SNP markers may serve as predictors of implant survival and aid in pharmacogenomic prevention of total joint replacement failure should be further investigated. A more comprehensive analysis of MMP and TIMP SNPs is thus required, and given the coverage by existing genome-wide association study (GWAS) platforms, a candidate gene approach is justified. Regarding TLRs, strong evidence indicated that macrophages, which are the most important cellular targets of wear debris, are the main TLR-expressing cells in periprosthetic microenvironment. The increased secretion of MMPs by combined TLR activation may be an important factor that should also be considered during treatment of periprosthetic loosening and osteolysis.

Extended information is available regarding the action of several nonsteroidal anti-inflammatory drugs (NSAIDs) upon significant for the loosening process effector molecules, which though originates from *in vitro* studies with articular chondrocytes and synovial or dental pulp fibroblasts [168–173]. However, little information is available in the literature about their possible role in retarding the periprosthetic loosening and bone resorption process. To this aim, we tested the effect of four widely used NSAIDS (i.e., aceclofenac, piroxicam, tenoxicam, and indomethacin) on cytokine, MMP, TIMP, and prostanoid production by IFT from patients with aseptic loosening of total arthroplasty [174]. The results showed that all the tested drugs exerted uniformly an inhibitory effect on IL-6 and TNF-$\alpha$, both known to directly cause osteoclastic bone resorption, independently of PGE2 [175–177]. Moreover, all of them modified specific MMPs (MMP-1, MMP-2, MMP-3, and MMP-9) expression and activity, although these drugs did not have a statistically clear effect on MMPs, which might reflect individual responses in terms of susceptibility to osteolysis. However, NSAIDs had a profound stimulatory effect on TIMP-1 production. Interestingly, paracetamol, which was used as a neutral drug, significantly decreased the synthesis of TNF-$\alpha$ and gelatinases (MMP-2 and MMP-9). Considering these observations, NSAIDs could reduce the ability of periprosthetic membrane to cause bone resorption, which is in line with previous reports that have shown that piroxicam, which exhibited about the same effects as the other tested NSAIDs, significantly decreased the IFT-induced resorptive process [178]. Consequently, *in vivo* long-term clinical trials may shed light on the possibility of a beneficial effect of specific NSAIDs on the loosening process.

## 5. Concluding Remarks

The elucidation and understanding of the cellular and molecular mechanisms that control the composition, turnover, and activity of matrix macromolecules within the periprosthetic microenvironment exposed to wear debris is highly important for the development of novel therapeutic approaches to the treatment of periprosthetic loosening and osteolysis. One ultimate target would be to disrupt the vicious cycle between the inflammatory response to wear debris particles induced by the secreted proinflammatory and osteoclastogenic cytokines and the periprosthetic osteolytic cascade governed by the uncontrolled action of MMPs. Considering the multicomplex biological mechanisms underlying the particle-induced periprosthetic loosening and osteolysis described in the present review, it may be crucial to develop and use combinations of conventional therapeutic agents as well as new approaches targeting specific extracellular, cell surface, and intracellular molecular effectors and apply them in clinical practice.

## Acknowledgment

The authors wish to thank Dr. Nikos Karamanos for valuable discussions and suggestions to improve the quality of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 214864, 10 pages
http://dx.doi.org/10.1155/2013/214864

*Research Article*

# Expression of Syndecan-4 and Correlation with Metastatic Potential in Testicular Germ Cell Tumours

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Received 30 April 2013; Accepted 28 May 2013

Academic Editor: Martin Götte



Although syndecan-4 is implicated in cancer progression, there is no information for its role in testicular germ cell tumours (TGCTs). Thus, we examined the expression of syndecan-4 in patients with TGCTs and its correlation with the clinicopathological findings. Immunohistochemical staining in 71 tissue specimens and mRNA analysis revealed significant overexpression of syndecan-4 in TGCTs. In seminomas, high percentage of tumour cells exhibited membranous and/or cytoplasmic staining for syndecan-4 in all cases. Stromal staining for syndecan-4 was found in seminomas and it was associated with nodal metastasis ($P = 0.04$), vascular/lymphatic invasion ($P = 0.01$), and disease stage ($P = 0.04$). Reduced tumour cell associated staining for syndecan-4 was observed in nonseminomatous germ cell tumours (NSGCTs) compared to seminomas. This loss of syndecan-4 was associated with nodal metastasis ($P = 0.01$), vascular/lymphatic invasion ($P = 0.01$), and disease stage ($P = 0.01$). Stromal staining for syndecan-4 in NSGCTs did not correlate with any of the clinicopathological variables. The stromal expression of syndecan-4 in TGCTs was correlated with microvessel density ($P = 0.03$). Our results indicate that syndecan-4 is differentially expressed in seminomas and NSGCTs and might be a useful marker. Stromal staining in seminomas and reduced levels of syndecan-4 in tumour cells in NSGCTs are related to metastatic potential, whereas stromal staining in TGCTs is associated with neovascularization.

## 1. Introduction

Testicular germ cell tumour (TGCT), although relatively rare, is the most common malignancy in men between 15 and 35 years old age group with increasing incidence in the past decades [1, 2]. TGCTs have become one of the most curable solid neoplasms, due to the advantage of diagnostic and therapeutic methods, but still the prognosis of highly advanced cases with bulky metastatic lesions is generally poor. Histologically, the TGCTs can be classified as seminomas germ cell tumours, which originate from undifferentiated germ cells, and nonseminomatous germ cell tumours (NSGCTs), which are arise from undifferentiated (embryonal carcinoma) and differentiated multipotent cells [3]. NSGCTs are generally more aggressive and the histological classification to seminoma or NSGCTs is the most important criterion for the selection of the treatment strategy. In patients with clinical stage I NSGCTs other biological markers apart from the percentage of embryonal carcinoma and the presence of vascular invasion, which are reliable prognostic indicators to identify patients at high risk for occult retroperitoneal disease, have not yet been shown to be of prognostic significance [4]. It has been shown that the presence of vascular invasion is associated with gain of a region at 17q12 and more specifically with the expression of inflammatory cytokine CCL2 in NSGCTs of stage I [5]. We demonstrated recently that the aggressiveness of testicular germ cell tumour cell lines is associated with increased expression of matrix

metalloproteinases (MMPs) and reduced expression of tissue inhibitors of matrix metalloproteinases (TIMPs) [6]. Hence it is important to evaluate novel markers for the development and prognosis of TGCTs.

Several studies have already focused on the role of proteoglycans in human tumours [7–11]. Accumulation of versican, an extracellular matrix proteoglycan, has been shown to correlate to the metastatic potential of testicular tumours [12]. Syndecans are integral membrane proteoglycans that are implicated in cell-cell recognition and cell-matrix interactions [11, 13]. Syndecans have a short cytoplasmic domain, one transmembrane, and one extracellular domain. The latter bearing heparan sulphate and or chondroitin sulphate glycosaminoglycan chains are capable of binding various growth factors and matrix molecules [13]. Syndecan-1 is the most thoroughly investigated member of the syndecan family and downregulation of cell membrane syndecan-1 is regarded as initial step towards malignant transformation in various malignancies [11, 13]. Although various studies have focused on the role of other syndecans in malignancies, little is known about the role of syndecan-4 in tumour development. Syndecan-4 mediates breast cancer cell adhesion and spreading [14] but also binds proangiogenic growth factors and cytokines and modulates growth factor/growth factor receptor interactions regulating angiogenic processes [15, 16]. Syndecan-4 potentiates Wnt5a signaling and enhances invasion and metastasis of melanoma cells [17]. The cell surface levels of syndecan-4 are reduced by Wnt5a signaling that promotes its ubiquitination and degradation thus regulating cell adhesion and migration [18]. Syndecan-4 interacts with chemokines through HS chains and promotes tumour cell migration and invasion [19, 20] but also regulates the invasion of K-ras mutant cells in collagen lattice together with integrin $\alpha 2\beta 1$ and MT1-MMP [21]. Taken into account the proved role of syndecans in malignancies and the structure/function similarities among syndecans we aimed to study the expression profile of syndecan-4 in TGCTs as well as its association with the metastatic potential of these tumours.

## 2. Material and Methods

*2.1. Cell Lines and Cultures.* The human seminoma cell line JKT-1 was a gift from Patrick Fenichel (University of Nice-Sophia-Antipolis, Faculty of Medicine, Nice, France) [22]. JKT-1 cells were cultured up to 38 passages to avoid the drift of these cells. The molecular signature of JKT-1 cells used in our study was described previously concerning the expression of seminoma markers (placenta alkaline phosphatase, NANOG, OCT3/4, AP2$\gamma$, and HIWI) [6]. Early passages of JKT-1 cells used in our study express a signature of markers which is still near from the one expressed by seminoma cells allowing their use as a model to study seminomas. Human embryonal carcinoma cell line NTERA-2 and teratocarcinoma cell line NCCIT were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The NTERA-2 and JKT-1 cell lines were cultured in DMEM supplemented with 10% fetal calf serum. The NCCIT cell line was cultured in RPMI 1640 supplemented with 10% fetal calf serum. All culture media contained 100 UI/mL penicillin and 100 UI/mL streptomycin. The cell lines were cultured in a humidified atmosphere containing 5% $CO_2$ at 37°C.

*2.2. Patients and Tissue Samples.* Primary tumours were obtained at surgery from nine patients with TGCTs (five with seminoma and four with NSGCTs). Six control healthy testicular tissues were taken from autopsies. All tissue samples were frozen immediately and subjected to RNA extraction.

A retrospective study was performed including 71 patients with TGCTs who had undergone orchiectomy in our hospital. Patients were further treated according to their stage, the histological type, and specific predictive and prognostic factors. Patients with stage I seminoma were treated with 2 cycles of adjuvant chemotherapy based on carboplatin, while patients with stage I NSGCTs were treated with 2–4 cycles of chemotherapy based on bleomycin, etoposide, and carboplatin. Patients with stage II disease were treated with 4 cycles of adjuvant chemotherapy based on bleomycin, etoposide, and carboplatin, while in patients with stage III disease ifosfamide was added in the therapeutic pattern. RPLND was selected for the treatment of patients with NSGCTs with identified residual disease after completion of adjuvant chemotherapy. Tissue samples were selected from the archives of the Pathology Department of the University Hospital of Patras. None of the patients had received prior chemotherapy or irradiation. The median age of the patients at the time of surgery was 30 years, with a range of 17–78 years. All experiments were performed after obtaining informed consent according to the institutional guidelines. Tumour staging and histopathologic findings were assessed according to the American Joint Committee on Cancer. Clinicopathological characteristics of the patients are summarized in Table 1. After an initial review of all available hematoxylin-eosin stained slides of surgical specimens, serial sections from a representative paraffin block of each case were immunostained. The study was performed in accordance with the precepts established by the Helsinki Declaration, approved by Ethic Committee of Patras University Hospital and patients were enrolled after giving written consent. All data were analyzed anonymously.

*2.3. Immunohistochemistry.* Syndecan-4 expression was examined immunohistochemically by using the D-16 goat polyclonal antibody (sc 9499, Santa Cruz, USA) and avidin-biotin-peroxidase complex (Dako Co., Copenhagen, Denmark). Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Serial 5 $\mu$m sections were taken and deparaffinized with xylene and dehydrated with 98% ethanol. Antigen retrieval was performed in a microwave oven in 10 mM citric acid buffer (pH 6.0). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 5 min at room temperature. Nonspecific protein binding of the antibodies was blocked by incubation with 3% normal swine serum in PBS for 20 min at room temperature. Slides were incubated with anti-syndecan-4 polyclonal antibody

Table 1: Clinicopathological characteristics of the 71 patients with TGCTs.

| Variable | *n* | % |
|---|---|---|
| Histological type | | |
| Seminoma | 33 | 46.5 |
| Median age: 35 years | | |
| Nonseminoma | 38 | 53.5 |
| Median age: 26 years | | |
| Embryonal carcinoma | 8 | 11.3 |
| Teratoma | 5 | 7.0 |
| Mixed type | 25 | 35.2 |
| Tumour size ($T$) | | |
| $T_1$ | 26 | 36.6 |
| $T_2$ | 41 | 57.7 |
| $T_3$ | 4 | 5.6 |
| Vascular-lymphatic invasion | | |
| Negative | 32 | 45.1 |
| Positive | 39 | 54.9 |
| Nodal status ($N$) | | |
| $N_0$ | 36 | 50.7 |
| $N_1$ | 9 | 12.7 |
| $N_2$ | 22 | 31.0 |
| $N_3$ | 4 | 5.6 |
| Distant metastases ($M$) | | |
| $M_0$ | 63 | 88.7 |
| $M_1$ | 7 | 9.9 |
| $M_2$ | 1 | 1.4 |
| Stage | | |
| I | 36 | 50.7 |
| II | 27 | 38.0 |
| III | 8 | 11.3 |

diluted 1 : 150 in PBS containing 1% normal swine serum for 1 hr at room temperature. Obtained antigen-antibody complexes were visualized by 30 min incubation at room temperature, using biotinylated rabbit anti-mouse antibody diluted 1 : 200 and the avidin-biotin-peroxidase technique (Dako Co., Copenhagen, Denmark). The staining was developed with 3,3-diaminobenzidine (DAB)/hydrogen peroxide for 5 min at room temperature and slides were counterstained with hematoxylin. A positive tissue control and a negative reagent control (without primary antibody) were run in parallel. The level of syndecan-4 immunoreactivity in epithelial and stromal cells was expressed by scoring the percentage of syndecan-4 positive cells into three groups: high staining >30% of the cells stained; low staining 10–30% of the cells stained; and negative staining <10% of the cells stained. Syndecan-4 immunoreactivity in the tumour stroma was scored as follows: 0, no staining; 1+, moderate; 2+, strong staining. The level of stromal components immunostaining was graded by scoring the percentage of positivity into two groups: negative (<10% of stromal cells and negative staining of the stroma) and positive (>10% of stromal cells or/and moderate or strong staining of the stroma). Three independent researchers randomly evaluated the specimens using this method.

Endothelial cells in tumour tissues were stained immunohistochemically as described previously [12]. After examination of the slides, six random fields at high magnification (×250) were chosen to be evaluated for the number of microvessels in each slide. The number of microvessels in each section represents the mean of the six independent measurements. The evaluation was performed by three independent investigators blinded to the clinicopathological characteristics and syndecan-4 expression in the corresponding tissues.

*2.4. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis.* Total RNA was isolated from cell cultures and tissues using the NucleoSpin RNA/Protein extraction kit from Macherey-Nagel (MN GmbH & Co., Germany) following DNase treatment to remove DNA contaminations according to the manufacturers' instructions. Total RNA (1 $\mu$g) was reverse transcribed using the PrimeScript 1st strand cDNA synthesis kit (Takara Inc.) using random 6 mers primers provided according to standard protocol suggested. For PCR, primers for syndecan-4 "CTCCTAGAAGGCCGATACTTCT and GGACCTCCGTTCTCTCAAAGAT" and the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) "ACATCATCCCTGCCTCTACTGG and AGTGGGTGTCGCTGTTGAAGTC" were used.

PCR was performed for 35 cycles (initial denaturation at 94°C for 2 min, denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min in each cycle and final extension at 72°C for 10 min) using 50 ng of template according to DyNAzyme II kit (Finnzymes, Finland). PCR products for syndecan-4 and GAPDH were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The amounts of PCR products were determined by measuring the fluorescence of the bands using UNIDocMV program (UVI Tech). Relative fluorescence for syndecan-4 was obtained by dividing the fluorescence value for syndecan-4 by that of GAPDH.

*2.5. Statistical Analysis.* Data were analyzed using GraphPad Prism (Version 3.0 GraphPad Software Inc., San Diego, CA, USA). Statistical analyses were performed using the Fisher's exact tests to evaluate the associations between clinicopathologic variables and syndecan-4 expression. All tests were two tailed and statistical significance was set at $P < 0.05$. To estimate statistical significance of the differences in RT-PCR analyses as well as of microvessel number with stromal expression of syndecan-4, a two-tailed Student's $t$-test was used.

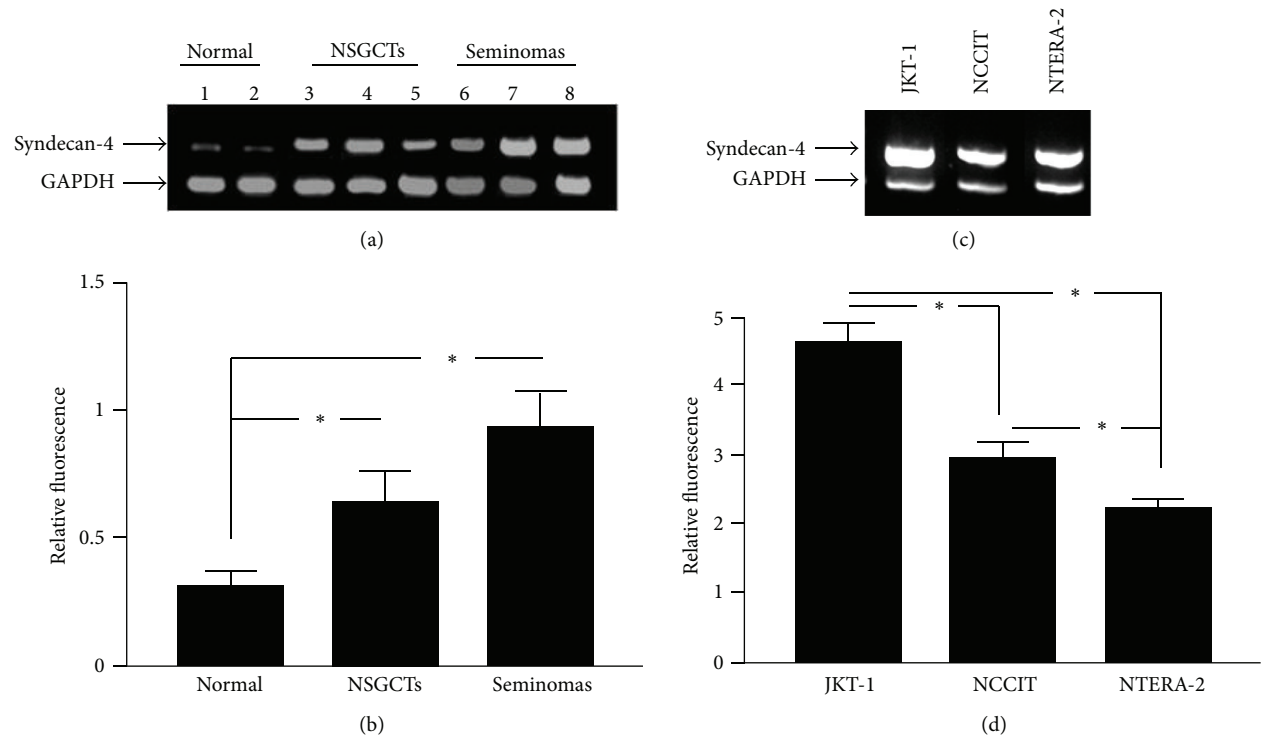


Figure 1: Expression of syndecan-4 in testicular germ cell tumours and cell lines. (a) Indicative RT-PCR analyses of syndecan-4 compared to reference gene GAPDH in two control normal testicular tissues (lanes 1 and 2), three NSGCTs (lanes 3, 4, and 5), and in three seminomas (lanes 6, 7 and 8). (b) Semiquantitative analysis of syndecan-4 expression in normal testicular tissues ($n = 6$), NSGCTs ($n = 4$), and seminomas ($n = 5$). (c) RT-PCR analyses of syndecan-4 compared to GAPDH in testicular GCT cell lines. (d) Semiquantitative analysis of syndecan-4 in TGCT cell lines. The data are presented as the median ± SE and analysed using two-tailed Student's $t$-test ( $^{*}P < 0.05$).

## 3. Results

*3.1. RT-PCR Analysis for Syndecan-4 Expression in GCTs.* A limited number of tissue samples obtained from patients with TGCTs as well as normal testicular tissues were analyzed for the expression level of syndecan-4 by RT-PCR. As shown in Figures 1(a) and 1(b) low expression for syndecan-4 was found in normal testicular tissues (relative fluorescence median ± SE, 0.30 ± 0.06). Statistically significant increase in the expression for syndecan-4 was detected in both NSGCTs (relative fluorescence median ± SE, 0.64 ± 0.11) and seminomas (relative fluorescence median ± SE, 0.79 ± 0.14) (Figures 1(a) and 1(b)), suggesting a higher expression of syndecan-4 by tumour cells or activated stromal cells. To evaluate the expression levels of syndecan-4 in TGCT cell lines, we performed RT-PCR analysis in three tumour cell lines (Figures 1(c) and 1(d)). Syndecan-4 is highly expressed in seminoma cell line JKT-1 (relative fluorescence median ± SE, 4.64 ± 0.26), whereas statistically significant lower expression was detected in teratocarcinoma cell line NCCIT (relative fluorescence median ± SE, 2.90 ± 0.31) and embryonal carcinoma cell line NTERA-2 (relative fluorescence median ± SE, 2.2 ± 0.19).

*3.2. Histological Overview of the Patients.* A retrospective study for the expression of syndecan-4 in testicular TGCTs was performed in a population of 71 patients. The histological review (Table 1) of the primary tumours revealed 33 patients (46.5%) with seminoma and 38 patients (53.5%) with NSGCTs. The median age at the time of surgery was 35 years (range 21–78 year) for the patients with seminoma and 26 years (range 17–65) for the patients with NSGCTs. Patients with NSGCTs were divided into three groups: 8 (11.3%) with embryonal carcinoma, 5 (7.0%) with teratoma, and 25 (35.2%) with mixed type TGCTs. Twenty-six of the patients were of $T_1$ stage, whereas 41 and 4 patients were of $T_2$ and $T_3$ stage, respectively. Among the 71 patients with TGCTs, 39 patients (54.9%) were positive for vascular and/or lymphatic invasion and in 35 patients (49.3%) nodal spread of the disease was observed. Only 8 patients (11.3%) were positive for distant metastases ($M_1$ and $M_2$). Finally, the categorization of the patients showed that 36 patients (50.7%) were of stage I, whereas 27 (38.0%) and 8 (11.3%) patients were of stage II and stage III, respectively.

