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REVIEW ARTICLE

TERM

Signaling of Extracellular Matrices for Tissue Regeneration and Therapeutics

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Cells receive important regulatory signals from their extracellular matrix (ECM) and the physical property of the ECM regulates important cellular behaviors like cell proliferation, migration and differentiation. A large part of tissue formation and regeneration depends on cellular interaction with its ECM. A comprehensive understanding of the mechanistic biochemical pathway of the ECM components is necessary for the design of a biomaterial scaffold for tissue engineering. Depending on the type of tissue, the ECM requirement might be different and this would influence its downstream intracellular cell signaling. Here, we reviewed the ECM and its signaling pathway by discussing: 1) classification of the ECM into hard, elastic and soft tissue based on its physical properties, 2) proliferation and differentiation control of the ECM, 3) roles of membrane receptor and its intracellular regulation factor, 4) ECM remodeling via inside-out signaling. By providing a comprehensive overview of the ECM's role in mechanotransduction and the self-regulatory effect of cells back on the ECM, we hope to provide a better insight of the physical and biochemical cues from the ECM. A sound understanding on the *in vivo* ECM has implication on the choice of materials and surface coating of biomimetic scaffolds used for tissue regeneration and therapeutics in a cell-free scaffold.

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INTRODUCTION

The goal of tissue engineering is to replace damaged or diseased tissues of the body, and restore healthy tissue function. Tissue engineering is successful because of its combinatory approach involving critical elements that include the scaffold, inductive growth factors and the progenitor/stem cells. The biomimetic scaffold supports cell adhesion, and together with inductive growth factors (e.g., chemokines, cytokines) potentiate various cellular activities like stem cell differentiation and proliferation. Using appropriate culturing methods (e.g., modified bioreactors), these tissue-engineered constructs can be made to closely replicate the native tissue [1,2].

A key step to the advancement of tissue engineering requires an elucidated understanding of the interaction between the cells and the extracellular matrix (ECM) since the physical properties of the biomaterial scaffold can regulate progenitor/stem cells activities. *In vivo*, different tissue types each have their own molecular composition and structures that are optimized to perform their respective biological and mechanical functions. The ECM is an important component of tissues because it offers structural support to the cells, and regulates many cellular behaviors like cell migration, apoptosis or maintenance [3-5].

Cells adhere to the ECM through cell adhesion molecules and this interaction triggers downstream signaling events that enable cells to respond to changes in the microenvironments [6]. Advances in stem cell biology demonstrated the importance of ECM in regulating stem cell fate through physical (e.g., matrix stiffness, mechanical forces) and biochemical (matrix-bound growth factors) cues [7-10]. Recent studies have used ECM components to regulate progenitor/stem cell behavior, particularly tissue-specific differentiation. Engineered and native ECM have been manipulated for this purpose in *in vitro* and *in vivo* experiments, and showed positive level of success [11-13]. However, how this can be applied to a biomimetic scaffold will first require a clear understanding of the ECM and its interaction

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with stem cells.

In this review, we begin by summarizing the native properties of ECM in the various tissue types and the role of these ECM components in regulating cell proliferation and differentiation. We will then elaborate the mechanism of outside-in and inside-out signaling of ECM-mediated factors on tissue modelling. These will form the basis of our proposition that an optimum ECM condition can act as a regulatory tool for tissue regeneration and other therapeutic applications.

ECM COMPONENTS FOR DIFFERENT TISSUES

Properties of the ECM

The ECM can be classified into three broad categories based on its matrix stiffness: hard, elastic and soft [14]. Hard tissues like the bone have high elastic modulus (~100 kPa) and are strong and rigid. Elastic tissues like the muscle and skin have moderate elastic modulus (~10 kPa) and shows elastic properties of certain degree. Soft tissues like the brain and lungs have low elastic modulus (~1 kPa) and are very susceptible to deformation. The physical property of ECM is an important consideration for the stem cell niche since it determines differentiation of mesenchymal stem cells (MSCs) [15,16]. Correspondingly, MSCs respond to the characteristic of the matrix it is placed upon. Matrices that mimic the soft gel-like brain tissue, elastic muscle tissue and hard bone surfaces induces a neurogenic, myogenic and osteogenic phenotype respectively [10]. Recent studies have shown the critical role of ECM's mechanical characteristics in directing differentiation (Fig. 1) of MSC and therefore, varying the ECM composition is one way to optimize the functionality of cell carriers for stem cell therapy.