*3.3. Immunohistochemical Expression of Syndecan-4 in GCTs and Correlation with Clinicopathological Variables.* To evaluate the expression of syndecan-4 by tumour and stromal cells, we performed immunohistochemistry in tissue sections. In normal tests ($n = 4$) weak staining for syndecan-4 was observed in the normal seminiferous tubules showing a cytoplasmic as well as membranous localization with the prominent staining to be detected in the basal cells. No staining was

Table 2: Syndecan-4 expression in 71 patients with testicular tumours.

| Histological type | Syndecan-4 positive tumour cells | | | Syndecan-4 stromal staining | |
|---|---|---|---|---|---|
| | <10% | 10–30% | >30% | Negative | Positive |
| Seminoma | 0 | 1 | 32 | 18 | 15 |
| NSGCTs | 2 | 15 | 21 | 16 | 22 |

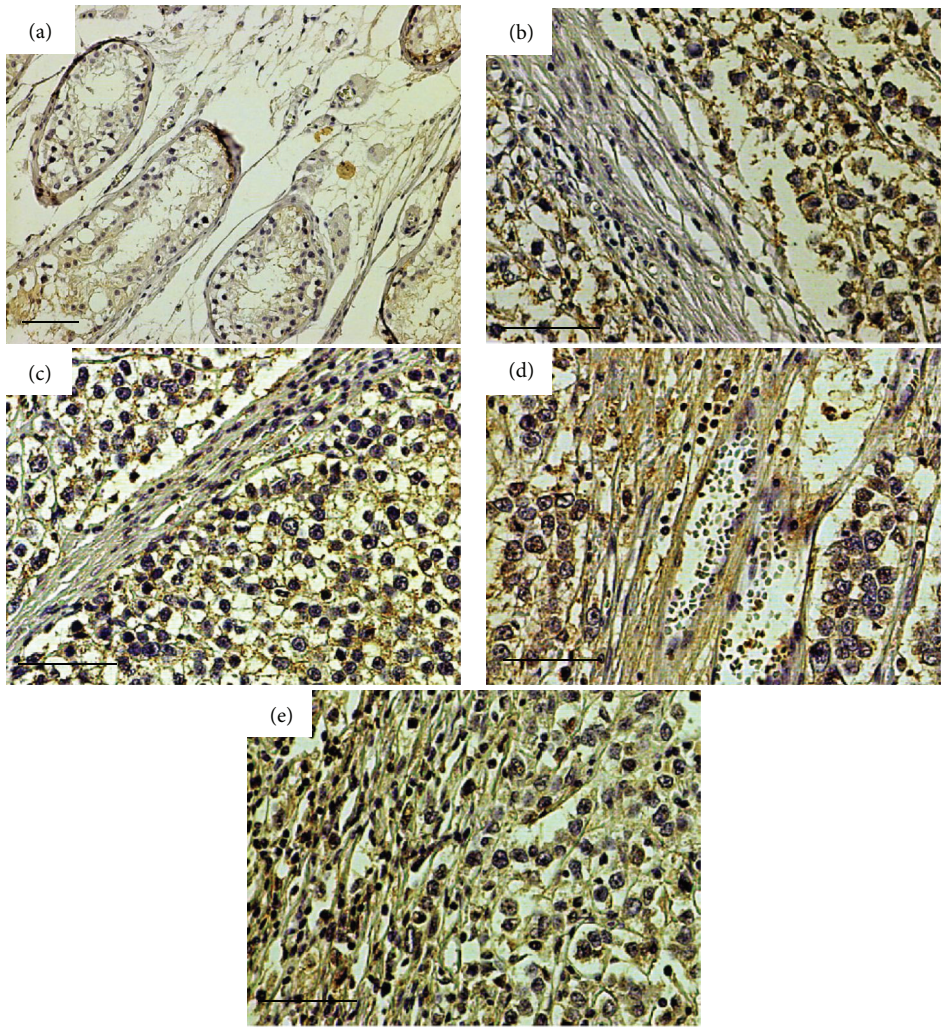


Figure 2: Syndecan-4 is highly expressed in seminomas. Weak staining for syndecan-4 in the seminiferous tubules in normal testicular tissue (a). Tumour cell associated staining for syndecan-4 in stage I seminomas ((b) and (c)) and tumour cell associated and stromal staining for syndecan-4 in stage II seminomas ((d) and (e)). Scale bar denotes 50 $\mu$m.

observed in the interstitial connective tissue in the interlobular septa surrounding the seminiferous tubules (Figure 2). Syndecan-4 expression in seminoma (Figures 2(b)–2(e)) and NSGCTs (Figures 3(a)–3(d)) was observed in tumour cells, stromal components, or both. High percentage (>30%) of tumour cells positive for syndecan staining was found in 32/33 (97.0%) patients with seminoma and in 21/38 (55.2%) patients with NSGCTs (Table 2). Stromal syndecan-4 staining was observed in 15/33 (45.5%) patients with seminoma and in 22/38 (57.9%) patients with NSGCTs (Table 2). Syndecan-4 was present in both cell membrane and cytoplasm of tumour cells in seminoma (Figures 2(b)–2(e)) and NSGCTs (Figures 3(a)–3(d)). Stromal syndecan-4 staining was seen both in stromal cells and in collagen tissue mainly in seminomas of advanced stage (Figures 2(d) and 2(e)), and in NSGCTs independently of disease stage (Figure 3). Since high staining for syndecan-4 was observed in tumour cells in all patients with seminoma (Figures 2(b)–2(e)), no correlation with the various clinicopathological variables was demonstrated. In contrast, the stromal expression of syndecan-4 in patients with seminoma was associated with the nodal status ($P = 0.04$), vascular/lymphatic invasion ($P = 0.01$), and stage of

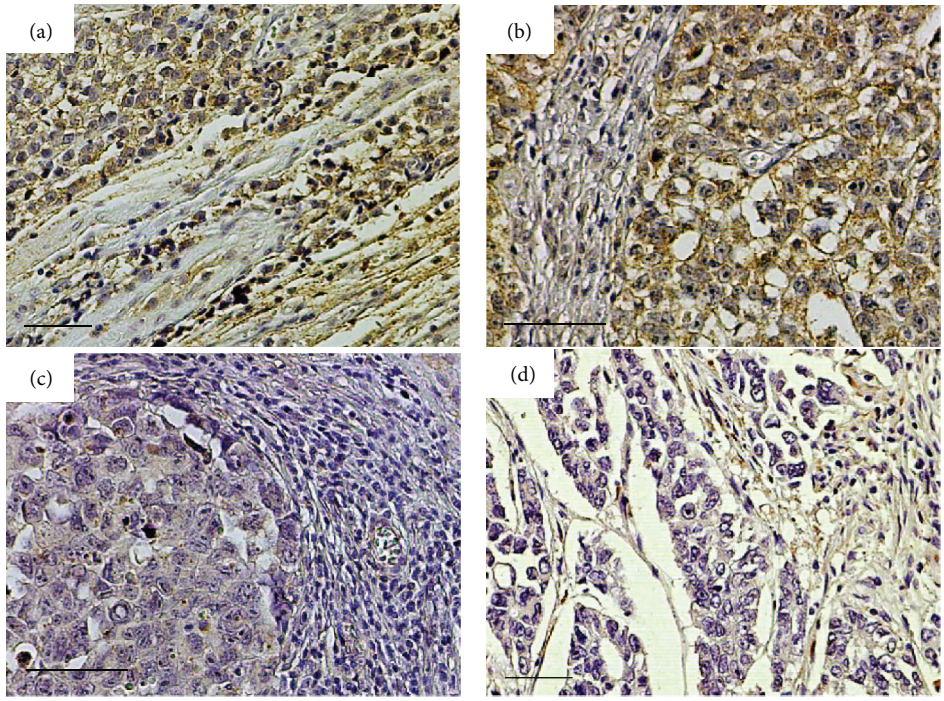


Figure 3: Loss of syndecan-4 staining in aggressive NSGCTs. Tumour cell associated staining and variable stromal staining for syndecan-4 in stage I teratoma/seminoma (a) and stage I embryonal/seminoma (b). Variable stromal staining and loss of tumour cell associated immunoreactivity for syndecan-4 in stage II embryonal/yolk sac (c) and embryonal stage III tumours (d). Scale bar denotes 50 $\mu$m.

Table 3: The association between syndecan-4 stromal staining and the clinicopathologic variables of 33 patients with seminoma.

| Variable | Negative | Positive | Statistics |
|---|---|---|---|
| Tumour size ($T$) | | | |
| $T_1$ | 9 | 6 | $P = 0.73$ |
| $T_2 + T_3$ | 9 | 9 | |
| Nodal status ($N$) | | | |
| $N_0$ | 14 | 6 | $P = 0.04$ |
| $N_1 + N_2$ | 4 | 9 | |
| Vascular-lymphatic invasion | | | |
| Negative | 15 | 6 | $P = 0.01$ |
| Positive | 3 | 9 | |
| Disease stage | | | |
| I | 14 | 6 | $P = 0.04$ |
| II | 4 | 9 | |

disease ($P = 0.04$) (Figures 2(b)–2(e) and Table 3). Stromal syndecan-4 staining was not related to any of the clinicopathologic variable in NSGCTs (Table 4). In NSGCTs less tumour cell associated staining for syndecan-4 was observed in patients with advanced disease stage (Figures 3(c) and 3(d)). In NSGCTs 17/38 patients showed low syndecan-4 expression in contrast to seminoma where 22/23 patients exhibited high syndecan-4 staining (Table 2). This loss of syndecan-4 by tumour cells in NSGCTs was associated with nodal metastasis ($P = 0.01$), vascular and lymphatic invasion ($P = 0.01$), and disease stage ($P = 0.01$) (Figure 3 and Table 4). A clear trend for correlation of lower tumour cells associated staining for syndecan-4 with tumour size and distant metastases was observed as well, but no statistical significance was reached (Table 4).

*3.4. Correlation between the Stromal Expression of Syndecan-4 and Microvessel Density.* The expression of syndecan-4 in the tumour stroma was correlated with the microvessel density in TGCTs. Figure 4 shows that increased staining for syndecan-4 in the tumour stroma was significantly associated with increased microvessel numbers in TGCTs, suggesting an implication in neovascularization.

## 4. Discussion

Syndecans are directly implicated in cancer progression [11, 13]. The aim of the present study was to investigate the expression of syndecan-4 in seminomatous and NSGCTs and to examine all possible associations with the malignant behavior of these tumours. In both seminomatous TGCTs and NSGCTs, significantly increased expression for syndecan-4 was detected in tumour cells. Previously, syndecan-4 has been reported to correlate significantly with high histological grade and negative estrogen receptor status [23], suggesting it to be a marker of poor prognosis in breast cancer. Another study failed to confirm this but instead found syndecan-4 expression to be independent of histological tumour grade and histological tumour type [24]. In our previous study, we demonstrated that estradiol does not affect the levels of syndecan-4 in breast cancer cells through ER$\alpha$ signaling

Table 4: The association between syndecan-4 stromal and tumour cells staining and the clinicopathologic variables of 38 patients with NSGCTs.

| Variable | Stromal staining | | Statistics | Syndecan-4 positive tumour cells | | Statistics |
|---|---|---|---|---|---|---|
| | Negative | Positive | | ≤ 30% | >30% | |
| Tumour size ($T$) | | | | | | |
| $T_1$ | 6 | 4 | $P = 0.27$ | 2 | 8 | $P = 0.14$ |
| $T_2 + T_3$ | 10 | 18 | | 15 | 13 | |
| Nodal status ($N$) | | | | | | |
| $N_0$ | 8 | 8 | $P = 0.51$ | 3 | 13 | $P = 0.01$ |
| $N_1 + N_2 + N_3$ | 8 | 14 | | 14 | 8 | |
| Distant metastases ($M$) | | | | | | |
| $M_0$ | 14 | 16 | $P = 0.43$ | 11 | 19 | $P = 0.11$ |
| $M_1 + M_2$ | 2 | 6 | | 6 | 2 | |
| Vascular-lymphatic invasion | | | | | | |
| Negative | 6 | 5 | $P = 0.47$ | 1 | 10 | $P = 0.01$ |
| Positive | 10 | 17 | | 16 | 11 | |
| Disease stage | | | | | | |
| I | 8 | 8 | $P = 0.51$ | 3 | 13 | $P = 0.01$ |
| II + III | 8 | 14 | | 14 | 8 | |

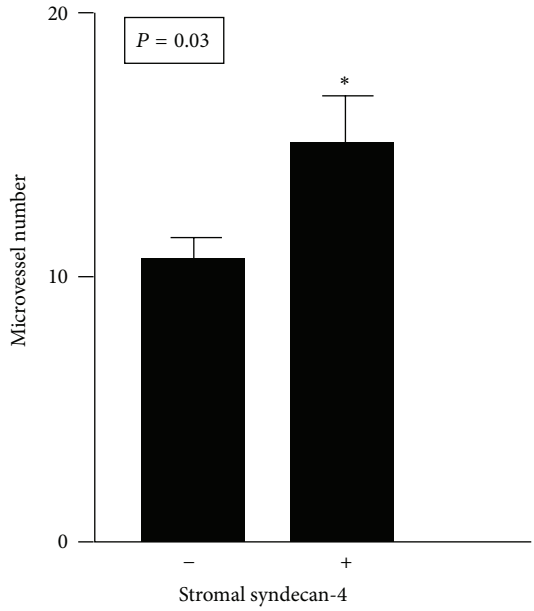


Figure 4: Stromal syndecan-4 promotes angiogenesis. Correlation between stromal syndecan-4 expression and microvessel number in TGCTs. Two-tailed $P$ value was obtained by Student's $t$-test.

although the levels of syndecan-2 are regulated by hormonal treatment [25]. The effects of syndecans in tumour progression may be dependent on organ and tumour type. In this study, the overexpression of syndecan-4 in tumour cells may facilitate the transmission of growth signals in these cells since syndecans are important coreceptors for various growth factors. It has been shown that soluble and membrane-bound forms of syndecan-1 play different roles at different stages of breast cancer progression. The release of soluble syndecan-1 from cell membrane by proteolytic degradation marks a switch from a proliferative to an invasive phenotype in cancer cells [26].

The reduction of syndecans by cancer cell surface is associated with reduced levels of E-cadherin and induction of epithelial to mesenchymal transition (EMT) [26–28]. EMT results in the conversion of malignant epithelial cells into cells with a mesenchymal phenotype and clinically more aggressive tumours. Decreased expression of epithelial syndecan-1 has been reported to be associated with dedifferentiating cancer cells or increasing metastatic potential and to correlate with a poor prognosis in head and neck, gastric, colorectal, laryngeal, cholangiocarcinoma, malignant mesothelioma, hepatocellular, and non-small-cell lung tumours [28–36].

Syndecan-4 is a focal adhesion component in a range of cell types, adherent to several different matrix molecules [37, 38], activating protein kinase C-alpha (PKCa), focal adhesion kinase (FAK), and small GTPase Rho to promote cell adhesion and migration [39–46]. FGF-2 treatment of melanoma cells resulted in the reduction in syndecan-4 expression and downregulation of FAK Y397-phosphorylation thus decreasing cell attachment on FN and promoting their migration [47]. Syndecan-4 overexpressing cells form larger and denser focal adhesions, correlated to stronger attachment and decreased cell migration [48], whereas lack of syndecan-4 engagement promotes amotile fibroblast phenotype where FAK and Rho signaling are downregulated and filopodia are extended [49]. These results suggest that a directed homeostasis in syndecan-4 levels supports optimal migration. Our study revealed that seminomatous TGCTs are characterized by much higher staining of syndecan-4 in tumour cells compared to NSGCTs. The lower staining of syndecan-4 in tumour cells is significantly correlate,

with nodal metastasis, vascular and lymphatic invasion, and disease stage in NSGCTs. Identical results were obtained by analysis of syndecan-4 expression in TGCT cell lines. Less aggressive seminoma cells JKT-1 [6] exhibited higher expression levels of syndecan-4 more than aggressive NSGCT cell lines such as embryonal carcinoma cell line NTERA-2 and teratocarcinoma cell line NCCIT. Although increased levels of syndecan-4 in tumour cells may promote cell growth, the imbalanced upregulation of syndecan-4 in seminomas may be related to the lower metastatic potential of these cells, which is a general characteristic of this type of testicular tumours. The lower expression of syndecan-4 in NSGCTs compared to seminomas but still higher than that found in the corresponding normal cells is significantly correlated to the metastatic potential of these tumours. These results strengthen the current opinion that the balanced expression of syndecans by tumour cells regulates their spreading.

Both seminomas and NSGCTs have shown stromal staining for syndecan-4. The presence of syndecan-4 in the tumour stroma was associated with nodal metastasis, vascular and lymphatic invasion, and disease stage only in seminomas. Such stroma immunoreactivity was also reported for syndecan-1 in reactive stromal cells [30, 50, 51]. Since many epithelial mitogens, including FGFs, hepatocyte growth factor (HGF), and heparin-binding epidermal growth factor (HB-EGF), bind to glycosaminoglycan chains of syndecans, it is speculated that syndecans store several growth factors within the tumour stroma and the accumulation of syndecans may contribute to the extensive angiogenesis and stromal proliferation. The expression of syndecans by stromal fibroblasts may create a favorable microenvironment for accelerated tumour cell growth by storing and presenting growth factors to the carcinoma cells. Furthermore, experimental and clinical data have shown that the expression of syndecan-1 by the stromal fibroblasts promotes breast carcinoma growth *in vivo* and stimulates tumour angiogenesis [52, 53]. Our study demonstrates for first time the stromal distribution of syndecan-4 in malignancies. Syndecan-4 present not only in extracellular matrix but also in stromal cells may play a tumour promoting role in TGCTs. Syndecan-4 stromal staining is significantly associated with neovascularization in TGCTs and the metastatic potential only in seminomas and may be involved in the proliferation of reactive stroma, the promotion of angiogenesis, and the formation of chemotactic gradient of growth factors within tumour stroma. In contrast, the stromal expression of syndecan-4 in NSGCTs, which are more aggressive in general, is not important for tumour cells dissemination and this may be only regulated by lower expression of syndecan-4 in tumour cells that directly affects their migratory ability.

## 5. Conclusions

In conclusion, seminomas and NSGCTs are two different categories of testicular tumours with different expression profiles for syndecan-4. Loss of syndecan-4 overexpression on the surface of tumour cells in NSGCTs is correlated with aggressiveness in contrast to less aggressive seminomas where syndecan-4 is highly expressed constantly. Furthermore, stromal expression of syndecan-4 promotes angiogenesis in TGCTs and metastatic potential only in seminomas. Our data suggest that syndecan-4 represents a biological marker in patients with TGCTs and further studies can be performed in order to determine the clinical utility of syndecan-4 expression in predicting occult lymph node disease in patients with stage I NSGCTs. The identification of reliable prognostic risk factors for those patients remains one of the most challenging issues in assigning patients to the best therapeutic options according to their individual risk profiles for metastasis.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 205948, 9 pages
http://dx.doi.org/10.1155/2013/205948

*Research Article*

# Urine Bikunin as a Marker of Renal Impairment in Fabry's Disease

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Received 22 February 2013; Revised 2 May 2013; Accepted 20 May 2013

Academic Editor: Achilleas D. Theocharis



Fabry's disease is a rare lysosomal storage disorder caused by the deficiency of $\alpha$-galactosidase A that leads to the accumulation of neutral glycosphingolipids in many organs including kidney, heart, and brain. Since end-stage renal disease represents a major complication of this pathology, the aim of the present work was to evaluate if urinary proteoglycan/glycosaminoglycan excretion could represent a useful marker for monitoring kidney function in these patients at high risk. Quali-quantitative and structural analyses were conducted on plasma and urine from 24 Fabry's patients and 43 control subjects. Patients were sorted for presence and degree of renal impairment (proteinuria/renal damage). Results showed that levels of urine bikunin, also known as urinary trypsin inhibitor (UTI), are significantly higher in patients with renal impairment than in controls. In this respect, no differences were evidenced in plasma chondroitin sulfate isomers level/structure indicating a likely direct kidney involvement. Noteworthy, urine bikunin levels are higher in patients since early symptoms of renal impairment occur (proteinuria). Overall, our findings suggest that urine bikunin level, as well as proteinuria, could represent a useful parameter for monitoring renal function in those patients that do not present any symptoms of renal insufficiency.

## 1. Introduction

Fabry's disease (FD) is a panethnic, X-linked lysosomal storage disorder due to deficiency of $\alpha$-galactosidase A [1]. This lysosomal enzyme normally breaks down neutral glycosphingolipids, particularly globotriaosylceramide (Gb3), catalyzing the hydrolytic cleavage of the terminal molecule of galactose. The consequent accumulation of these glycosphingolipids in many cell types and tissues results in several clinical signs and symptoms [1]. The prevalence of Fabry's disease has been estimated to range from 1 in 117,000 to up to 1 in 40,000, but it might be much higher since it is likely that many patients are not identified, because of either the nonspecificity of clinical features or the scarce suspicion of the clinician for the disease [1]. The $\alpha$-galactosidase A gene (GLA-gene) is located on the long arm of chromosome X in position Xq22, and it has recently been sequenced [1]. More than 400 mutations have been identified so far. Depending on the type of mutation there may be different clinical forms of the disease. In particular, GLA-gene mutations resulting in a total absence of $\alpha$-galactosidase A activity usually lead to a more severe form of FD [1]. Disease manifestations usually start in childhood, with intermittent acroparesthesias, sometimes associated with episodic fever, hypohidrosis, gastrointestinal symptoms, typical vascular skin lesions named angiokeratomas, and corneal opacities [2]. After the 3rd decade of life the progression of the pathology frequently leads to renal damage [3], cardiac manifestations, and high propensity to develop brain ischemic stroke, resulting in decreased life expectancy [4]. In particular, end-stage renal disease, with proteinuria and progressive renal failure, is a major cause of morbidity and mortality in FD. Renal damage seems mainly

to be caused by diffuse deposition of glycosphingolipids in glomeruli, tubular system, and vasculature. In routine clinical practice, general proteinuria and microalbuminuria are considered the best biomarkers of FD nephropathy [3], although, recently, many new markers, including Gb3 or specific proteins such as N-acetyl-$\beta$-D-glucosaminidase and cystatin C, have been suggested to improve decision making [5]. However, the efficacy of all of the biomarkers currently in use for Fabry's nephropathy remains uncertain.

Several studies evidenced variations in plasma/urine glycosaminoglycans in physiological and pathological conditions. Glycosaminoglycans (GAGs) are linear and complex polysaccharides, composed of a variable number of repeating disaccharide units, each containing a hexuronic acid glycosidically linked to a hexosamine residue. GAGs have been found in many tissues and in biological fluids such as blood, plasma and urine [6]. Plasma GAGs represent components of intact proteoglycans (PGs) mainly of hepatic and endothelial origin, secreted into blood as well as products of tissue PG degradation. Chondroitin sulfate (CS), the main GAG type in plasma, is derived from both the cell surfaces and the extracellular matrix. A portion of CS is covalently bound to a protein core to form bikunin that is principally synthesized and secreted by the liver [7]. CS chains are short, consisting of 12–18 disaccharides units, and present a charge density of about 30–40% as previously reported [8, 9]. Most of circulating bikunin is present as the light chain of Inter-$\alpha$-Inhibitor (I$\alpha$I) family molecules [10]. Bikunin is a serine protease inhibitor and it occurs in plasma as well as in many tissues [10]. It is also excreted in urine and referred to as urinary trypsin inhibitor (UTI). In urine, GAGs consist mainly of heparan sulfate (HS), CS, and, in negligible quantity, dermatan sulfate [6]. In the general population, bikunin has been reported to occur at higher levels in various pathological conditions exhibiting chronic inflammation, including cancer [11], chronic glomerulonephritis [12, 13], kidney transplantation [14], type 1 diabetes [15], and systemic lupus erythematosus [16]. Moreover, we reported variations of urine bikunin levels during the physiological menstrual cycle in fertile women [17]. It has also been suggested that bikunin may be a useful marker for renal damage [18], liver disease [19], and brain contusion [20], suggesting a potential application in patients with FD. Since kidney damage is a frequent and severe complication of FD, we thought of evaluating plasma and urine GAGs levels in this pathology to assess if their urinary excretion could represent a useful early marker of kidney impairment in patients with Fabry's disease.

## 2. Methods

*2.1. Samples.* Analyses were conducted on fasting blood-plasma and first-morning urine samples from 24 Fabry's patients of both sexes, aged from 20 to 61 years, and 43 age- and gender-matched healthy controls. All FD patients were diagnosed by identifying the mutation in the GLA-gene and showed reduced activity of $\alpha$-galactosidase A enzyme in plasma (Table 1). Among patients, 13 did not present evidence of chronic renal damage (NRD); the other 11, instead, presented, for more than 3 months, pathological abnormalities indicative of chronic renal damage (RD), including abnormalities in blood or urine tests (i.e., serum creatinine; proteinuria/microalbuminuria; and abnormalities in urine sediment) or imaging studies (e.g., renal ultrasound). More in detail, 5 RD patients presented with only proteinuria, whereas 6 presented with overt renal damage. In both FD patients and controls the serum levels of three classic markers of inflammation such as erythrocyte sedimentation rate (ESR) (by means of stopped flow capillary microspectrophotometry), C-reactive protein (CRP) (by means of chemiluminescence assay), and $\alpha$1- and $\alpha$2-globulins (by means of capillary electrophoresis) were evaluated. Informed consent was obtained before enrolment. Institutional review board approval was obtained. The study was conducted in accordance with the ethical principles of the current Declaration of Helsinki.

*2.2. Plasma CS Isomers Analysis.* GAGs isolation was performed by a microanalytic preparative method, as previously described [8]. Briefly, 500 $\mu$L of plasma samples was subjected to proteolytic treatment with papain. Plasma CS isomers were isolated by anion exchange chromatography (DEAE-Sephacel) and precipitated with 5 volumes of ethanol at −20°C for 24 h. Subsequently, they were subjected to depolymerization by using chondroitin ABC lyase (0.1U per 100 $\mu$g hexuronic acid), and the unsaturated disaccharides were derivatized with 12.5 mmol/L 2-aminoacridone (AMAC).

Separation of CS-derived unsaturated disaccharides ($\Delta$Di) was performed by fluorophore-assisted carbohydrate electrophoresis (FACE). Images were acquired by means of Gel Doc XR system (Bio-Rad) and analyzed by using Quantity One v4.6.3 software (Bio-Rad). A calibration curve was set up by using home-made CS isomers obtained from a pool of plasma samples, assayed for hexuronate content [21], and subjected to disaccharides analysis [8]. CS levels were reported as $\mu$g of hexuronic acid per mL of plasma ($\mu g_{UA}$/mL), and CS charge density was evaluated as ratio between 4-sulfated $\Delta$-disaccharides ($\Delta$Di-4S) and total unsaturated disaccharides ($\Delta$Di-4S + $\Delta$Di-nonS).

*2.3. Urine GAGs/Bikunin Analysis.* First-morning urine samples (about 50 mL) were collected and, after centrifugation at 3000 g for 15 min at 4°C, the sediment of broken cells or tissues and other solid materials was discarded. Urine GAGs/bikunin containing fraction was obtained by anion exchange chromatography (DEAE-Sephacel resin) as previously described [15]. Briefly, clarified urines were applied to a column packed with about 6 mL of resin, previously equilibrated with a buffer containing 0.02 M Tris-HCl, 0.15 M NaCl (pH 8.6). After exhaustive washing, urinary GAGs/bikunin were eluted with a buffer containing 0.02 M Tris-HCl, 2 M LiCl (pH 8.6), and assayed for hexuronate content by the method of Bitter and Muir, using glucuronic acid as a standard [21]. Hexuronate levels were normalized for the urinary creatinine concentration, formerly determined by the Jaffè method (Sentinel CH, Milan, Italy). Urinary GAGs/bikunin composition was determined by discontinuous electrophoresis on cellulose

Table 1: Clinical and genetic features of Fabry's patients.

| Patient | Age | Gender | Renal involvement | GLA mutations | $\alpha$-galactosidase A (nmol/mL/h)* | ERT |
|---|---|---|---|---|---|---|
| 1 | 59 | F | RD | Cys172Tyr | 1.70 | Y |
| 2 | 26 | F | RD | Cys172Tyr | 4.68 | Y |
| 3 | 30 | M | NRD | Cys172Tyr | 3.70 | Y |
| 4 | 38 | M | NRD | Cys172Tyr | 5.30 | Y |
| 5 | 33 | M | proteinuria | Cys172Tyr | 2.80 | Y |
| 6 | 27 | M | RD | Cys172Tyr | 3.40 | Y |
| 7 | 46 | F | RD | 846_847delTC | 3.50 | Y |
| 8 | 24 | F | proteinuria | 846_847delTC | 3.50 | Y |
| 9 | 20 | F | proteinuria | 846_847delTC | 3.30 | Y |
| 10 | 58 | F | RD | 846_847delTC | 3.90 | Y |
| 11 | 36 | M | proteinuria | 846_847delTC | 0.48 | Y |
| 12 | 36 | F | NRD | Arg112His | 2.80 | N |
| 13 | 61 | F | NRD | Gln57Arg | 11.10 | N |
| 14 | 62 | F | NRD | Gln57Arg | 14.80 | N |
| 15 | 23 | F | NRD | Gln57Arg | 10.40 | N |
| 16 | 58 | F | NRD | Gln57Arg | 9.80 | Y |
| 17 | 53 | F | NRD | Asp313Tyr | 15.40 | Y |
| 18 | 52 | F | NRD | Arg227Gln | 8.10 | N |
| 19 | 31 | M | NRD | Arg227Gln | 2.50 | Y |
| 20 | 42 | M | RD | Arg227Gln | 0.10 | Y |
| 21 | 32 | M | proteinuria | Arg227Gln | 0.70 | Y |
| 22 | 25 | M | NRD | Arg227Gln | 2.50 | Y |
| 23 | 19 | F | NRD | Arg227Gln | 6.60 | Y |
| 24 | 43 | F | NRD | IVS3+G>A | 9.70 | Y |

NRD: no-renal disease; RD: renal disease; ERT: enzyme replacement therapy; Y: under ERT therapy; N: no ERT therapy.
*At the time of diagnosis.

acetate plates [12–17], according to Cappelletti et al. [22]. Analytes were resolved by three electrophoretic steps in 0.25 M barium acetate running buffer, pH 5.0. Titan III-H cellulose acetate plate (6.0 × 7.5 cm, Helena BioSciences) was first soaked in distilled $H_2O$ for about 1.5 cm and immediately blotted between filter papers. Then, the opposite end was soaked in 0.1 M barium acetate buffer, pH 5.0, for 5.5 cm, leaving a narrow band (2–4 mm large), apparently dry, where 5 $\mu$g as uronic acid of each sample was loaded. Electrophoresis was carried out at 5 mA for about 6 minutes followed by incubation of the plate in 0.1 M barium acetate, pH 5.0, for 2 minutes. The second electrophoretic step was carried out at 15 mA for 14 minutes. Subsequently, the plate was soaked in 0.1 M barium acetate buffer, pH 5.0, containing 15% (v/v) ethanol for 2 minutes. A third electrophoretic step was carried out at 12 mA for 17 minutes. Finally, electrophoretic profiles were detected following 0.1% (w/v) Alcian Blue staining. Images were acquired by means of GS-800 calibrated densitometer (Bio-Rad) and analyzed by using Quantity One v4.6.3 software (Bio-Rad). GAGs were expressed as relative percentages.

The GAGs/bikunin fractions were assessed for both urine bikunin protein content and presence of urine bikunin fragments by performing SDS-PAGE followed by highly sensitive Coomassie Brilliant Blue G-250 staining (limit of detection: 20 ng of protein) [23] on untreated samples and after chondroitin ABC lyase digestion. The latter was performed in a buffer containing 0.1 M ammonium acetate (pH 8.0) using 0.1 U of chondroitin ABC lyase (Sigma Aldrich) per 100 $\mu$g of hexuronate at 37°C overnight. Samples were added with 4X SDS-buffer containing 250 mM Tris (pH 6.8), 8% SDS (w/v), 8% DTT (w/v), 40% glycerol (v/v), and 0.0008% bromophenol blue (w/v) and boiled for 5 minutes before electrophoresis. Urine bikunin was resolved by Tris-glycine SDS-PAGE in 1 mm thick 15% T, 3% C running gel, using a MiniProtean II cell vertical slab gel electrophoresis apparatus (Bio-Rad). Electrophoresis was carried out at 50 V for 15 minutes and subsequently at 150 V until the bromophenol dye front reached the lower limit of the gel. Then, gels were fixed in 30% ethanol (v/v), 2% phosphoric acid (v/v) for 1 h, washed twice in 2% phosphoric acid (v/v) for 10 minutes, equilibrated in 18% ethanol (v/v), 2% phosphoric acid (v/v), and 15% ammonium sulfate (w/v) for 30 minutes, and stained in the same solution containing 0.02% Coomassie Brilliant Blue G-250 (w/v) for 48 h. Gel images were acquired by using GS-800 calibrated densitometer (Bio-Rad) at 63 $\mu$m resolution.

*2.4. Statistical Analysis.* Student's $t$-test for unpaired samples was used to compare plasma and urinary GAGs levels between Fabry's patients and control subjects, using

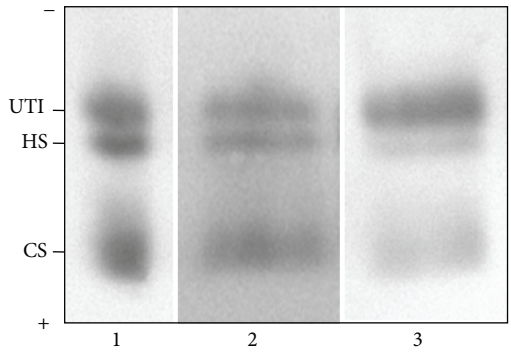



Figure 1: Representative cellulose acetate electrophoretic profiles of urine glycosaminoglycans/UTI from control subjects (lane 2) and Fabry's patients with renal disease (lane 3). Lane 1: mixture of standard GAGs/UTI (UTI: urinary trypsin inhibitor/urine bikunin; HS: heparan sulfate; CS: chondroitin sulfate).

the software package Sigma Stat 3 (Systat Software). Pearson's correlation analysis was performed to evaluate the association between plasma CS and UTI levels and between UTI and serum creatinine levels. Significance was set at $P < 0.05$.

## 3. Results

Quali-quantitative GAGs analyses were conducted in plasma and urine from 24 Fabry's patients and 43 control subjects. Patients were sorted in RD patients, either with only proteinuria ($n = 5$) or with overt renal damage ($n = 6$), and NRD patients, with no evidence of chronic renal damage ($n = 13$). Qualitative analysis by electrophoresis on cellulose acetate (Figure 1) followed by image analysis allowed evaluating percentages of each purified urinary glycosaminoglycan/proteoglycan, namely, urine bikunin (UTI), HS, and CS. Quali-quantitative results with regard to urine are reported in Table 2 and Figure 2. Hexuronate content analysis evidenced higher urinary GAGs levels (+48%) in patients group in respect to controls ($P = 0.03$). After sorting the group of patients as mentioned previously, it was evident that the major differences found had to be ascribed to the occurrence of renal damage since NRD patients showed urinary GAGs levels not significantly different from controls ($P = 0.99$). The patients group showed quite different electrophoretic profiles with respect to controls (Table 2, Figure 2). After integration, quali-quantitative results evidenced levels of urine bikunin 2.8 times higher in the patients group in respect to controls ($P = 0.005$). Notably, this difference was significant for RD patients only, who showed about 3.8 times higher levels of urine bikunin as compared with control subjects ($P = 0.0001$). To evaluate if urine bikunin levels in RD patients were associated with the degree of renal impairment, the results for RD patients were analyzed after sorting the group in RD patients with only proteinuria (early renal impairment) and RD patients with overt renal damage (Table 3, Figure 3). In this respect, no major differences were evidenced either in GAGs/bikunin levels or in their distribution among the two subgroups, indicating that the increase of bikunin excretion in RD patients is likely an early biochemical event that occurs at the onset of renal impairment. In order to verify if urine bikunin was in its intact form, we performed SDS-PAGE analysis on GAGs/bikunin fractions as a whole and after chondroitin sulfate removal by chondroitin ABC lyase treatment. In this respect, no significant bikunin fragmentation was evidenced in urine samples from either patients or controls (Figure 4). Furthermore, the SDS-PAGE analysis allowed confirming the higher urine bikunin levels in RD patients.

To rule out the possibility that higher urine bikunin levels in patients could be ascribed, at least partly, to higher levels of plasma bikunin, we assayed plasma CS isomers by FACE analysis evidencing no differences in either plasma CS levels or charge density between patients and control subjects (Table 4, Figures 5 and 6). Furthermore, no correlation between plasma CS and urine bikunin levels (Figure 7) or between serum creatinine and urine bikunin levels was found suggesting a direct kidney involvement in the higher UTI excretion of Fabry's patients. In absence of overt infection, eleven FD patients out of twenty-four (45.8%) presented with at least one marker of inflammation altered in serum (ESR or CRP, or $\alpha$-1 and $\alpha$-2 globulins). These markers were altered in only 10% of controls.

## 4. Discussion

Fabry's disease is a multisystemic disorder in which progressive renal impairment, along with cardiac and central nervous system involvement, plays a major role in lowering life quality and expectancy [3, 4]. In a retrospective study on Fabry's patients with renal involvement, Branton et al. showed that 50% of patients had proteinuria by 35 years of age and 100% by 52 years of age [24]. Moreover, 50% of male patients presented with chronic renal insufficiency (CRI) by 42 years of age. These authors evidenced that after the development of CRI, there was a rapid decline in glomerular filtration rate leading to end-stage renal disease within 4.1 years. The enzyme replacement therapy (ERT) seems to represent a valid tool to partly counteract the natural progression of Fabry's disease in combination with renoprotective treatments, such as ACE inhibitors, which are known to be effective in slowing disease progression in other chronic proteinuric kidney diseases [25, 26]. It has also been shown that ERT may be effective in preserving normal renal function in children [27]. Nevertheless, in FD, the diagnosis of an early renal dysfunction is likely of primary importance to aid the clinician in decision making, in designing therapeutic interventions, and in following the natural disease progression or the effects of specific treatments [28].

This paper is the first report to point out that urine bikunin levels are significantly higher in FD patients with renal impairment compared to healthy controls. This finding suggests that the amount of this proteoglycan in urine, as well as proteinuria, could represent an early biomarker of renal impairment in Fabry's patients, useful in monitoring renal functionality also in those patients without overt renal damage.

Interestingly, in our study also several FD patients treated with ERT showed elevated levels of bikunin in urine.

Table 2: Urine GAGs/UTI levels and distribution in Fabry's patients and healthy control subjects.

| | All patients ($n = 24$) | RD Patients ($n = 11$) | NRD Patients ($n = 13$) | Controls ($n = 43$) | All patients versus Controls ($P$) | RD Patients versus NRD patients ($P$) | RD Patients versus Controls ($P$) | NRD Patients versus Controls ($P$) |
|---|---|---|---|---|---|---|---|---|
| Uronic acid ($\mu g_{UA}$/mg Cr) | 3.83 ± 1.69 | 4.61 ± 1.63 | 2.60 ± 0.88 | 2.59 ± 1.59 | **0.03** | **0.009** | **0.004** | 0.99 |
| UTI (%) | 52.8 ± 20.9 | 63.2 ± 18.3 | 36.6 ± 12.9 | 28.5 ± 17.6 | **0.0009** | **0.004** | **<0.0001** | 0.29 |
| HS (%) | 20.1 ± 9.1 | 17.4 ± 10.0 | 24.4 ± 5.6 | 30.3 ± 9.3 | **0.003** | 0.113 | **0.002** | 0.14 |
| CS (%) | 27.0 ± 14.4 | 19.4 ± 9.0 | 39.0 ± 13.2 | 41.2 ± 11.9 | **0.004** | **0.002** | **<0.0001** | 0.70 |
| UTI ($\mu g_{UA}$/mg Cr) | 2.29 ± 1.79 | 3.15 ± 1.79 | 0.93 ± 0.44 | 0.82 ± 0.81 | **0.005** | **0.006** | **0.0001** | 0.74 |
| HS ($\mu g_{UA}$/mg Cr) | 0.66 ± 0.26 | 0.67 ± 0.28 | 0.63 ± 0.24 | 0.72 ± 0.37 | 0.57 | 0.77 | 0.71 | 0.58 |
| CS ($\mu g_{UA}$/mg Cr) | 0.89 ± 0.36 | 0.79 ± 0.23 | 1.03 ± 0.50 | 1.04 ± 0.65 | 0.38 | 0.18 | 0.23 | 0.97 |

GAGs/PGs levels are reported as $\mu$g of uronic acid (UA) per mg of creatinine.
UTI, HS and CS levels are calculated from total UA content and relative percentages of each GAG.
RD: renal disease (proteinuria/renal damage); NRD: no-renal disease.
Significant differences are reported in bold ($P < 0.05$).

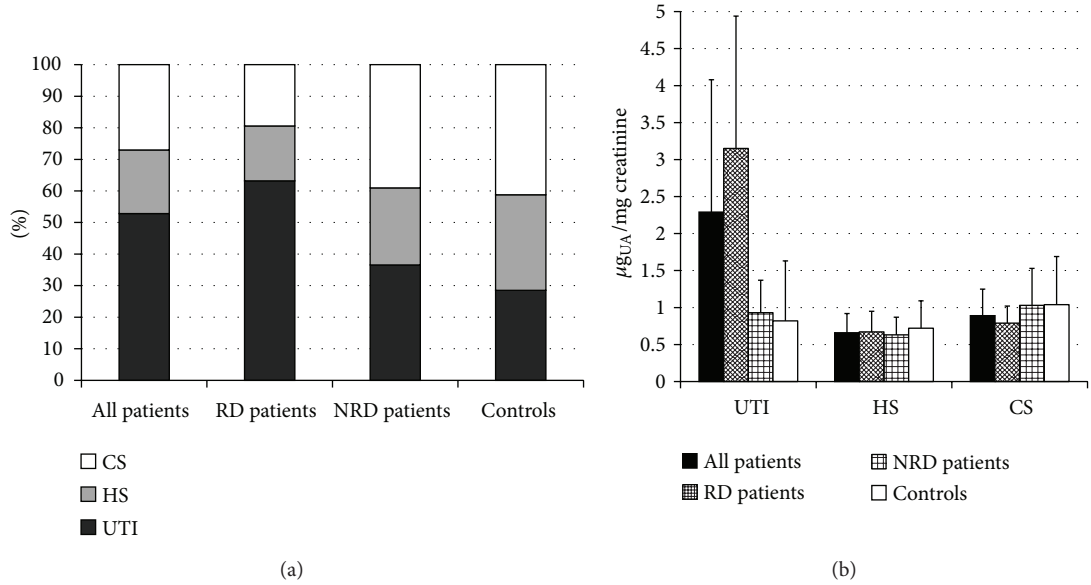



Figure 2: Diagrams reporting percentages (a) and levels (b) of urinary trypsin inhibitor (UTI), heparan sulfate (HS), and chondroitin sulfate (CS) in the totality of patients, patients with renal disease (RD), patients without renal disease (NRD), and controls. UA: uronic acid.