At the heart of the ECM is a complex structure comprising of molecular components that can be further divided into two classes, namely the fibrous proteins and proteoglycans [17]. There are two types of fibrous proteins: structural and specialized proteins. Examples of structural proteins include collagen and elastin, and they influence the structural properties of the ECM. Specialized proteins like fibronectin and laminin perform specialized functions to mediate cell adhesion to the ECM. Proteoglycans are found on cell surface near the ECM and they form the attachment of proteins to glycosaminoglycans (GAGs). GAGs and be classified into four groups with different properties depending on their core disaccharide structure. The hardness of the ECM depends on GAG composition, which then regulate cellular processing for proliferation and differentiation of stem cells [18,19]. *In vivo*, ECM component and arrangement is very tightly regulated depending on the development stage of the tissue and the environmental condition required to maintain homeostasis [20]. Therefore, the application of appropriate bioactive scaffolds is imperative for optimizing the secretion of cytokines and growth factors from stem cells.

At the interface of the cells and ECM are cell adhesion receptors that trigger intracellular signaling events in response to changes in the topography of the microenvironment. To date, various micro and nano-patterns on ECMs have been used to regulate progenitor/stem cell behavior, particularly tissue-specific proliferation and differentiation (Fig. 1). These engineered and native ECMs have shown promising success in *in vitro* experiments. Hence, optimizing the topography of stem cell carriers can provide important cues for soluble factor-guided communication between stem cells and cells of the immune system (e.g., T- and B-cells). This will modulate cell regulation and function, depending on the topographical features and the type of surface molecules present.

The stiffness, molecular components and topography of the ECM are regulatory factors of stem cell. In tissue engineering, biomimetic scaffold developed for tissue repair aims to closely replicate the *in vivo* ECM physical property of the desired tissue

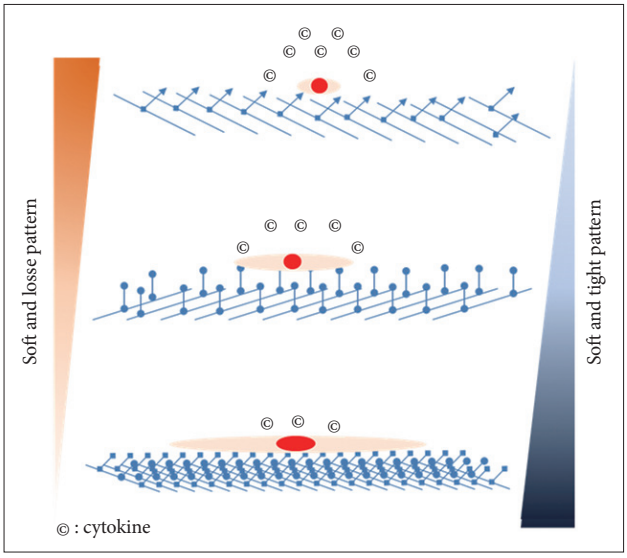

Figure 1. Mechanical and topographical properties of bioactive scaffold regulate cell behavior and its cytokine release pattern. The mechanical properties of the ECM depend on its elastic modulus, which influence the directional differentiation of MSC. Various micro and nano patterns of the ECM topogrpahy regulate progenitor/stem cell behavior, particularly tissue-specific proliferation and differentiation. These properties determine the soft and loose, or stiff and tight pattern of the ECM and its interface. Through adhesion receptors such as integrins, the ECM interface plays a key role in cell adhesion and triggers intracellular signaling events that enable cells to respond to changes in their environmental cues. Physical properties of ECM are important consideration for engineering tissue constructs that mimic host tissues. These physical cues not only regulate proliferation and differentiation, but also cytokine release and the downstream immunoreactions. ECM: extracellular matrix, MSC: mesenchymal stem cell.

[21,22]. For a better understanding, we will specifically look at the native ECM components of hard, elastic and soft tissue since all tissues can be categorized into one of them.