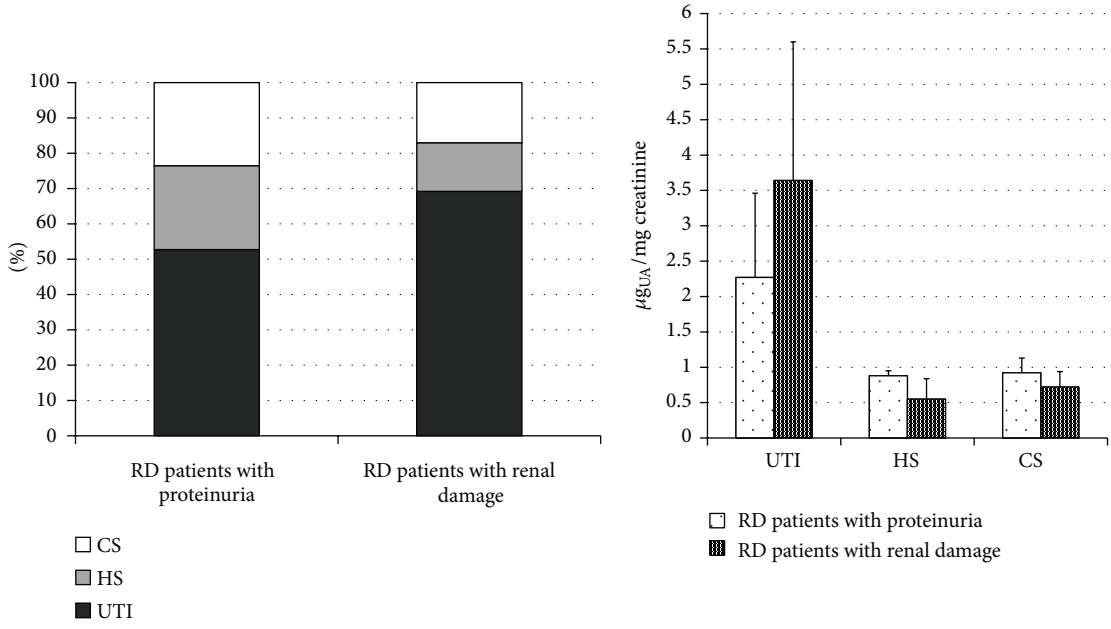
Although in this study we did not plan a formal design aimed to evaluate the urinary bikunin levels at baseline and after a period of ERT, this finding may be supportive of several clinical data indicating that ERT may change significantly the natural progression of the disease if started before the establishment of irreversible organ lesion [25, 29].

Plasma and urine levels of bikunin are related to its anti-inflammatory activity [18], and several studies have associated high plasma and/or urine levels of this proteoglycan with various diseases exhibiting chronic inflammation [11–16]. Notably, several clinical and laboratory findings, as the occurrence of episodic and unexplained fever in some patients with FD, and/or the increased serum levels of ESR, CRP, and $\alpha$1- and $\alpha$2-globulin (observed also in 45.8% of our patients with FD) indicate the likely occurrence of both systemic and local inflammation in this pathology [30, 31]. The activation of the inflammatory biochemical pathways in FD, as it may occur in other lysosomal storage disorders (LSDs), is probably related to secondary inappropriate activation of the immune system, in response to storage, resulting in chronic inflammation [32, 33]. It is noteworthy that in LSDs, the occurrence of systemic inflammation contributes to pathogenesis, predates the onset of clinical signs, and may determine the appearance in plasma and/or urine of secondary metabolites which may act as biomarkers that could be useful in following disease progression and may become a target for adjunctive therapy [32, 33]. Importantly, in FD, the ERT is frequently only partially effective in many patients, either due to a late start of this therapy or because of the secondary activation of biochemical mechanisms other than glycosphingolipids storage that also contribute to the pathogenesis of FD. A better understanding of the secondary biochemical pathways involved in FD pathogenesis, as the ones involved in chronic inflammation, may foster the discovery of new therapeutic

Table 3: Urinary glycosaminoglycans/UTI levels and distribution in Fabry's patients presenting with either only proteinuria or renal damage.

| | RD Patients with proteinuria ($n = 5$) | RD Patients with renal damage ($n = 6$) | ($P$) |
|---|---|---|---|
| Uronic acid ($\mu g_{UA}$/mg Cr) | 4.08 ± 1.40 | 4.91 ± 1.77 | 0.44 |
| UTI (%) | 52.7 ± 13.6 | 69.2 ± 18.8 | 0.16 |
| HS (%) | 23.8 ± 8.9 | 13.8 ± 9.2 | 0.11 |
| CS (%) | 23.5 ± 4.8 | 17.0 ± 10.3 | 0.27 |
| UTI ($\mu g_{UA}$/mg Cr) | 2.27 ± 1.19 | 3.64 ± 1.96 | 0.24 |
| HS ($\mu g_{UA}$/mg Cr) | 0.88 ± 0.07 | 0.55 ± 0.29 | 0.05 |
| CS ($\mu g_{UA}$/mg Cr) | 0.92 ± 0.21 | 0.72 ± 0.22 | 0.18 |

GAGs/PGs levels are reported as $\mu$g of uronic acid (UA) per mg of creatinine.
UTI, HS and CS levels are calculated from total UA content and relative percentages of each GAG.




(a) (b)

Figure 3: Diagrams reporting percentages (a) and levels (b) of urinary trypsin inhibitor (UTI), heparan sulfate (HS), and chondroitin sulfate (CS) in RD patients with proteinuria and RD patients with renal damage. UA: uronic acid.

Table 4: Plasma CS isomers levels and structure in Fabry's patients and healthy control subjects obtained by FACE analysis of fluorophore-labeled unsaturated disaccharides.

| | Patients ($n = 24$) | Controls ($n = 43$) | Patients versus Controls ($P$) |
|---|---|---|---|
| CS isomers ($\mu g_{UA}$/mL plasma) | 6.07 ± 2.96 | 5.61 ± 2.99 | 0.66 |
| *CS charge density (%) | 41.9 ± 5.9 | 42.8 ± 8.6 | 0.72 |

*CS charge density was evaluated as ratio between ΔDi-4S and the sum of ΔDi-nonS and ΔDi-4S.

approaches, adjunctive to ERT, potentially of benefit in this pathology.

Since in our Fabry's patients no correlation was found between plasma CS and urine bikunin levels and no differences were evidenced in plasma CS level/structure between patients and controls, the origin of higher levels of bikunin in urine may imply a direct kidney involvement. Recent RT-PCR analysis studies documenting that several human organs, including the kidney, express bikunin fit this possibility [34]. Moreover, no significant correlation was found between urine bikunin levels and serum creatinine; thus the only impairment of renal function in FD patients seems insufficient to explain higher urine bikunin levels. Nevertheless, the origin of urine bikunin levels and the mechanisms by which urine levels are elevated in our Fabry's patients remains unclear and need to be further evaluated.

## 5. Conclusions

Our data indicate that urine bikunin levels may be an early biomarker of renal impairment in patients with FD. Moreover, higher urine levels of this secondary metabolite in FD patients suggest the secondary activation, in response to glycosphingolipids storage, of biochemical pathways related to inflammation. Further studies are necessary to test our

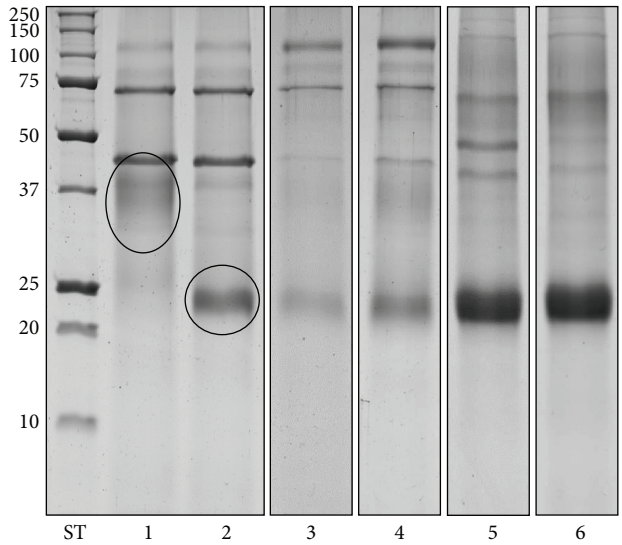



Figure 4: Representative SDS PAGE profiles of nontreated (lane 1) and Chase ABC-treated GAGs-containing fractions from controls (lane 2). Chase ABC-treated GAGs-containing fractions from controls (lane 3), NRD patients (lane 4), RD patients with proteinuria (lane 5), and RD patients with renal damage (lane 6). In lanes 3, 4, 5, and 6, aliquots corresponding to 2 mg of creatinine were loaded. Ovals indicate intact UTI (lane 1) and UTI depleted of chondroitin sulfate chains (lane 2). ST: molecular weight standards (kDa).

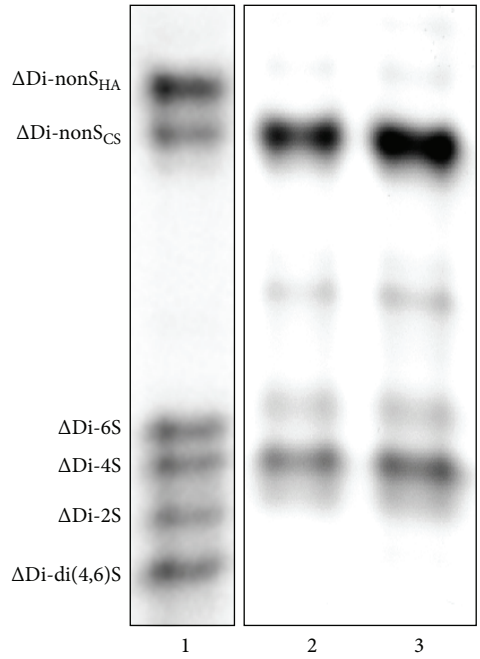



Figure 5: Representative FACE profiles of fluorophore-labeled unsaturated disaccharides (ΔDi) obtained from plasma CS isomers of both controls (lane 2) and Fabry's patients (lane 3). Lane 1: mixture of commercial standard ΔDi (ΔDi-nonS$_{HA}$, 2-acetamido-2-deoxy-3-O-(4-deoxy-$\alpha$-L-threo-hex-4-enepyranosyluronic acid)-4-D-glucose; ΔDi-nonS$_{CS}$, 2-acetamido-2-deoxy-3-0-(4-deoxy-$\alpha$-L-threo-hex-4-enepyranosyluronic acid)-4-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-0-(4-deoxy-$\alpha$-L-threo-hex-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-0-(4-deoxy-$\alpha$-L-threo-hex-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose; ΔDi-mono2S, 2-acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulpho-$\alpha$-L-threo-hex-4-enepyranosyluronic acid)-D-galactose; ΔDi-di(4,6)S, 2-acetamido-2-deoxy-3-O-(4-deoxy-$\alpha$-L-threo-hex-4-enepyranosyluronic acid)-4,6-O-sulpho-D-galactose).

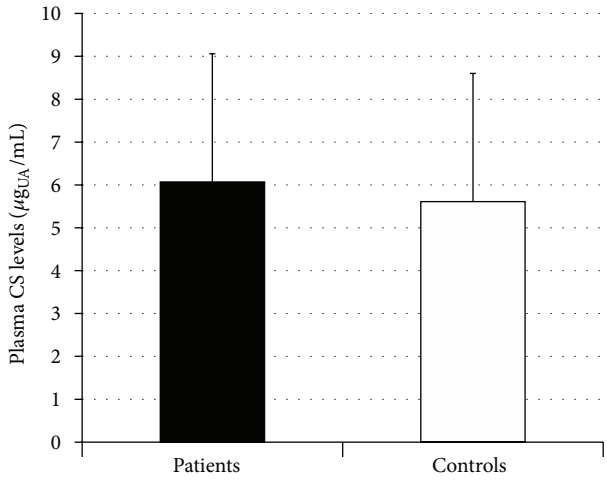



Figure 6: Diagram showing levels of plasma chondroitin sulfate isomers in Fabry's patients and controls. UA: uronic acid.

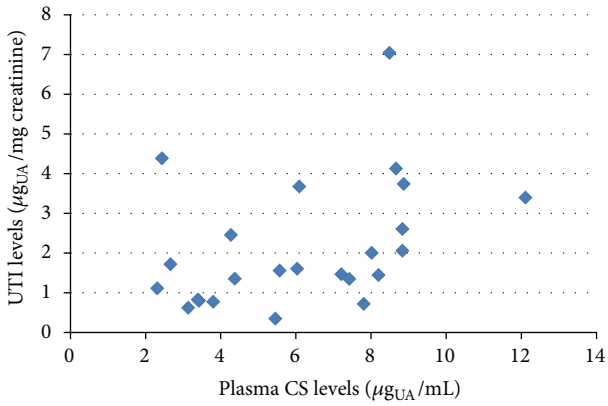



Figure 7: Scatter plot showing levels of urine bikunin (UTI), as $\mu$g of uronic acid (UA) per mg of creatinine, in relation to plasma CS isomers levels, as $\mu$g of UA per mL of plasma, in Fabry's patients.

findings in a larger cohort of FD patients and to investigate any existing correlation between urine bikunin levels, progression of FD, and changes in response to ERT.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Acknowledgments

This study was supported by Regione Autonoma della Sardegna (P.O.R. Sardegna F.S.E. 2007/2013, Asse IV Capitale Umano, Obiettivo operativo l.3-Linea di attività l.3.1) and a free research grant from Shire Italy-Human Genetic Therapies Unit.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 101979, 10 pages
http://dx.doi.org/10.1155/2013/101979

*Review Article*

# Regulation of Tissue Fibrosis by the Biomechanical Environment

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Received 6 May 2013; Accepted 10 May 2013

Academic Editor: Mauro S. G. Pavao



The biomechanical environment plays a fundamental role in embryonic development, tissue maintenance, and pathogenesis. Mechanical forces play particularly important roles in the regulation of connective tissues including not only bone and cartilage but also the interstitial tissues of most organs. *In vivo* studies have correlated changes in mechanical load to modulation of the extracellular matrix and have indicated that increased mechanical force contributes to the enhanced expression and deposition of extracellular matrix components or fibrosis. Pathological fibrosis contributes to dysfunction of many organ systems. A variety of *in vitro* models have been utilized to evaluate the effects of mechanical force on extracellular matrix-producing cells. In general, application of mechanical stretch, fluid flow, and compression results in increased expression of extracellular matrix components. More recent studies have indicated that tissue rigidity also provides profibrotic signals to cells. The mechanisms whereby cells detect mechanical signals and transduce them into biochemical responses have received considerable attention. Cell surface receptors for extracellular matrix components and intracellular signaling pathways are instrumental in the mechanotransduction process. Understanding how mechanical signals are transmitted from the microenvironment will identify novel therapeutic targets for fibrosis and other pathological conditions.

## 1. Introduction

Mechanical forces play integral roles in embryonic development, homeostasis, and pathogenesis. All cells in multicellular organisms are exposed to mechanical forces of varying degrees. Endothelial cells, for instance, are exposed to shear stress due to the passage of fluid through the cardiovascular system. Chondrocytes and other cells in joints are exposed to repetitive compressive forces. The effects of mechanical forces on cells and tissues have received greater attention as models have been developed to systematically analyze these effects. Many of the early studies in this regard were focused on cells and tissues that are influenced by obvious mechanical force including the cardiovascular and musculoskeletal systems. Early investigations in the mechanobiology field relied on relatively simple and imprecise systems. For instance, studies have utilized a hanging-drop culture system to examine the effects of tensile forces on connective tissue cells [1]. As interest grew in the mechanobiology field, innovative systems were developed to apply tensile strain to rat calvarial cells cultured on ribbons of collagen [2] and compressive forces to chick long bones [3].

The mechanobiology field began to move forward rapidly as *in vitro* model systems were developed to more precisely isolate the effects of mechanical forces on cellular processes. Various systems were engineered to apply uniaxial or multiaxial distension or stretch to cells grown on deformable substrata. These systems date back several decades to studies conducted on smooth muscle cells that were cultured on deformable elastin matrices [4, 5]. Among other responses, these studies illustrated a role for mechanical force in the growth and maintenance of skeletal and cardiovascular cells [6–9]. It has become increasingly clear that many aspects of cell behavior can be modulated by mechanical force including cell proliferation, differentiation, migration, and gene expression. The realization that most cells respond to mechanical stimuli has resulted in enhanced interest in the contribution of these forces to pathogenesis including tissue fibrosis and in the mechanisms whereby cells detect and respond to these forces.

Studies by Leung et al. [5] were among the first to illustrate that cyclic mechanical loading promotes the production of extracellular matrix (ECM) components by vascular smooth muscle cells. The ECM is a dynamic network composed primarily of collagens, noncollagenous glycoproteins, and proteoglycans. The ECM was historically appreciated for its function as a three-dimensional scaffold that played an essential role in tissue development and function. Alterations in ECM composition, organization, and accumulation can deleteriously impact embryonic development and organ homeostasis in adults. For instance, deficits in collagen production result in vascular weakness and aneurysms [10]. On the other extreme, increased accumulation of ECM components or fibrosis results in dysfunction of many organs.

The expression of ECM components is regulated by diverse biochemical factors including growth factors, cytokines, and hormones (see [11, 12] for recent reviews). In addition, ECM production can be modulated by electrical and mechanical stimuli. Until relatively recently, the role of mechanical forces in regulating gene expression and cell behavior has received little attention. This has changed as it has been realized that all cells are exposed to mechanical forces, and with the advent of *in vitro* testing systems the effects of these forces and the mechanisms of their actions have been and continue to be investigated.

## 2. Mechanical Stretch and Promotion of Tissue Fibrosis

Cells can be exposed to diverse types of extrinsic mechanical forces including mechanical stretch (tension), compression, and shear stress. A number of early studies utilized cells cultured on deformable membranes to examine the cellular effects of mechanical stretch. These studies illustrated that mechanical stretch of isolated cells mimicked many of the responses that had been characterized to increased load *in vivo*. For instance, mechanical stretch of skeletal myotubes elicited a hypertrophic response that included increased general protein synthesis and enhanced accumulation of contractile proteins [13].

Alterations in mechanical load *in vivo* had been known for some time to impact synthesis and deposition of the ECM. For instance, increased cardiovascular load has for some time been correlated to increased deposition of ECM components. The period immediately after birth is associated with increased cardiovascular load and rapid growth of the heart [14]. This period of "physiological hypertrophy" is also associated with rapid deposition and organization of ECM components, particularly interstitial collagens [15–18]. Increased mechanical load as seen during aortic constriction or stenosis also promotes myocardial hypertrophy and fibrosis in the adult heart [19, 20]. While a number of mechanical stretch devices have been utilized to mimic changes in mechanical forces seen *in vivo*, all have generally illustrated that mechanical stretch of matrix-producing cells (largely fibroblasts and smooth muscle cells) results in increased production of ECM components or a profibrotic response [21–24].

To more accurately mimic the *in vivo* environment, apparatuses are being developed to investigate mechanical forces in three-dimensional *in vitro* systems. Several recent studies have applied mechanical loads to cells cultured in three-dimensional scaffolds [25, 26]. The use of three-dimensional constructs provides important insight into the effects of complex mechanical forces on tissue properties, and the development of systems to apply and analyze mechanical load to these constructs will also be advantageous to efforts to engineer functional tissue constructs.

## 3. Effects of Tissue Stiffness on Fibrosis

While two-dimensional *in vitro* systems have been invaluable in elucidating the effects of mechanical forces on cells and the mechanisms of mechanotransduction, cells function within a three-dimensional environment whose mechanical properties can change during development [27] or various pathological conditions including fibrosis [28, 29], cancer [30–34], and atherosclerosis [35]. Due to accumulation of ECM components and cross-linking of these components, alterations in tissue stiffness are a common feature of fibrosis. For instance, pathological scars are stiffer relative to unwounded normal skin and typically consist of thicker collagen bundles [36]. Accumulation of ECM components alters the tissue mechanical properties, which in turn can deleteriously impact organ function [37]. Component cells sense and respond to ECM rigidity, which can regulate cell growth [38], shape [39], migration [40, 41], and differentiation [42, 43].

Seminal studies by Mauch et al. [44] were among the first to evaluate the effects of the biomechanical microenvironment on the expression of ECM components. The expression of ECM components and ECM-modifying enzymes was compared between cells cultured on tissue culture plastic, a rigid substratum, and three-dimensional collagen gels, a more flexible substratum. These studies illustrated that collagen expression is markedly decreased in fibroblasts cultured in three-dimensional collagen scaffolds compared to cells grown on tissue culture plastic. This effect was, at least in part, regulated at the mRNA level as $\alpha$1(I), $\alpha$2(I), and $\alpha$1(III) collagen mRNAs were diminished in cells cultured in the three-dimensional scaffolds. Further studies by this group of investigators illustrated that collagenase activity is enhanced by culture in three-dimensional scaffolds promoting a collagenolytic phenotype in the less rigid environment of the collagen gels [45]. A number of studies have subsequently supported the concept that matrix rigidity propagates the profibrotic response. Culture of human colon fibroblasts on matrices that mimic the mechanical properties of the normal colon or the pathologically stiff colon of Crohn's disease patients demonstrated enhanced expression of ECM components and increased proliferation of fibroblasts on the stiffer matrix [46]. Similarly, culture of human dermal fibroblasts in collagen gels that were made stiffer by prestraining resulted in enhanced expression of collagen by dermal fibroblasts relative to that in unstrained scaffolds [47]. Liu et al. [48] have utilized a novel photopolymerization approach to generate

polyacrylamide scaffolds with stiffness gradients that span the range of normal and fibrotic lung tissue (0.1 to 50 kPa). In this system, proliferation of lung fibroblasts was induced by increased scaffold stiffness. In contrast, matrix stiffness protected cells from apoptosis in response to serum starvation. The patterns of collagen $\alpha$1(I) and $\alpha$1(III) mRNA expression paralleled proliferation with increasing expression in stiffer regions of the scaffold. The expression of prostaglandin, which is an endogenous antifibrotic factor, was opposite to that of the collagens with increased levels in the less rigid portions of the construct. These studies and others indicate that the biomechanical properties of the microenvironment can direct the expression of ECM components and ECM-modifying enzymes with stiffer tissue properties contributing to enhanced ECM production. Less rigid matrices appear to promote an anti-fibrotic environment that includes increased production of matrix-degrading proteases and anti-fibrotic agents like prostaglandin.

Matrix rigidity impacts not only the expression of ECM components but also other parameters associated with fibrosis including the deposition and organization of these components. Studies by Halliday and Tomasek [49] illustrated that fibroblasts cultured in stabilized three-dimensional collagen gels generate stress that is transmitted throughout the collagen scaffold. These cells develop large actin microfilament bundles and organize fibronectin into extracellular fibrils. Fibroblasts cultured in free-floating collagen gels generate less stress and lack fibronectin-containing fibrils. More recently, Carraher and Schwarzbauer [50] utilized a polyacrylamide model to evaluate the role of matrix stiffness on fibronectin organization. Polyacrylamide scaffolds have become popular three-dimensional models as their rigidity can be modulated by altering the ratios of the components contributing to polymerization of the scaffold. Similar to previous studies, this work illustrated that growth of cells on more rigid substrates promoted fibronectin assembly and activation of focal adhesion kinase (FAK). Furthermore, activation of ECM receptors of the integrin family by $Mn^{2+}$ on softer substrates stimulated fibronectin assembly illustrating that integrin activity is an important mediator of this process (discussed further below). Previous studies have illustrated that the conformation of fibronectin on more rigid substrata is extended, which exposes additional binding sites for cells to fibronectin [51]. This is consistent with other studies illustrating that multiple proteins that are involved in mechanotransduction become extended in response to mechanical force thus revealing cryptic interaction sites that mediate activity of the proteins. Indeed, providing exogenous unfolded fibronectin to cells in "soft" polyacrylamide gels increases FAK activation to a similar degree as culture in more rigid gels [50].

## 4. ECM Density and Myofibroblast Formation

An important step in tissue fibrosis of many organs is the formation of myofibroblasts or myofibroblast-like cells. These cells are characterized by enhanced contractile activity, formation of stress fibers, and expression of $\alpha$-smooth muscle actin. Myofibroblasts are responsible for alterations to connective tissues including increased synthesis of ECM components. In addition, these cells produce cytokines and growth factors that promote the fibrotic response in an autocrine/paracrine manner. Myofibroblasts are derived from a variety of cells in response to tissue damage and stress including quiescent fibroblasts, blood-derived fibrocytes, mesenchymal stem cells, stellate cells of the liver, and others [52, 53]. Regardless of their origin, myofibroblasts likely arise as an acute and beneficial response to repair damaged tissue. Continued myofibroblast contraction and production of ECM components become deleterious and in many cases yield to stiff fibrotic tissue that obstructs and destroys organ function [54]. Stiffened tissue further promotes myofibroblast formation perpetuating scar formation.

Studies using a three-dimensional collagen scaffold system illustrated that collagen deformability or compliance is inversely related to the transformation of cells into a myofibroblast phenotype [55]. Culture of cells on plastic coated with thin films of collagen (minimal compliance and maximal generation of intracellular tension) resulted in the highest levels of $\alpha$-smooth muscle actin expression, routinely used as a marker for myofibroblast formation. Culture of cells in free-floating collagen gels (maximal compliance and least generation of intracellular tension) yielded the lowest relative level of $\alpha$-smooth muscle actin expression. Similar results have been obtained in experiments examining matrix rigidity and differentiation of bronchial fibroblasts to a myofibroblast phenotype [56]. Culture of bronchial fibroblasts on polydimethylsiloxane substrates of variable stiffnesses (1–50 kPa) was performed to evaluate the effects of matrix mechanical properties on myofibroblast formation [56]. Increased scaffold stiffness promoted myofibroblast formation and increased $\alpha$-smooth muscle actin and interstitial collagen expression. In the former studies, the expression of the $\alpha$1 and $\alpha$2 integrins, which are collagen receptors, correlated to enhanced myofibroblast formation on collagen-coated plastic [55]. Incubation of cells with function-blocking antibodies to these integrins attenuated myofibroblast formation indicating that generation of intracellular tension via integrin-ECM interactions is critical to the transformation process. More recent studies have illustrated an interaction between the mechanical properties of three-dimensional collagen gels and the biochemical environment [57]. In these studies, there was no difference in $\alpha$-smooth muscle actin expression between cells in free-floating and constrained collagen gels cultured in low serum (5%); however, enhanced $\alpha$-smooth muscle actin expression was seen in constrained gels at higher serum levels (10%). These studies and others illustrate integration of mechanical and biochemical signals by cells.

The conversion of hepatic stellate cells to a myofibroblast phenotype is a critical step in liver fibrosis and is part of the pathway to cirrhosis in chronic liver disease. Culture of hepatic stellate cells on tissue culture plastic and in high levels of serum results in their spontaneous conversion to a myofibroblast phenotype [58]. Culture of hepatic cells on Matrigel, a relatively soft basement membrane-like matrix, retains the quiescent nature of hepatic stellate cells [59]. Furthermore, culture of differentiated hepatic myofibroblasts

on Matrigel results in loss of myofibroblast characteristics [60]. The mechanisms of the dedifferentiation of these cells are not well understood. Recent studies by Olsen et al. [61] to evaluate the role of substrate stiffness on differentiation of hepatic stellate cells utilized polyacrylamide scaffolds coated with various ECM substrates. These studies illustrated that increased matrix stiffness is capable of promoting myofibroblast formation independent of growth factor or cytokine stimulation. However, addition of TGF-$\beta$ to the culture medium enhanced differentiation on stiff scaffolds, again indicating interactions between the mechanical and biochemical environments. These studies also illustrated that interactions between the cells and the surrounding ECM and generation of mechanical tension are critical to the conversion to a myofibroblast phenotype. That is, coating of polyacrylamide scaffolds with collagen or fibronectin promoted myofibroblast formation to a much greater degree than polyacrylamide scaffolds coated with poly-L-lysine. Cell adhesion to poly-L-lysine is through electrostatic charges and not via specific integrin receptors. Studies with foreskin fibroblasts have illustrated that alterations in integrin expression accompany changes in substrate rigidity and myofibroblast formation [62]. In these studies, cells cultured on less rigid polyacrylamide gels expressed little $\alpha$-smooth muscle actin and primarily the $\alpha 2\beta 1$ integrin. Culture of cells on more rigid substrata resulted in enhanced expression of $\alpha$-smooth muscle actin and a switch to expression primarily of $\alpha v\beta 3$ integrin.

Fibroblasts isolated from diseased patients or animal models typically retain characteristics of their altered phenotype *in vitro* [63]. Indeed, comparison of fibroblasts from normal individuals and individuals with idiopathic pulmonary fibrosis illustrated differences in proliferation and contractile activity on rigid substrates [64]. However, the fibroblasts from idiopathic pulmonary fibrosis patients remained responsive to alterations in matrix rigidity with decreased proliferation and contractile properties when plated in soft matrices. This suggests that the myofibroblast phenotype is not a permanent state but can be reversed by alterations in the matrix properties. In contrast to this, studies culturing fibroblasts for prolonged periods on matrices of different mechanical properties suggest the conversion to a myofibroblast phenotype is a more "permanent" condition [65]. Culture of cells on a rigid matrix for three weeks resulted in sustained fibrotic activity, even after moving the cells to softer matrices. Understanding the plasticity of the fibrotic phenotype is critical to development of novel therapeutic approaches to fibrosis.

Recent studies have been carried out utilizing a novel photodegradable cross-linker-polyethylene glycol scaffold in which exposure to ultraviolet light can modulate the mechanical properties of the substratum to evaluate the effects on myofibroblast conversion of heart valve interstitial cells [66]. Similar to studies with other cell types, increased elastic modulus of the scaffold yielded an enhanced proportion of $\alpha$-smooth muscle actin-containing cells. Interestingly, and of potential therapeutic significance, the proportion of myofibroblasts in the scaffolds decreased by approximately half when the elastic modulus was decreased by photodegradation. This coincided with a reduction in connective tissue growth factor and in proliferation. The classic dogma has been that once fibrosis has begun, it cannot be reversed; however, recent studies have illustrated that fibrosis can be halted or even reversed depending upon the extent of its progression [67]. The above studies suggest that alteration in the ECM biomechanical properties may be an important therapeutic target that is able to modulate myofibroblast formation and fibrosis.

Recent studies with gold nanoparticles have shown that they can be used for both measuring cell-induced deformation of the ECM as well as modulating matrix stiffness and formation of myofibroblasts. Stone et al. [68] described a method using the light scattering properties of gold nanorods as a pattern marker to track cardiac fibroblast deformation of a two-dimensional collagen matrix using digital image correlation. This study detected areas of both tensile and compressive strain within the collagen films and displacements on the order of 18 $\mu$m [68]. Recently this method was applied to examine age-dependent differences in cellular mechanical behavior. Cardiac fibroblasts isolated from neonatal and adult rats were examined for their ability to deform a two-dimensional collagen film and three-dimensional collagen gels [69]. While no significant differences in strain were detected between the cell populations on the two-dimensional films, neonatal fibroblasts were significantly more contractile in three-dimensional collagen gels and expressed higher levels of $\alpha$-smooth muscle actin compared to adult fibroblasts. Inclusion of negatively charged, polyelectrolyte-coated gold nanorods within three-dimensional collagen gels significantly reduced the ability of neonatal cardiac fibroblasts to contract these gels and was accompanied by a significant decrease in both the expression of $\alpha$-smooth muscle actin and type I collagen [70]. This study suggested that the presence of the surface-modified nanorods impaired the ability of the fibroblasts to transform into myofibroblasts. In addition, it has been shown that negatively charged nanorods accelerated the *in vitro* assembly to type I collagen, and rheological characterization of the mechanical properties of these constructs revealed that these gels were stiffer and more elastic than controls or gels containing positively charged gold nanorods [71]. These latter studies would suggest that nanomaterials may hold promise as a means to both alter the mechanical properties of the ECM and the formation of the myofibroblast phenotype associated with pathological fibrosis.

Another mechanism to take advantage of matrix mechanical properties therapeutically is in targeting death of cells via alterations in matrix rigidity. It has long been known that interactions with the ECM are necessary for survival of normal cells. However, the effects of the mechanical properties of the ECM on cell survival are only recently being addressed. Using polyacrylamide gels of varying rigidity coated with type I collagen, Wang et al. [72] illustrated that proliferation of NIH 3T3 cells is enhanced on stiffer scaffolds. These studies also illustrated that apoptosis of NIH 3T3 cells was increased by almost two fold on less rigid collagen-coated polyacrylamide gels. The effect of matrix stiffness on apoptosis was absent in H-ras-transformed cells. A similar increase in apoptosis was seen in cells from the

rat annulus fibrosis when cultured on softer polyacrylamide scaffolds [73]. These studies suggest that decreasing local matrix stiffness will result in apoptosis, potentially of matrix-producing myofibroblasts or other cells.

The ability of matrix mechanical properties to direct cell behavior is also being integrated into novel tissue engineering approaches, particularly in attempting to develop vascularized tissue constructs [74]. Examination of the invasive activity of endothelial cells plated onto the surface of collagen scaffolds has been used as an angiogenic model. Increasing the stiffness of the collagen scaffolds by cross-linking with microbial transglutaminase resulted in increased numbers of angiogenic sprouts and enhanced cell invasion independent of ECM pore size or density [75]. Under the appropriate biochemical and mechanical conditions, endothelial cells are able to form three-dimensional networks. Utilizing polyacrylamide gels functionalized with peptide sequences derived from cell adhesion sequences, the effect of scaffold mechanical properties on network formation was evaluated [76]. Endothelial cells formed stable networks on relatively soft functionalized polyacrylamide gels (Young's modulus of 140 Pa) in the absence of angiogenic biochemical factors (bFGF or VEGF). On stiffer polyacrylamide scaffolds (2500 Pa), endothelial cells failed to assemble into networks in the presence or absence of angiogenic factors. Thus, the elastic modulus of hydrogels is able to direct the migration and organization of vascular cells [74].

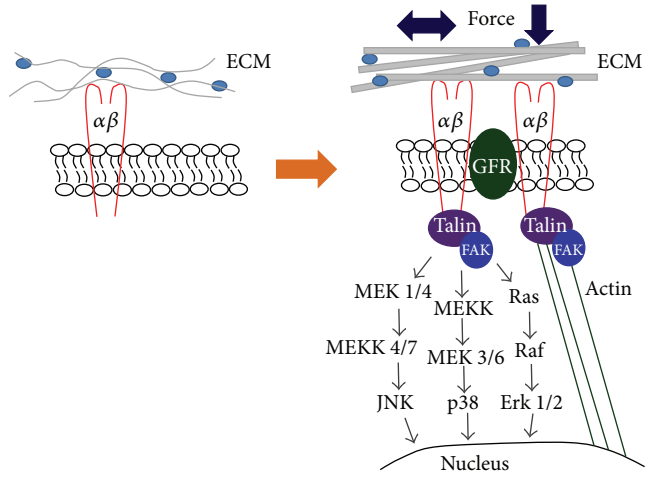


Figure 1: This schematic illustrates the transduction of mechanical force from the microenvironment to the cell. Extrinsically applied force results in alteration in the three-dimensional structure of the ECM and activation of integrin-associated signaling and transmission of signals via the actin cytoskeleton. These forces subsequently result in accumulation of ECM components and a stiffer ECM, which exacerbates the fibrotic response.

## 5. Transduction of Mechanical Signals

Studies utilizing *in vitro* systems have provided fundamental information regarding the molecular mechanisms whereby cells detect and respond to mechanical forces. During the past two decades, extensive progress has been made in understanding "mechanotransduction" or the mechanisms whereby physical stimuli are converted into chemical signals by cells [77, 78]. Despite the fact that the types of mechanical forces cells experience are variable, including externally applied forces (stretch, shear stress, compression, etc.) and forces generated by cells themselves, the molecular mechanisms whereby this information is transduced appear to have similarities. Alterations in the three-dimensional conformation of mechanosensitive proteins or adhesion structures are often at the foundation of this process. Studies utilizing mechanical stretch systems were fundamental in implicating cell surface integrins as central components of cell adhesion complexes and fundamental to mechanotransduction [79]. Integrins are heterodimers composed of an alpha and a beta chain that serve as the primary family of receptors for ECM components [80–82]. There are over twenty different $\alpha/\beta$ heterodimer combinations, and specific $\alpha/\beta$ heterodimers serve as receptors for particular ECM ligand(s). The response of cells to mechanical stretch varies depending upon the ECM substratum suggesting a role for specific integrin heterodimers [79, 83]. Utilizing function-blocking antibodies to specific integrins ($\alpha$4 and $\alpha$5 chains) or arginine-glycine-aspartic acid (RGD) peptides to prevent integrin-ECM interactions, MacKenna et al. [79] were among the first to show roles for specific integrins in the response of fibroblasts to mechanical stretch.

These early studies set the stage for extensive research focused on the mechanisms whereby cells detect mechanical changes in the microenvironment and transduce these into biochemical and molecular alterations in the cytoplasm and nucleus. The cell-ECM linkage involving integrins and a myriad of associated proteins is a critical component of this process (Figure 1). It has become increasingly clear that integrin-based adhesions are dynamic and complex structures that transmit information from the ECM to the cell and vice versa [84]. Integrins, which lack intrinsic enzyme activity, provide a physical linkage from the ECM to the actin cytoskeleton and to a wide array of signaling proteins. In fact, integrin complexes can contain over a hundred different proteins, many that bind in a force-dependent manner [85, 86]. The characterization of the ECM-integrin-cytoskeletal linkage has contributed to the concept of tensegrity in which signals can be transmitted from the ECM to the cytoplasm and nucleus via these physical connections [87, 88]. Several proteins can simultaneously bind integrins and actin and are thus thought to participate in mechanotransduction via the physical ECM-integrin-cytoskeleton linkage including vinculin, talin, and $\alpha$-actinin [89, 90].

A number of signaling molecules associate directly or indirectly with the integrin cytoplasmic domain including focal adhesion kinase (FAK). FAK was initially identified as a Src kinase substrate [91, 92]. As integrins do not have intrinsic enzyme activity, FAK is a critical mediator of integrin-induced signaling events. The activation of FAK is initiated by autophosphorylation of tyrosine at position 397 and can be induced by clustering of integrins [93, 94]. In turn, FAK can activate integrins, which strengthens cell adhesions with

the ECM [95]. Activated FAK can act independently or as part of a Src-containing complex to phosphorylate other signaling proteins or act as a scaffold in the recruitment of additional proteins to cell adhesions.

Exposure of cells to mechanical force results in activation of numerous intracellular signaling pathways including protein kinases such as protein kinase C, c-Jun N-terminal kinases (JNK), extracellular signal-regulated kinases (Erk), and others (see [96] for recent review). Activation of these pathways ultimately leads to activation of transcription factors and cell activities that comprise the response of a given cell to mechanical events.

While there appear commonalities in signaling pathways induced by various types of mechanical forces, *in vitro* studies illustrate that cells respond differently to diverse types of mechanical perturbations. The type of mechanical force can modulate differentiation of connective tissue cells. The ratio between tensile and compression type forces can promote either differentiation into cartilage or bone [97]. Exposing heart fibroblasts to constant versus cyclic mechanical stretch resulted in differences in collagen gene expression [98]. Similarly, exposing vascular endothelial cells to cyclic stretch resulted in differences in growth factor expression and branch formation compared to constant stretch [99]. Application of steady mechanical force on aortas resulted in more pronounced FAK activation compared to pulsatile stretch [100]. These studies suggest that while generalities may be developed regarding the response of cells to mechanical force, the details of this response likely vary depending on the type of force and in a cell- or tissue-specific manner.

## 6. YAP/TAZ as Mechanotransducers

Recent studies have illustrated that signals from the ECM and cell adhesion sites converge on two components of the Hippo pathway, Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) [101, 102]. Analysis of the expression of YAP and TAZ illustrated that the levels of these proteins were enhanced in endothelial cells cultured on stiff fibronectin-containing polyacrylamide hydrogels (10–40 kPa) compared to cells growing on soft hydrogels (0.7–1.0 kPa) [101]. The expression of YAP and TAZ on stiff hydrogels was similar to that seen in cells cultured on plastic culture dishes. In addition, the subcellular localizations of YAP and TAZ are altered by the ECM mechanical environment. These proteins are predominantly located in the cytoplasm of cells grown in softer matrices but are translocated to the nucleus in cells cultured in stiff substrates. YAP and TAZ modulate the activity of transcription factors, including LEAD, RUNx, and Smads in the nucleus. Among the transcriptional targets of the YAP and TAZ system are connective tissue growth factor and TGF-$\beta$, two important biochemical factors that promote fibrosis, and transglutaminase-2, an important component of ECM deposition and turnover [103].

Several recent studies have begun to evaluate the functional roles of YAP and TAZ in mediating the response of cells to mechanical forces. In humans, the trabecular meshwork of the eye is approximately twentyfold stiffer in individuals with glaucoma than in normal individuals [104]. Cells from the trabecular meshwork have been cultured on hydrogels of varying stiffness representing normal and glaucomatous conditions (5 kPa and 75 kPa, resp.) to evaluate the role of the YAP/TAZ system in the progression of fibrosis associated with glaucoma. Similar to the above studies, culture of trabecular meshwork cells on stiffer ECM resulted in enhanced expression of TAZ and transglutaminase-2. Interestingly, YAP expression was decreased relative to that on softer scaffolds suggesting that there may be cell-specific regulation of YAP and TAZ in response to altered mechanical properties of the microenvironment.

## 7. Conclusions and Future Directions

It has become increasingly clear that most cells in the vertebrate body are exposed to varying degrees of mechanical forces. These forces impact embryonic development, homeostasis, and pathological conditions including fibrosis. Historically most of the studies that focused on mechanical force as a profibrotic stimulus utilized two-dimensional stretch or compression models with isolated matrix-producing cells. These studies have provided substantial knowledge regarding the responses of cells to mechanical force and the underlying mechanisms of this response. However, these systems do not adequately mimic the *in vivo* three-dimensional environment. This has led to development of three-dimensional models to evaluate the effects of mechanical forces in a more *in vivo*-like environment. The realization that the biomechanical properties of the microenvironment can promote fibrosis and other responses has led to renewed interest in the effects of mechanical forces on cell and tissue behavior.

While extensive knowledge has been gained regarding the effects of the mechanical environment on cells and tissues, many questions remain regarding the molecular mechanisms of these effects. Identification of novel mechanoresponsive proteins such as YAP and TAZ will provide new therapeutic targets to modulate the deleterious effects of increased mechanical force. As it is becomingly increasing clear that tissue stiffness may precede fibrosis or at least contribute to ongoing fibrosis, identifying methods to modulate the mechanical properties of the microenvironment may also yield novel therapeutic approaches. Along these lines, specific nanomaterials may provide such reagents. However, the mechanisms whereby these materials regulate tissue properties have not been elucidated.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 352370, 7 pages
http://dx.doi.org/10.1155/2013/352370

*Research Article*

# Cardiac Fibroblast-Derived Extracellular Matrix (Biomatrix) as a Model for the Studies of Cardiac Primitive Cell Biological Properties in Normal and Pathological Adult Human Heart

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Received 23 February 2013; Accepted 11 April 2013

Academic Editor: Martin Götte



Cardiac tissue regeneration is guided by stem cells and their microenvironment. It has been recently described that both cardiac stem/primitive cells and extracellular matrix (ECM) change in pathological conditions. This study describes the method for the production of ECM typical of adult human heart in the normal and pathological conditions (ischemic heart disease) and highlights the potential use of cardiac fibroblast-derived ECM for *in vitro* studies of the interactions between ECM components and cardiac primitive cells responsible for tissue regeneration. Fibroblasts isolated from adult human normal and pathological heart with ischemic cardiomyopathy were cultured to obtain extracellular matrix (biomatrix), composed of typical extracellular matrix proteins, such as collagen and fibronectin, and matricellular proteins, laminin, and tenascin. After decellularization, this substrate was used to assess biological properties of cardiac primitive cells: proliferation and migration were stimulated by biomatrix from normal heart, while both types of biomatrix protected cardiac primitive cells from apoptosis. Our model can be used for studies of cell-matrix interactions and help to determine the biochemical cues that regulate cardiac primitive cell biological properties and guide cardiac tissue regeneration.

## 1. Introduction

Advances in stem cell biology and recent reports of stem cells and progenitors cells residing in the adult human heart gave origin to cardiovascular regenerative medicine, and this approach to the heart failure treatment immediately captured the attention of clinicians [1]. Numerous preclinical studies and clinical trials have aimed at the use of stem cells in the therapy of infrcted heart or ischemic cardiopathy [2], evolving from cell therapy to refined bioengineering. In the era of tissue engineering, there is an ongoing interest in the development of biomimetic materials that would be able to elicit specific cellular responses and direct tissue formation based on either intrinsic tissue regeneration capacity (provided by resident stem cells) or scaffold-incorporated or scaffold-seeded stem cells [3]. Several such approaches to cardiac tissue regeneration have been developed and are still under investigation (reviewed in [4]).

All cells exist *in vivo* in a specialized environment in which their survival and function are assured, while their biological activity is controlled. Extracellular matrix (ECM), with growth factors stored within it, contributes to this microenvironment. While the expression of some ECM components, among which matricellular proteins, is

typical of tissue development and organogenesis [5, 6], a number of cardiac diseases, including myocardial ischemia, are associated with qualitative and quantitative alterations in ECM proteins [7]. Cardiac resident or homing stem cells, as well as cells injected in or applied onto myocardium in cell-populated scaffolds interact with ECM components and respond to the local tissue conditions accordingly [8]. Therefore it seems reasonable that the role of ECM and, even more importantly, the effects of the modifications of its composition ongoing in pathological conditions should be studied and taken into consideration when planning the use of cardiac primitive cell-mediated tissue regeneration.

This study describes the method for the *in vitro* production of ECM typical of adult human heart in the normal and pathological conditions (ischemic heart disease) and highlights the potential use of cardiac fibroblast-derived ECM for *in vitro* studies of the interactions between ECM components and cardiac primitive cells responsible for tissue regeneration.

## 2. Materials and Methods

*2.1. Cardiac Tissue Samples.* Cardiac tissue samples were obtained from the left atrium of hearts from patients with end-stage heart failure due to ischemic heart disease, undergoing heart transplantation ($n = 9$, mean age $55.8 \pm 3.1$ years, 7 males, 2 females, mean ejection fraction $25 \pm 1\%$). Samples of atrial appendages from normal hearts ($n = 9$, mean age $50.4 \pm 4.1$ years, 6 males, 3 females) were collected from the donor heart waste fragments, that is, tissue trimmed off from the heart while adjusting atrium size and form at the time of organ transplantation. Specimens were collected without patient identifiers following protocols approved by Monaldi Hospital and in conformity with the principles outlined in the Declaration of Helsinki.

*2.2. Isolation of Fibroblasts and Cardiac Primitive Cells.* Cardiac tissue samples were dissected, minced, and enzymatically disaggregated by incubation in 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% (w/v) collagenase II (both from Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37°C. The digestion was stopped by adding double volume of HBSS supplemented with 10% FBS. This preparation was further disaggregated by pipetting and tissue debris and cardiomyocytes were removed by sequential centrifugation at 100 g for 2 minutes, passage through 20 $\mu$m sieve, and centrifugation at 400 g for 5 minutes.

Obtained cell population was used for isolation of fibroblasts and cardiac primitive cells by immunomagnetic cell sorting based on Miltenyi Biotec (Bergisch Gladbach, Germany) protocol. Fibroblasts were purified by positive selection with antifibroblast MicroBeads and passage through MS columns placed in magnetic field, followed by incubation of the collected negative fraction with anti-human-CD117 MicroBeads and positive selection of CD117-positive cardiac primitive cells.

*2.3. Extracellular Matrix Deposition and Decellularization In Vitro.* The previously published protocols [9, 10] have been modified and optimized for the culture of cardiac primitive cells from adult human heart. Cardiac fibroblasts were seeded on gelatin-coated plates in DMEM supplemented with 10% fetal bovine serum and cultured in confluent state ($15 \times 10^3$ cells per cm$^2$) for up to 21 days, allowing for extracellular matrix (ECM) deposition. Then, fibroblasts were removed by incubation with a solution of 0.25% Triton X-100 and 10 mM $NH_4OH$ in PBS prewarmed to 37°C. The decellularization process was observed at an inverted phase contrast microscope (Olympus Italia, Segrate, Italy). After 1-2 minutes, when cells were no longer discernible, the decellularization solution was diluted and removed, followed by gentle washing of culture plates with PBS. Fibroblast-derived ECM was photographed with a computer-assisted digital camera connected to the microscope (Color View IIIu, Soft Imaging System, Muenster, Germany) and stored at +4°C.

*2.3.1. Immunofluorescence.* Both fibroblasts during ECM secretion and fibroblast-derived ECM after decellularization (biomatrix) were fixed in 4% paraformaldehyde for 20 minutes at room temperature. After blocking with 10% donkey serum, plates were incubated with primary antibody against fibronectin (rabbit polyclonal anti-human, Sigma-Aldrich), collagen IV (mouse monoclonal anti-human, Sigma-Aldrich), tenascin-C (rabbit polyclonal anti-human, Santa Cruz Biotechnology, Dallas, TX, USA), or laminin (mouse monoclonal anti-human, Sigma-Aldrich) and specific secondary antibodies conjugated with fluorescein or rhodamine (Jackson ImmunoResearch Europe, Newmarket, UK); F-actin was stained with rhodamine phalloidin (Sigma-Aldrich). Nuclei were counterstained with DAPI (Merck Millipore, Billerica, MA, USA), and the stained area of culture dish was mounted in Vectashield (Vector Labs, Burlingame, CA, USA). Microscopic analysis was performed with a Leica DMLB microscope equipped with epifluorescence EL6000 system (Leica Microsystems). Pictures were taken with digital camera connected to the microscope (Leica DFC345FX) and then merged with the software Leica Application Suite 3.6.

*2.3.2. Electrophoresis and Immunoblotting.* Control plates were used to evaluate the number of fibroblasts when cultured in confluent state: no statistically significant difference in cell number was observed between plates with normal and pathological fibroblasts. Protein extracts were prepared from biomatrix secreted by normal (Bm-N) and pathological (Bm-P) heart-derived fibroblasts. Lysates containing 30 $\mu$g of proteins were size fractionated by electrophoresis on 8% SDS-polyacrylamide gel and transferred onto a PVDF membrane (Merck Millipore). Molecular weight markers were loaded onto each gel as a weight indicator. The membranes were blocked and then incubated with one of the following antibodies: tenascin-X, laminin $\alpha$1, laminin $\alpha$2, fibronectin, and collagen I, followed by horseradish peroxidase-labelled secondary IgG (all from Santa Cruz Biotechnology). Antibody binding was visualized by chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) and autoradiography (Eastman Kodak Company, Rochester, NY, USA).

*2.4. Cardiac Primitive Cell Culture.* Pathological heart-derived CD117-positive cells were plated on biomatrix-covered culture dishes at a density of $8.5 \times 10^3$ cells per $cm^2$ in Dulbecco's modified Eagle's medium-Ham's F-12 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma-Aldrich), basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA), glutathione (Sigma-Aldrich), penicillin, and streptomycin (Life Technologies, Paisley, UK). For evaluation of their proliferation, apoptosis, and migration, cells cultured on surface treated with bovine serum albumin (CTR), biomatrix secreted by normal (Bm-N), and pathological (Bm-P) heart-derived fibroblasts were starved in serum-free medium for 24 hours. All *in vitro* experiments were repeated a minimum of three times, in triplicate.

*2.4.1. Proliferation.* For evaluation of proliferation, quiescent cells were incubated with the complete medium for 24 hours, and 5-bromo-2′-deoxyuridine (BrdU) was added (1 : 1.000) for 1 hour before cell fixation. Incorporation of BrdU was evaluated by immunofluorescence with the use of BrdU Labeling and Detection Kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. Nuclei were counterstained with DAPI (Merck Millipore), and the stained area of culture dish was mounted in Vectashield (Vector Labs). Microscopic analysis was performed with a Leica DMLB microscope equipped with epifluorescence EL6000 system (Leica Microsystems).

*2.4.2. Apoptosis.* For evaluation of apoptosis, cells were incubated with 200 $\mu$M hydrogen peroxide in the complete medium for 24 hours and fixed in 1% paraformaldehyde. The fragmentation of DNA was detected using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Merck Millipore) based on terminal transferase dUTP nick end labeling, according to the manufacturer's protocol. Nuclei were counterstained with DAPI (Merck Millipore), and the stained area of culture dish was mounted in Vectashield (Vector Labs). Microscopic analysis was performed with a Leica DMLB microscope equipped with epifluorescence EL6000 system (Leica Microsystems).

*2.4.3. Migration.* For the evaluation of migration of cardiac primitive cells in the presence of biomatrix, cells were grown to confluence, and a thin scratch was introduced on culture plates with a pipette tip, producing a cell-free zone [11]. The plates were observed at an inverted phase contrast microscope (Olympus Italia) and photographed with computer-assisted digital camera (Soft Imaging System) at five distinct points, previously marked along the scratch on every culture plate, at various time points. Cell migration was quantified by measuring the width of the cell-free zone (distance between the edges of the scratched cell monolayer) at every time point with Cell$^A$ Imaging Software for Life Sciences Microscopy (Soft Imaging System).

*2.5. Statistical Analysis.* All numerical data are presented as mean ± SEM. Statistical differences between groups were evaluated with Student's two-tailed unpaired $t$-test; $P < 0.05$ was considered significant.

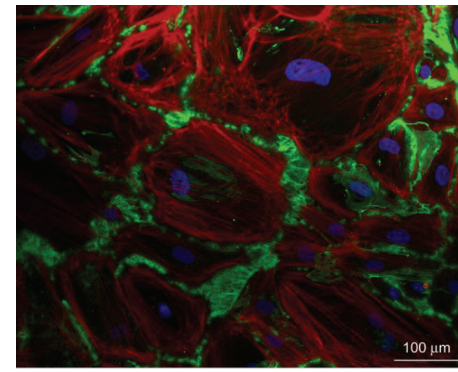


Figure 1: Biomatrix synthesis. Fibroblasts isolated from samples of adult human heart were cultured in confluent state allowing for ECM deposition *in vitro*. Representative image obtained by immunofluorescent labelling of actin filaments (red), cell nuclei (blue), and fibronectin (green).

## 3. Results and Discussion

*3.1. Biomatrix Production and Characterization.* Fibroblasts isolated from samples of adult human heart were cultured in confluent state allowing for ECM deposition *in vitro* (Figure 1). Incubation with basic solution of Triton X-100 induced membrane permeabilization and cell lysis, resulting in the removal of fibroblasts. Decellularized matrix adhered to culture plate; its presence was observed at phase contrast microscope (Figure 2(a)), while its composition was revealed by indirect immunofluorescent staining of representative extracellular matrix proteins and glycoproteins (Figure 2(b)): fibronectin, collagen, laminin, and tenascin.

An organized ECM is necessary for the arrangement of cells and thus for the maintenance of structure in any given tissue; this intricate interlocking mesh of fibrillar and nonfibrillar proteins and glycosaminoglycans also determines tissue biomechanical properties. Numerous cell types are able to synthesise and secrete ECM components, and their activity is regulated and changes in response to various stimuli, such as inflammation, biomechanical stress, and tumorigenesis [12]. Similarly, already deposited ECM proteins are targeted by specific enzymes, metalloproteinases, that are responsible for the continuous remodeling of ECM and allow cell movement and size adaptation. In any necrotic tissue, fibronectin and collagen deposition is responsible for scar formation, which preserves wall integrity and thickness. Life-threatening pathologies ensue when ECM remodelling becomes exacerbated by chronic stimuli. Also in the ischemic heart disease or cardiac pressure overload, cardiomyocyte hypertrophy is accompanied by interstitial fibrosis, while the necrotic tissue is substituted by a scar [13]. However, in the chronic conditions such ECM remodelling increases wall stiffness, contributes to cardiomyocyte slippage, and worsens the contractile properties of the myocardium [14]. On these bases, it is becoming increasingly evident that secretory

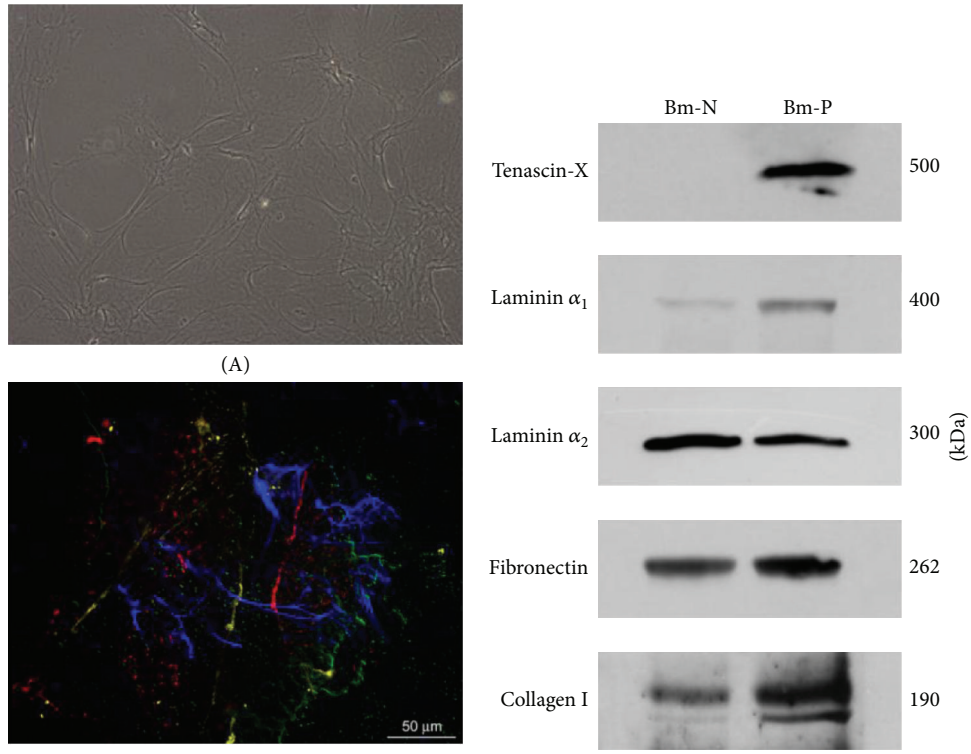


Figure 2: Decellularized biomatrix. (a) After nonenzymatic removal of fibroblasts, ECM was observed at phase contrast microscope (A). Its composition was revealed by indirect immunofluorescent staining of representative extracellular matrix proteins (B) collagen IV (red), laminin (green), fibronectin (blue), and tenascin-C (yellow). (b) Electrophoresis of biomatrix, followed by immunoblotting, revealed semiquantitative differences in biomatrix composition.

activity of fibroblasts, which exceed in number any other cell type in the myocardium, influences myocardial function: it is determinant in normal, but detrimental in pathological conditions.

The synthetic and secretory activity of cardiac fibroblasts isolated from normal or pathological adult human heart can be studied in our model of fibroblast cell culture *in vitro*. The soluble factors condition the medium used for fibroblast maintenance, while the fibrillar and nonfibrillar proteins are deposited as biomatrix on culture plates. Both ECM components can be further analyzed by analytical methods, and their biological function can be tested on various cell types cultured in the presence of fibroblast-conditioned medium or on decellularized biomatrix. The choice of decellularization method is essential for ECM preservation [15] and subsequent use of extracellular matrix as a substrate for cell biology studies *in vitro*. Similarity in the composition and biological properties of the decellularized substrates to those of the native tissue is a prerequisite, as it grants the preserved role in the remodelling of tissue structure and modulation of cell function. In fact, many protein components of extracellular matrix react with each other and with the specific membrane receptors based on their secondary, tertiary, or quaternary structure. In our study, decellularization with nondenaturing solution allowed the preservation of protein structure. Further removal of cellular debris was accomplished by gentle washing. If molecular studies of cells cultured on naturally derived matrix are programmed, biomatrix should be treated with endonuclease before cell seeding.

*3.2. Cardiac Primitive Cell Culture on Biomatrix.* Recently, ECM proteins with a role that goes beyond the structural and mechanical support have been distinguished among other ECM components and termed matricellular proteins [16]. This group encompasses proteins that modulate cell function by interacting directly with cells or by modulating the activity of soluble factors present in extracellular microenvironment, thus influencing cell migration, proliferation, and differentiation [17]. Moreover, these proteins present unique expression pattern, with high levels during organogenesis, virtual absence in normal adult tissue, and reexpression in response to injury and tissue regeneration. In the heart, osteopontin, osteonectin, thrombospondins, tenascin, and CCN family are the matricellular proteins identified so far [18], but laminin-1 also fulfills the requirements [19]. Given these properties, extracellular matrix, and matricellular proteins in particular, can drive cardiac tissue regeneration, described in adult human heart in infarction or pressure overload [20]. Such regeneration depends on cardiac stem cells and their progenies-cardiac primitive cells committed to

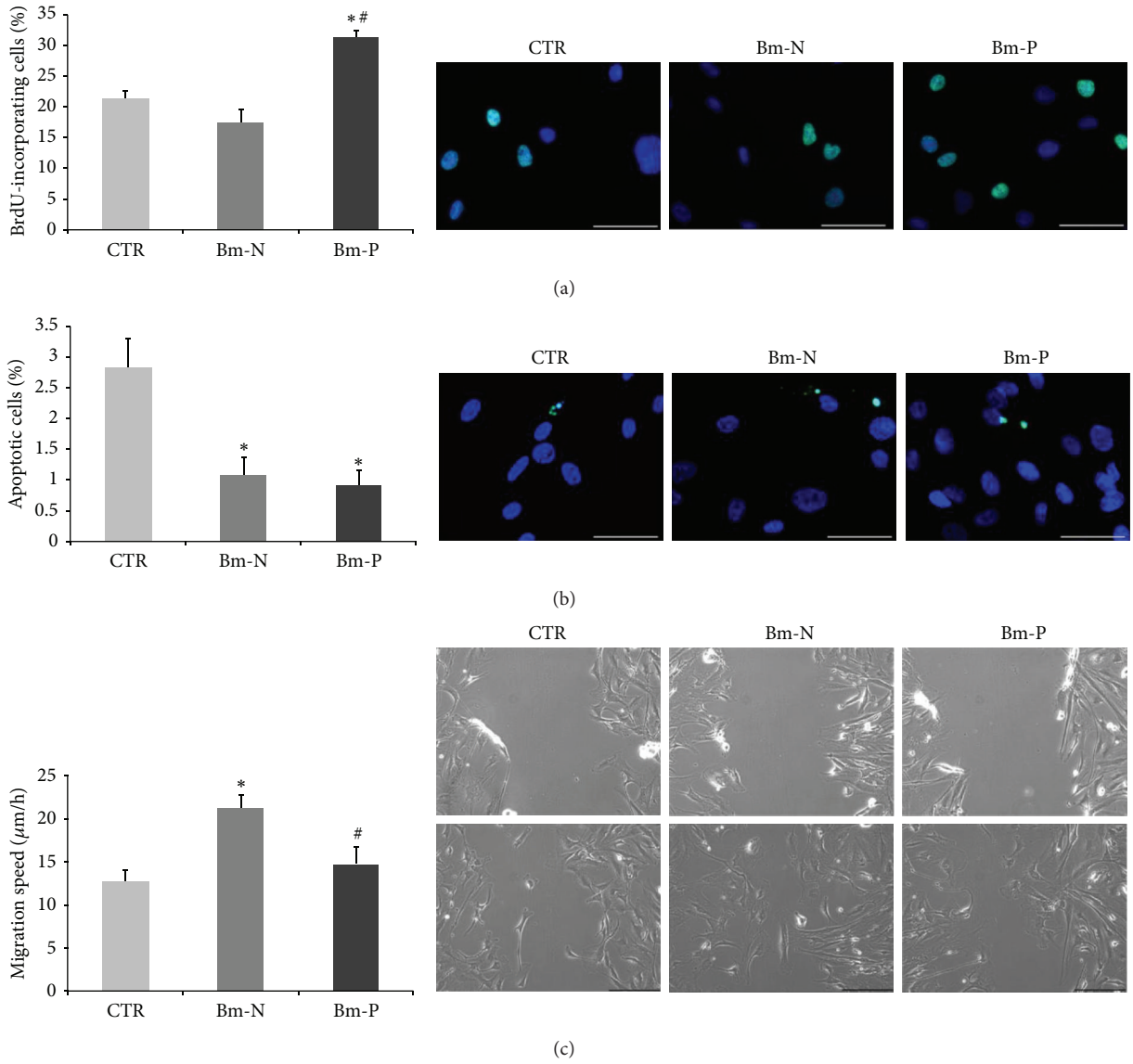


FIGURE 3: Biological characteristics of cardiac primitive cells isolated from adult human heart with ischemic heart disease cultured in the presence of ECM typical of normal and pathological adult human heart. Proliferation (a), apoptosis (b), and migration (c) were influenced by the type of the substrate. Representative images of incorporated BrdU (for evaluation of proliferation), nick end-incorporated nucleotides (for evaluation of apoptosis) immunofluorescent staining (scale bar corresponds to 50 $\mu$m), and scratch wound assay at baseline and after 6 hours (for evaluation of migration, scale bar corresponds to 100 $\mu$m) are shown beside the corresponding graphs. CTR: cells grown on bovine serum albumin; Bm-N: cells grown on biomatrix synthesised by fibroblasts from normal adult human hearts; Bm-P: cells grown on biomatrix synthesised by fibroblasts from hearts with ischemic cardiopathy. $^{*}P < 0{,}05$ versus CTR; $^{\#}P < 0{,}05$ versus Bm-N.

cardiomyocyte, endothelial, and smooth muscle cell lineage [21].

The biomatrix, obtained in our study by culturing fibroblasts derived from adult human heart, contained various extracellular components, including laminin and tenascin protein family. Consequently, we used this fibroblast derived-ECM as a substrate for the culture of cardiac primitive cells *in vitro*. Given the differences in biomatrix composition, revealed by western blotting (Figure 2), two types of biomatrix—Bm-N, produced by fibroblasts isolated from the fragments of adult human normal heart of donors died for reasons other than cardiovascular disease, and Bm-P, produced by fibroblasts isolated from the fragments of adult human hearts of patients with end-stage heart failure due to ischemic cardiopathy, undergoing heart transplantation—were used in this part of the study, enabling us to test the integrity and functionality of biomatrix and to compare the effects of ECM typical of normal and pathological conditions on cardiac primitive cell proliferation, apoptosis, and migration *in vitro*.

Proliferation of cardiac primitive cells (Figure 3(a)) on Bm-P was 147% of control ($n = 3$, $P < 0.01$) and 177% of that in the presence of Bm-N ($n = 3$, $P < 0.01$). The presence of biomatrix protected cells from apoptosis

(Figure 3(b)) provoked by oxidative stress ($n = 6$, $P < 0.01$), although no statistically significant advantage of specific biomatrix type was evident. Migration of cardiac primitive cells (Figure 3(c)) was the fastest on Bm-N; in the presence of Bm-P, it was similar to that of control but significantly slower when compared with the speed of migration on Bm-N ($n = 9$, $P < 0.05$).

It is arguable that the differences in cardiac primitive cell biological properties observed in the presence of different culture substrates (ECM deposited by normal and pathological heart-derived fibroblasts) reflect the changes in fibroblast synthesis and secretory activity, and thus in biomatrix composition. Several authors, including our group, have described the changes of cardiac stem/primitive cells biological properties in pathological conditions or aging [22, 23], but the contribution of microenvironment has not been considered and sufficiently acknowledged due to the lack of an appropriate model for cell-matrix interaction studies. The molecular constituents of ECM play major role in the responses of cells to their local microenvironment. Both direct stimulation of the specific receptors by growth factors stored in ECM and indirect activation by specific integrin expression and clustering can transmit extracellular biochemical inputs along the intracellular signaling pathways that regulate cell proliferation, survival, and migration [24]. As a matter of fact, the expression of integrin subunits in the same cardiac primitive cell population changes in qualitative and quantitative manner depending on our culture substrate (Bm-N or Bm-P, data not shown). From the above observations it follows that the composition of ECM must be taken into consideration when planning cardiac regeneration based on stem/primitive cell transplantation. Tissue regeneration can be accomplished by cells able to survive, proliferate, migrate, and differentiate in the host environment. While the methods of genetic engineering of stem cells and their application in human disease therapy are still under investigation [25], the aforementioned cell characteristics can be enhanced by cell-matrix interactions.

In mammals, nearly 300 proteins (among which collagen subunits, proteoglycans, and glycoproteins) have been identified as components of ECM [26]. So far, only few of them have been recombinantly expressed or purified and are available for the *in vitro* studies of their role in cell biology. Considering the interactions among ECM constituents, the possible influence on cardiac primitive cells is difficult to predict. Hence, our model of ECM production by cardiac fibroblasts and its use as a substrate for cardiac primitive cell culture may fill this gap and improve the results of cell transplantation. In the light of our ongoing study, the modification of biomaterials with bioactive molecules [27, 28], such as a native long chain proteins or short peptide sequences derived from intact ECM proteins, that can incur specific interactions with cell receptors should benefit from the description of biological characteristics of cells in the presence of naturally derived cardiac ECM.

In summary, this study highlights the potential use of cardiac fibroblast-derived ECM for *in vitro* studies of the interactions between components of ECM and cardiac primitive cells responsible for tissue regeneration. In the era of tissue engineering, the choice of biomimetic materials and natural biological components should be based on their specific role on cardiac cell biology. Ensuing knowledge would deepen our understanding of the biochemical cues that regulate cardiac primitive cell survival, proliferation, migration, and, possibly, differentiation, and, hence, guide cardiac tissue regeneration.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Authors' Contribution

Clotilde Castaldo, Franca Di Meglio, and Daria Nurzynska contributed equally to this work.

## Acknowledgment

This work was supported by the Future in Research (Futuro in Ricerca) Programme of the Italian Ministry of Education, University and Research (Ministero dell'Istruzione, dell'Università e della Ricerca), Grant RBFR10L0GK (University of Naples "Federico II").

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 656319, 9 pages
http://dx.doi.org/10.1155/2013/656319

*Research Article*

# Expression of *N*-Acetylgalactosamine 4-Sulfate 6-*O*-Sulfotransferase Involved in Chondroitin Sulfate Synthesis Is Responsible for Pulmonary Metastasis

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Received 27 November 2012; Accepted 20 December 2012

Academic Editor: Davide Vigetti



Chondroitin sulfate (CS) containing E-disaccharide units, glucuronic acid-*N*-acetylgalactosamine(4, 6-*O*-disulfate), at surfaces of tumor cells plays a key role in tumor metastasis. However, the molecular mechanism of the metastasis involving the CS chain-containing E-units is not fully understood. In this study, to clarify the role of E-units in the metastasis and to search for potential molecular targets for anticancer drugs, the isolation and characterization of Lewis lung carcinoma (LLC) cells stably downregulated by the knockdown for the gene encoding *N*-acetylgalactosamine 4-*O*-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST), which is responsible for the formation of E-units in CS chains, were performed. Knockdown of *GalNAc4S-6ST* in LLC cells resulted in a reduction in the proportion of E-units, in adhesiveness to extracellular matrix adhesion molecules and in proliferation *in vitro*. Furthermore, the stable downregulation of *GalNAc4S-6ST* expression in LLC cells markedly inhibited the colonization of the lungs by inoculated LLC cells and invasive capacity of LLC cells. These results provide clear evidence that CS chain-containing E-units and/or GalNAc4S-6ST play a crucial role in pulmonary metastasis at least through the increased adhesion and the invasive capacity of LLC cells and also provides insights into future drug targets for anticancer treatment.

## 1. Introduction

Chondroitin sulfate (CS) is a sulfated glycosaminoglycan (GAG) that covalently attaches to core proteins to form CS-proteoglycans (CS-PGs) [1, 2]. CS/DS-PGs are ubiquitous in extracellular matrices (ECMs) and at cell surfaces in various tissues and regulate various physiological events such as cell proliferation, cytokinesis, morphogenesis, and viral infections through interaction with various proteins [3, 4]. Furthermore, CS-PGs at the tumor cell surface and in the ECM are related to metastatic potential and facilitate tumor invasion by enhancing integrin-mediated cell adhesion, motility, and intracellular signaling [5–8]. Interestingly, the binding of P-selectin to a tumor cell surface depends on the expression of the *CHST11* gene encoding chondroitin 4-*O*-sulfotransferae-1 (C4ST-1), and CSPG4 (also known as melanoma-associated CSPG) serves as a P-selectin ligand through its CS side chains and participates in the binding of P-selectin to highly metastatic breast cancer cells [9]. Further, the expression of a PG, versican, is upregulated in various types of tumors including lung cancer, as a macrophage activator that acts through Toll-like receptor-2 and its co-receptors Toll-like receptor-6 and CD14 [10].

The sugar backbone of CS chains is a linear polysaccharide consisting of repeating disaccharide units, $[-4\text{GlcUA}\beta1\text{-}3\text{GalNAc}\beta1-]_n$, where GlcUA and GalNAc represent D-glucuronic acid and *N*-acetyl-D-galactosamine, respectively, [4]. CS chains are modified by specific sulfotransferases, which catalyze the transfer of a sulfate group from 3′-phosphoadenosine 5′-phosphosulfate to C-2 of GlcUA and C-4 and/or C-6 of GalNAc. The combination of sulfated positions results in several disaccharide units with different

sulfation patterns and yields enormous structural diversity in terms of sequence [3, 11]. For instance, monosulfated GlcUA-GalNAc(4-*O*-sulfate) and GlcUA-GalNAc(6-*O*-sulfate) disaccharides, abbreviated as A- and C-units, are involved in the differentiation of chondrocytes and the development of a spine, respectively [12–15]. Representative disulfated disaccharide units are GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) (D-units) and GlcUA-GalNAc(4-*O*-, 6-*O*-disulfate) (E-units), which function in neuritogenesis [16–18] and viral infections [19, 20], respectively.

Recently, it has been reported that the expression of E-unit-containing structures recognized by an anti-CS-E phage display antibody, GD3G7 [21, 22], is increased in ovarian and pancreatic cancer tissues, resulting in alterations in tumor growth and tumor cell motility through the regulation of the signaling of vascular endothelial growth factor (VEGF) and the cleavage of CD44, respectively [22, 23]. Further, the expression of GalNAc 4-*O*-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST), which transfers a sulfate group to position 6 of GalNAc(4-*O*-sulfate) in A-disaccharide units formed by C4ST and is responsible for the formation of E-units [24, 25], is increased in colorectal cancer tissues compared with paired normal mucosa [26]. Thus, these observations appear to suggest that E-units in CS chains are upregulated in tumor tissues compared to normal tissues.

Moreover, we demonstrated that the expression of *GalNAc4S-6ST* and the proportion of disulfated E-disaccharides are increased in highly metastatic compared to low metastatic Lewis lung carcinoma (LLC) cells [27]. The colonization by intravenously injected LLC cells of mouse lungs was efficiently inhibited by preinjected CS-E polysaccharides, rich in E-units, derived from squid cartilage and by the anti-CS-E phage display antibody, GD3G7 [27], suggesting *GalNAc4S-6ST* and/or E-unit-containing CS chains to be involved in the pulmonary metastasis of LLC cells. In addition, the GAG-binding receptor in mouse lung was recently identified as Receptor for Advanced Glycation Endproducts (RAGE), which showed high affinity toward CS-E and heparan sulfate chains [28]. However, the exact structural features of GAGs remain to be investigated especially because RAGE could interact with both CS-E and heparan sulfate with high affinity [28]. In the present study, to clarify the role of E-units in metastasis, the isolation and characterization of LLC cells stably downregulated for the gene encoding GalNAc4S-6ST by knockdown using short hairpin RNA were performed.

## 2. Materials and Methods

*2.1. Materials.* The following sugars and enzymes were purchased from Seikagaku Biobusiness Corp. (Tokyo, Japan): CS-A from whale cartilages; CS-E from squid cartilages; six unsaturated standard disaccharides derived from CS; chondroitinase ABC (EC 4.2.2.20) from *Proteus vulgaris*; chondroitinase AC-II (EC 4.2.2.5) from *Arthrobacter aurescens*. Short hairpin RNA- (shRNA-) expressing plasmids (cat no.: sc-145317-SH) specific to mouse GalNAc4S-6ST, which target 5′-CUACAAUGUGGGAUAACAA-3′, 5′-CAAGACACCCUUAGAAUGU-3′, and 5′-GAACACUCGUGCUUAUACU-3′ and scrambled nucleotide sequence-containing control-shRNA plasmids (cat no.: sc-108060), shRNA transfection reagent, and shRNA plasmid transfection medium were purchased from Santa Cruz Biotechnologies, Inc (Santa Cruz, CA, USA).

*2.2. Animals and Cell Lines.* Seven-week-old male C57BL/6J mice and LLC cells were obtained from Japan SLC (Hamamatsu, Japan) and RIKEN Cell Bank (Tsukuba, Japan), respectively. All the experiments were performed under the experimental protocol approved by the local animal care committee of Hokkaido University.

*2.3. Isolation of LLC Clones Stably Downregulated for the Expression of GalNAc4S-6ST.* The *GalNAc4S-6ST (Chst15)* and *control shRNA* plasmids were individually transfected according to the manufacturer's instructions. The resultant puromycin-resistant colonies were subcultured on a 96-well culture plate by limiting dilution at a low density (1 cell/well), and were propagated.

*2.4. Quantitative Real-Time PCR.* Total RNA was extracted from each clone using an RNA isolation kit, illustra RNAspin Midi (GE Healthcare, Buckinghamshire, UK). Each cDNA was synthesized from ~1 $\mu$g of the total RNA using Moloney murine leukemia virus-reverse transcriptase (Promega, Madison, WI, USA) and an oligo$(dT)_{16}$ primer (Hokkaido System Science, Sapporo, Japan). The primer sequences used were as follows: for *GalNAc4S-6ST* (153 bp), 5′-TATGACAACAGCACAGACGG-3′ (forward) and 5′-TGCAGATTTATTGGAACTTGCGAA-3′ (reverse); for *glyceraldehyde-3-phosphate dehydrogenase* (*G3pdh*) (205 bp), 5′-CATCTGAGGGCCCACTG-3′ and 5′-GAGGCCATGTAGGCCATGA-3′. Quantitative real-time PCR was performed using a Brilliant II SYBER Green QPCR master mix in Mx3005P real-time QPCR (Agilent Technologies, Santa Clara, CA, USA). The level of *GalNAc4S-6ST* mRNA was normalized to that of the transcript of *G3pdh*.

*2.5. Analysis of Disaccharide Composition of CS/DS Chains Isolated from LLC Cells.* To obtain evidence that the knockdown of *GalNAc4S-6ST* results in a reduction in E-units [GlcUA-GalNAc(4-*O*-, 6-*O*-disulfates)] in CS chains from LLC cells, the CS disaccharide composition of each clone was determined as described previously [29, 30].

*2.6. Assays for Lung Metastasis.* To investigate the effects of the knockdown of *GalNAc4S-6ST* on experimental tumor metastasis, the control shRNA- and GalNAc4S-6ST-shRNA/LLC cells ($1 \times 10^6$ cells/mouse) were injected into a lateral tail vein of C57BL/6 mice as described in [27]. Three weeks after the injection, the animals were sacrificed, and the number of visible and parietal nodules in the lung was counted by two observers in a blinded fashion.

*2.7. Cell Adhesion Assay.* Plastic cover slips (10 × 10 mm) were precoated with 10 $\mu$g/mL of laminin (Invitrogen), fibronectin, or type IV collagen (BD Biosciences, San Jose, CA, USA) overnight at 4°C and then washed with phosphate-buffered saline twice. The control shRNA and GalNAc4S-6ST-shRNA/LLC cells were seeded on cover slips in 24-well plates at $5 \times 10^4$ cells/mL in serum-free Dulbecco's modified Eagle's medium (DMEM) and incubated for 1~2 h at 37°C. The supernatant with nonadherent cells was removed by three washes with a warmed culture medium. Attached cells were fixed, stained with the Diff-Quik staining kit (Sysmex International Reagents Co., Kobe, Japan), and counted in an area of 2 $mm^2$.

*2.8. Cell Migration and Invasion Assays.* The ability of GalNAc4S-6ST-shRNA/LLC cells to invade and migrate was assessed using the BD BioCoat chamber with or without Matrigel (BD Biosciences) *in vitro*, respectively. The lower chambers were filled with DMEM containing 10% FBS, and single cell suspensions of LLC cells ($2 \times 10^4$ cells/500 $\mu$L) in serum-free DMEM were placed in the upper chamber. After incubation for 26 h, the cells, which migrated or invaded through the membrane alone or the Matrigel-coated membrane, respectively, and remained bound to the underside of the membranes, were stained with the Diff-Quik and counted in five random microscopic fields/filters.

*2.9. Cell Proliferation Assay.* The control shRNA and GalNAc4S-6ST-shRNA/LLC cells were seeded in 96-well plates at 2,000 cells/well in DMEM containing 10% FBS and cultured for various periods. The number of living cells was measured at each time point using TetraColor One (Seikagaku Biobusiness Co.) according to the manufacturer's instructions. Triplicate cultures were used for each sample. After incubation for 1 h at 37°C, the developed color was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

## 3. Results

*3.1. Isolation and Characterization of the LLC Cells Expressing shRNAs Specific for GalNAc4S-6ST.* To isolate the clones of LLC cells in which the *GalNAc4S-6ST* gene was suppressed, the vectors expressing shRNAs, which also contain the puromycin-resistance gene, specific for mouse *GalNAc4ST-6ST* were introduced into the cells. Fifty-seven LLC clones resistant to puromycin were isolated (LLC-4S6ST-shRNA). To examine the efficacy of the knockdown of *GalNAc4S-6ST* by specific shRNA, quantitative real-time PCR was conducted after the extraction of total RNA from ten randomly selected clones, followed by the synthesis of the cDNA. The isolated LLC-4S6ST-shRNA clones (nos. 7, 17, and 23) showed the downregulation of *GalNAc4S-6ST* (30~40% of the control-shRNA clones) (Figure 1(a)). Thus, these three clones were utilized for further analyses.

To further characterize the effects of the knockdown of the *GalNAc4S-6ST* gene on the amount of E-units, the disaccharide composition of CS chains, which were prepared from each clone as a GAG-peptide fraction, was determined. Representative chromatograms are shown in Figures 1(b) and 1(c), and the composition and amounts of the disaccharides are summarized in Table 1. The data obtained from the digest of the GAG-peptides using a mixture of chondroitinases ABC and AC-II revealed that the low sulfated disaccharide, HexUA-GalNAc(4-*O*-sulfate) (A), where HexUA represents hexuronic acid ($\beta$-GlcUA or $\alpha$-iduronic acid), was a major disaccharide unit, ~94% (Table 1), and HexUA-GalNAc(4-, 6-*O*-disulfate) (E) accounted for ~6% of all the disaccharides in the LLC-control-shRNA cells consistent with a previous report [28]. On the other hand, a drastic reduction in the proportion of E-units, to 0.5~1.9%, was observed in the LLC-4S6ST-shRNA clones (nos. 7, 17, and 23) compared to the LLC-control-shRNA cells (Table 1). Thus, these clones were utilized to further experiments. It should be noted that the amounts of total disaccharides recovered were less in GalNAc4S-6ST-shRNA clones (213, 323, and 246 pmol/mg acetone powder) compared to wild-type cells and control-shRNA clones (754, 1348, 924, and 534 pmol/mg acetone powder), suggesting that GalNAc4S-6ST or CS chains containing E-units may affect the amounts or lengths of CS chains, or other CS-biosynthetic enzymes. In contrast, bone marrow-derived mast cells from the knockout mice of GalNAc4S-6ST synthesized larger CS chains than the wild type, and levels of the chondroitin 4-*O*-sulfotransferase-1 and chondroitin 6-*O*-sulfotransferase-1 transcripts in the homozygous mutant mice were higher than those in the wild type [25]. Thus, the contrasting effect on the biosynthesis of CS by the expression of GalNAc4S-6ST in LLC cells remains to be elucidated.

*3.2. Effects of the Knockdown of the GalNAc4S-6ST Gene in LLC Cells on Pulmonary Metastasis.* To assess the influence of the knockdown of the *GalNAc4S-6ST* gene and the resulting reduction of E-units in LLC cells on pulmonary metastasis, the LLC-4S6ST-shRNA clones were individually inoculated into mice via a tail vein. Three weeks later, the mice were sacrificed, and pulmonary metastasis was evaluated by counting tumor foci on the lung surface and weighing the lung tissues. As expected, the knockdown of *GalNAc4S-6ST* drastically reduced the metastasis of LLC cells compared with that in mice injected with the LLC-control-shRNA (Figure 2), suggesting a crucial role for the cell surface CS chains containing E-units in the pulmonary metastasis of LLC cells.

*3.3. Characterization of the LLC-4S6ST-shRNA Cells In Vitro.* LLC cells are frequently utilized as a model for experimental lung metastasis [31]. After their inoculation into the tail of mice, LLC cells reach the lung and may bind to the surface of the vascular endothelium through adhesion to ECM molecules [32–34]. Hence, to assess the change in the adhesiveness of LLC cells by the knockdown of *GalNAc4S-6ST*, the adhesive capacity of the LLC-4S6ST-shRNA was examined using adhesion molecules in ECM including laminin, fibronectin, and type IV collagen. The number of LLC-4S6ST-shRNA cells adhering to laminin or fibronectin but not to type IV collagen was significantly

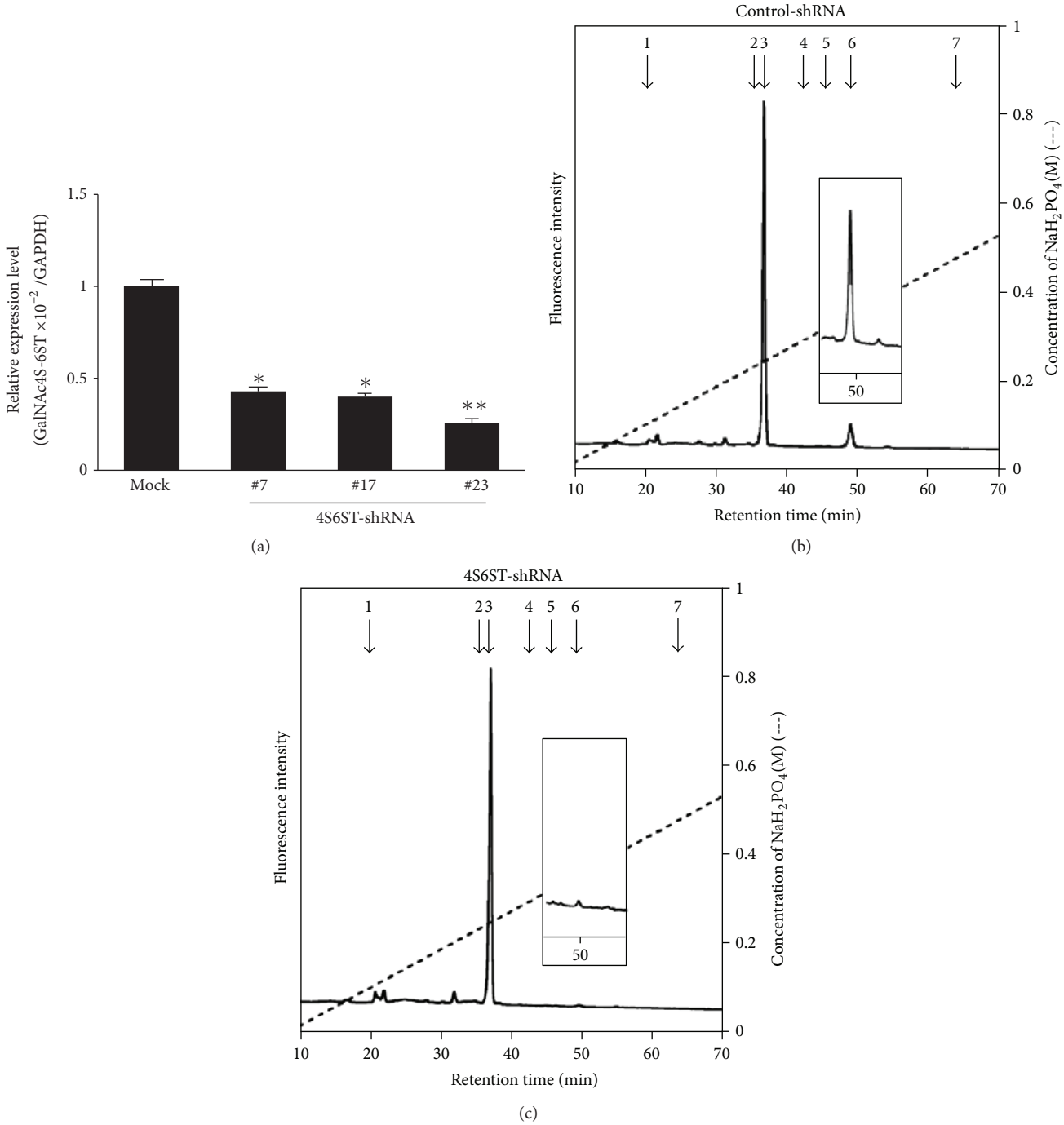


Figure 1: Quantitative real-time PCR analysis of the *GalNAc4S-6ST* transcript and profile of sulfation pattern of CS in the LLC-4S6ST-shRNA cells. (a) Total RNA was extracted from the control- (mock) or GalNAc4S-6ST-shRNA/LLC cells, and cDNA was synthesized by reverse transcriptase. Real-time PCR was conducted using the cDNAs, *Taq* polymerase, and SYBER Green. The expression of *GalNAc4S-6ST* was individually normalized to that of *G3pdh*. The assay was performed at least twice in triplicate, and representative results are shown. Values represent the mean ± SD. $^{*}P < 0.005$; $^{**}P < 0.001$ versus control by one-way ANOVA with Dunnett's adjustment. (b, c) Anion-exchange HPLC of disaccharides obtained from the digests of CS derived from control- and GalNAc4S-6ST-shRNA/LLC cells with a mixture of chondroitinases ABC and AC-II. The GAG-peptide preparations from LLC cells, which stably express control-shRNA (b) or *4S6ST-shRNA* (c), were individually digested with a mixture of chondroitinases ABC and AC-II. Each digest was labeled with a fluorophore 2AB as detailed in Section 2 and analyzed by anion-exchange HPLC on an amine-bound silica PA03 column using a linear gradient of $NaH_2PO_4$ as indicated by the dashed lines. The eluate was monitored by fluorescence intensity with the excitation and emission wavelengths of 330 and 420 nm, respectively. The insets show magnified chromatograms (10-fold) around the elution position of ΔE units. The positions of the 2AB-derivatized authentic disaccharides are indicated by numbered arrows: 1: ΔO, ΔHexUA-GalNAc; 2: ΔC, ΔHexUA-GalNAc(6-*O*-sulfate); 3: ΔA, ΔHexUA-GalNAc(4-*O*-sulfate); 4: ΔD, ΔHexUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate); 5: ΔB, ΔHexUA(2-*O*-sulfate)-GalNAc(4-*O*-sulfate); 6: ΔE, ΔHexUA-GalNAc(4-*O*-, 6-*O*-disulfate); 7: ΔT, ΔHexUA(2-*O*-sulfate)-GalNAc(4-*O*-, 6-*O*-disulfate).

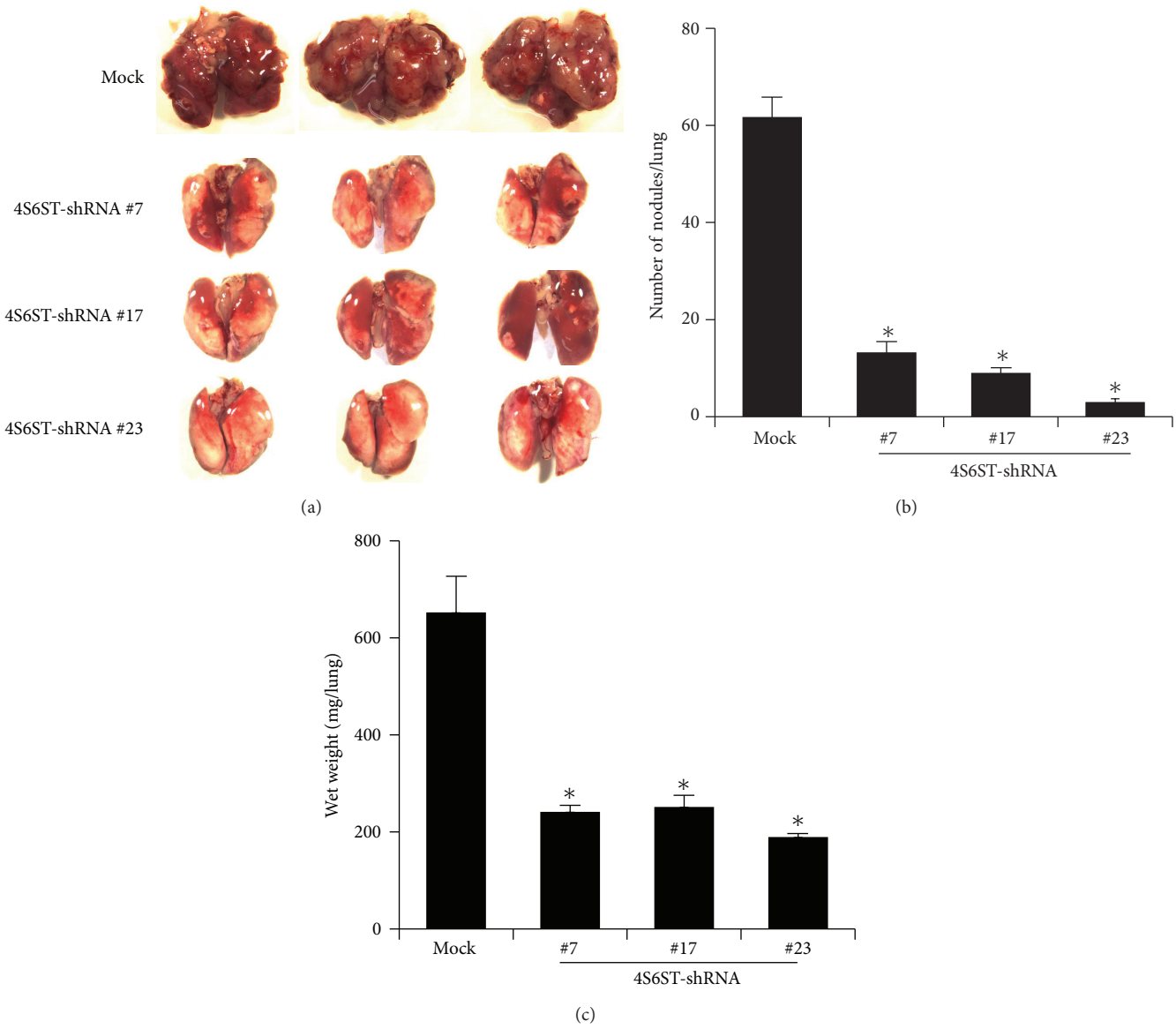


FIGURE 2: Effects of the knockdown of *GalNAc4S-6ST* on the pulmonary metastasis of LLC cells. Control- or GalNAc4S-6ST-shRNA/LLC-cell suspensions of $1 \times 10^6$ cells in 200 $\mu$L of DMEM were injected into a tail vein of C57BL/6J mice, and 21 days later, the number of tumor foci in the lungs was recorded. Six mice were used per group. Representative lungs from mice injected with the LLC cells stably expressing control- (mock) or *GalNAc4S-6ST-shRNA* (*4S6ST-shRNA*) are shown in (a). The number of colonies (b) and the wet weight (c) of the lungs from mice injected with each LLC clone were measured. Data represent the mean ± S.D. for two independent experiments. $^*P < 0.001$ versus control by one-way ANOVA with Dunnett's adjustment.

reduced (51 and 73% of the control-shRNA expressing LLC cells, resp.) (Figure 3). These observations indicate that CS chains at the LLC cell surface containing E-units may be involved in the initial cell adhesion to ECM molecules produced on the vascular endothelium in the lung during metastasis.

Along with the adhesion of LLC cells to a target tissue or cell, cell migration and invasion are also important to cancer progression and metastasis [35]. Next, to determine if the inhibition of the enzyme GalNAc4S-6ST using shRNA affects the migration and invasion of LLC cells; the invasive or migratory potential of LLC-4S6ST-shRNA cells was examined *in vitro* using a Boyden chamber coated with or without Matrigel, respectively. Although the migration was not significantly diminished by the knockdown of *GalNAc4S-6ST* when compared with that using a control-shRNA (Figure 4(b)), down-regulation of E-units in CS chains caused by the knockdown of *GalNAc4S-6ST* resulted in a significant decrease in the invasion of LLC-4S6ST-shRNA cells through Matrigel as compared with the control knockdown (Figure 4(a)), indicating that *GalNAc4ST-6ST* and/or the E-units in CS chains regulate invasion but not migration during the metastasis of LLC cells, being consistent with a previous report [27].

Table 1: Disaccharide composition of CS chains in the control- and 4S6ST-shRNA/LLC cells. The GAG-peptide preparation from each cell line was digested with a mixture of chondroitinases ABC and AC-II and analyzed by anion-exchange HPLC after labeling with a fluorophore 2AB as detailed in Section 2.

| | Wild type | GalNAc4S-6ST-shRNA | | | Control-shRNA | | |
|---|---|---|---|---|---|---|---|
| | | No. 7 | No. 17 | No. 23 | No. 5 | No. 10 | No. 14 |
| | pmol/mg acetone powder (mol%)[b] | | | | | | |
| ΔA[a] | 675 (93.6) | 209 (98.1) | 321 (99.4) | 243 (98.9) | 1,273 (94.4) | 867 (93.8) | 487 (91.0) |
| ΔE[a] | 46 (6.4) | 4 (1.9) | 2 (0.5) | 3 (1.1) | 75 (5.6) | 57 (6.2) | 48 (9.0) |
| Total | 721 (100) | 213 (100) | 323 (100) | 246 (100) | 1,348 (100) | 924 (100) | 535 (100) |

[a]ΔA and ΔE represent ΔHexUA-GalNAc(4-*O*-sulfate), and ΔHexUA-GalNAc(4-*O*-, 6-*O*-disulfate), respectively. No other disaccharide units including ΔO, ΔC, or ΔD were detected (data not shown). ΔO, ΔC, and ΔD stand for ΔHexUA-GalNAc, ΔHexUA-GalNAc(6-*O*-sulfate) and ΔHexUA(2-*O*-sulfate)-GalNAc(6-*O*-disulfate), respectively.

[b]Values are expressed in pmol of disaccharide per mg acetone powder as starting materials from the cells and calculated based on the peak areas of the disaccharides detected by anion-exchange HPLC (Figures 1(b) and 1(c)).

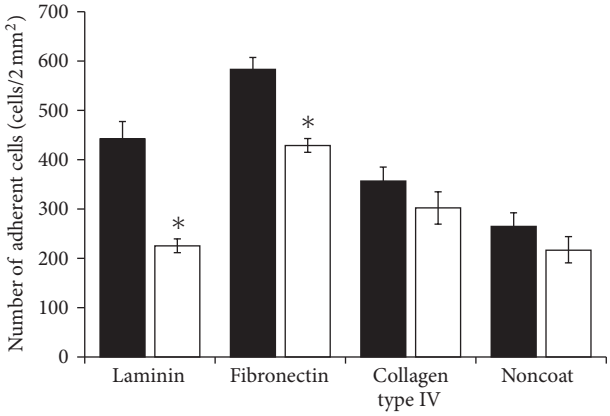


Figure 3: Effects of the knockdown of *GalNAc4S-6ST* on the adhesion of LLC cells. LLC cells stably expressing control-shRNA (filled bars) or *GalNAc4S-6ST-shRNA* (open bars) were seeded on laminin-, fibronectin-, or collagen type IV-precoated plastic cover slips for 1-2 h. The cells were stained with Diff-Quik, and the adherent cells were counted. Average values obtained from three clones of both control-shRNA (nos. 5, 10, and 14) and GalNAc4S-6ST-shRNA (nos. 7, 17, and 23) are shown, and the experiments were performed in triplicate. Error bars indicate ±S.D. of triplicate samples. $^{*}P < 0.05$ versus control-shRNA by Student's *t*-test.

It is possible that LLC-4S6ST-shRNA cells are not so metastatic as we expect because of a possibility that they may grow more slowly than LLC-control-shRNA cells, which also partly contributes the lower metastatic capcity of LLC-4S6ST-shRNA cells. To address this issue, the LLC cells were plated at a low density and the growth rate was determined. LLC-4S6ST-shRNA cells grew more slowly than control cells according to the results of a cell proliferation assay (Figure 5). These results indicate that the suppression of the metastasis of LLC cells by the knockdown of *GalNAc4S-6ST* shown in Figure 1(b) was partly due to a depression of cell growth potential.

## 4. Discussion

In the present study, to evaluate the involvement of E-units in CS chains expressed at the surface of LLC cells in tumor metastasis, we used an animal model of lung carcinoma and shRNA specific for the *GalNAc4S-6ST* gene, which is responsible for the formation of E-units [24, 25]. Firstly, *GalNAc4S-6ST* was overexpressed in LLC cells using several expression vectors such as pcDNA3.1/myc-His, pEF6/V5-His, and pIRESneo3, although the expression of *GalNAc4S-6ST* and E-units in CS chains was not enhanced for some unkown reason(s) (data not shown). Therefore, we assessed functions of E-units in the experimental metastatic model by knockdown of *GalNAc4S-6ST* using an shRNA-expressing vector in LLC cells.

CS-E interacts with heparin or heparan sulfate-binding proteins such as fibroblast growth factors, VEGF, pleiotrophin, and midkine [22, 36]. E-unit-containing structures of CS chains at the cell surface are important for the binding of LLC cells to laminin and fibronectin (Figure 3), which also interact with heparan sulfate [37–39]. Thus, E-units in CS chains in addition to heparan sulfate chains at the surface of LLC cells may also contribute the binding to laminin and/or fibronectin on the luminal side of the vascular endothelial cells in lungs.

Furthermore, endothelial carbohydrate-binding proteins, E- and P-selectins, play a role in the pulmonary metastasis of B16 melanoma cells [40]. It has also been reported that CS-E interacts with the adhesion molecules L- and P-selectins, and that CS-PGs at the surface of the metastatic breast cancer cell line are major P-selectin ligands on the endothelium [41, 42]. Hence, CS chains containing E-units may be involved in the adhesion of LLC cells through such cell adhesion molecules. In fact, most recently, we identified RAGE, which is a member of the immunoglobulin superfamily predominantly expressed in the lung, as a receptor for CS-E involved in pulmonary metastasis [28]. Together, the interaction of cell adhesion molecules or receptors including P-selectin and RAGE expressed on the endothelium at secondary target tissues with CS-containing E-units expressed on malignant cell surfaces play major or some roles in the targeting of tumor cells to lungs.

It has been demonstrated that matrix metalloproteinase-9 is critical for the invasion and metastasis of LLC cells [43], and that heparan sulfate-proteoglycan, syndecan-2, functions as a suppressor for matrix metalloproteinase-2 activation depending on the heparan sulfate side chains on LLC cells

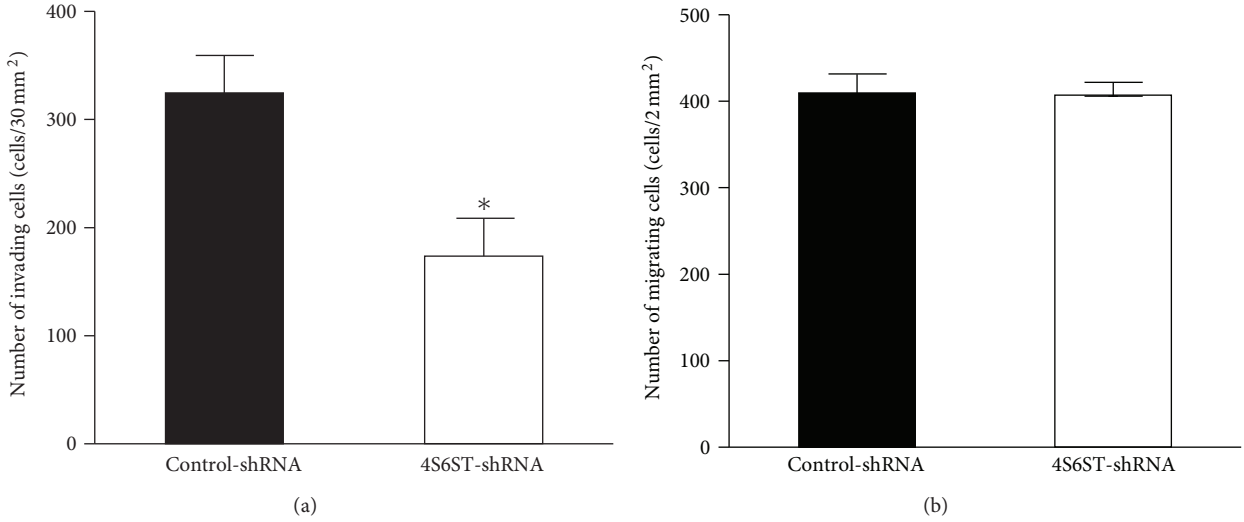


Figure 4: Effects of the knockdown of *GalNAc4S-6ST* on the invasion and migration of LLC cells. LLC cells stably expressing control-shRNA (filled bars) or *GalNAc4S-6ST-shRNA* (open bars) were plated on BD BioCoat chambers in the absence of fetal bovine serum. Cell invasion and migration were measured with or without Matrigel (BD Biosciences), respectively, as described in Section 2. Effects of the knockdown of *GalNAc4ST-6ST* on the invasion (a) and migration (b) of the LLC cells are summarized. The data represent the mean values ± S.D. for three clones of both control-shRNA (nos. 5, 10, and 14) and GalNAc4S-6ST-shRNA (nos. 7, 17, and 23). Three independent experiments were performed, and the representative results are shown. $^*P < 0.05$ versus control-shRNA by Student's *t*-test.

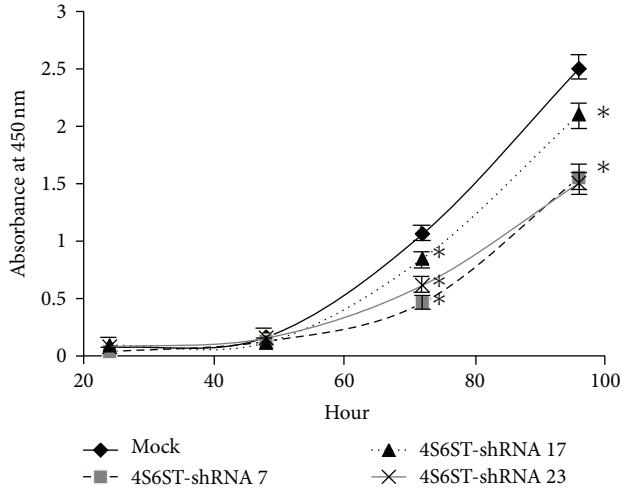


Figure 5: Effects of the knockdown of *GalNAc4S-6ST* on the proliferation of LLC cells. LLC cells stably expressing control-shRNA (mock) or *GalNAc4S-6ST-shRNA* (*4S6ST-shRNA*) were seeded on 96-well plates at $2 \times 10^3$ cells/well and incubated at 37°C for the assessed period. Cell numbers were measured every 24 h using TetraColor One reagent containing a tetrazolium and electronic carrier as described in Section 2. The data represent the mean ± S.D. for three clones of both control-shRNA (nos. 5, 10, and 14) and GalNAc4S-6ST-shRNA (nos. 7, 17, and 23). Three independent experiments were performed, and representative results are shown. $^*P < 0.001$ versus control-shRNA by Student's *t*-test.

[44]. Moreover, melanoma CS-PG regulates membrane-type 3 matrix metalloproteinase and invasion of melanoma cells [45]. The invasive ability of the LLC-4S6ST-shRNA cells in Matrigel was lower than that of the LLC-control-shRNA cells (Figure 4). Hence, CS-containing E-units as well as heparan sulfate may regulate metalloproteinases at the surfaces of LLC cells.

Metastasis is completed via processes involving growth, survival, and neoangiogenesis [32, 33]. CS-E interacts with various heparin-binding proteins such as fibrobrast growth factors, midkine, and pleiotrophin [36]. Further, VEGF binds to CS-E expressed in tumor blood vessels *in vitro* [22]. Our findings (Figure 5) together with these observations prompted us to speculate that CS chains containing E-units may participate as a regulator of VEGF signaling in the proliferation of tumor cells and tumor angiogenesis. In fact, GAG side chains including heparan sulfate and CS of neuropilin-1, a coreceptor for VEGF that augments angioplastic events through VEGF receptor-2, are critical for the reactivity to VEGF in endothelial cells and smooth muscle cells [46].

Interestingly, CS-PG, versican, functions as a macrophage activator that acts through Toll-like receptor-2, resulting in the production of tumor-necrosis factor-$\alpha$ and strong enhancement of the metastatic growth of LLC [10]. Notably, versican contains E-units in the CS side chains [42]. Thus, these observations with our results raise the possibility that E-unit-containing structures in the CS side chains of versican may contribute to the growth and/or metastasis of LLC cells.

## 5. Conclusions

In the present study, a reduction in the expression of *GalNAc4S-6ST* or the proportion of E-unit-containing CS chains effectively suppresses metastatic lung carcinoma through the reduction in adhesiveness, invasion, and proliferation but not the migration of LLC cells. Recently, we

identified RAGE, which is specifically expressed in the normal lung, as a receptor for CS-E involved in the pulmonary metastasis [28]. The siRNA of the *GalNAc4S-6ST* gene and CS-E mimetics including small molecular inhibitors that bind RAGE are potential targets for anticancer therapies.

## Abbreviations

| | |
|---|---|
| 2AB: | 2-Aminobenzamide |
| CS: | Chondroitin sulfate |
| GAG: | Glycosaminoglycan |
| GalNAc: | *N*-Acetyl-D-galactosamine |
| GalNAc4S-6ST: | *N*-Acetylgalactosamine 4-*O*-sulfate 6-*O*-sulfotransferase |
| GlcUA: | D-Glucuronic acid |
| LLC: | Lewis lung carcinoma |
| PG: | Proteoglycan |
| shRNA: | Short hairpin RNA |
| VEGF: | Vascular endothelial growth factor. |

## Authors' Contribution

S. Mizumoto and M. Watanabe contributed equally to this work.

## Acknowledgments

This work was supported in part by Grant-in-Aid for Scientific Research (B) 23390016 (to K. Sugahara), Future Drug Discovery and Medical Care Innovation Program (to K. Sugahara), and Young Scientists (B) 23790066 (to S. Mizumoto) from Japan Society for the Promotion of Science (JSPS). None of these sponsors were involved in the experimental design.

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