ECM of hard tissue

The ECM is an important scaffold for tissue formation and maintenance in hard tissues like the bone. Bone formation and regeneration follow a multiple phase process where a dramatic change in ECM components is observed in each phase [23]. In the initial phase, there is an increase in expression of type I collagen, vitrogen and fibronectin for osteoblast proliferation [24]. This is followed by the bone maturation phase where the ECM starts to develop, and bone-specific molecules like osteopontin (OPN) and alkaline phosphatase could be detected in the ECM [25]. For the last phase of bone formation, osteoblast starts to differentiate and mineralize. During mineralization, osteocalcin (OCN) and bone sialoprotein (BSP) can be found in the bone tissue [26].

The ECM of bone tissue is made up of three key molecular components that support those functions. The first is collagen, a major ECM component that consists about 90% of the bone mass and plays a very important role in the initial phase of bone formation [27]. Patient with metabolic disorder of collagen display imperfect osteogenesis of the bone tissue [28]. Within the composition of collagen, the percentage distribution of type I and type V of collagen is 95% and 5% respectively. Collagen promotes proliferation of osteoblast and accumulation of calcium for bone mineralization. Aside from collagen, the other remaining 10% of the ECM molecular components in bone are non-collagenous proteins and large molecules of proteoglycans made up of hyaluronan and chondroitin sulfate that are responsible for regulation of early stage morphogenesis in the bone. Biglycan and decorin are small molecules of proteoglycans that regulate late stage bone formation [29]. Following that, fibronectin becomes a key regulation factor for osteoblast mineralization. Fibronectin is a 440 kDa ECM glycoprotein with a dimer structure that contains multiple RGD (Arg-Gly-Asp), RGDS (Arg-Gly-Asp-Ser), LDV (Leu-Asp-Val), and REDV (Arg-Glu-Asp-Val) sequences on its cell adhesive site. They bind to transmembrane receptor like integrin as well as other ECM molecules like collagen, fibrin and heparan sulfate proteoglycans [30]. Fibronectin also plays an important role in osteoblast proliferation and migration in the damaged bone tissues. Hence, it is commonly coated on the surface of biomaterials used as a scaffold for bone regeneration.

Among the non-collagenous proteins are three proteins closely associated with the bone: OPN, OCN and BSP. OPN is a bone-specific differentiation marker expressed in the bone maturation stage of bone formation [31,32]. It is a highly negatively ECM protein that lacks an extensive secondary structure. At the moment, its role is still not fully understood. In general, OPN is acknowledged as a bone remodeling factor and how it regulates depend on the type of cells. In the bone, OPN is predominantly expressed by osteocytes, osteoblast (cells involved in bone formation) and osteoclasts (cells involved in bone resorption cells). They also assist in anchoring osteoclasts to the bone matrix during bone resorption. OCN is abundantly expressed in the bone as a Vitamin K-dependent protein, and it can bind to calcium for calcium ion homeostasis. Calcium ion is a key ingredient in bone mineralization and not surprisingly, OCN has an influence on its process However, the mechanism in which it influences bone mineralization remains unclear [33]. OCN is produced by osteoblast and accumulates at the bone ECM for bone mineralization. Specifically, it is present at the onset of mineralization during the last phase of bone regeneration. OCN activity may other have cross-talk effect on the endocrine system, including the regulation of insulin secretion for energy metabolism [26]. BSP is an important protein found in the bone maturation phase. It supports hydroxyapatite crystal formation in the hard tissue of the bone and teeth, and promotes several osteogenic gene expressions in the osteoblast that drives cell differentiation. It is mainly secreted by osteoclast, cells involved bone resorption for tissue remodeling and regeneration. When BSP is immobilized on biomaterial surface, significant increase in osteoblast adhesion rate is observed leading to bone regeneration. As a bone-specific ECM protein, it can also induce osteoblast differentiation [34]. Therefore, BSP is a valued protein for bone tissue engineering applications.

OPN, OCN, and BSP are bone-specific ECM components of the hard tissue that play an important role in bone maintenance and regeneration. Therefore, the use of these ECM proteins could be applicable in the construct of an artificial bone scaffold used for bone replacement.

ECM of elastic tissue

Smooth muscle, skeletal muscle and cardiac muscle are examples of elastic tissue of myogenic origin. In injured tissues, they develop from myoblast progenitor cells [35]. Muscle tissue has several types of collagen components such as major collagen type I, II and minor collagen type II, VI, IX, XI-XVI, and XVIII-XIX [36]. In the muscle tissue, cell-matrix interaction is important for muscle regeneration after an injury. The myogenic progenitor cells pick up microcellular environment cues from the ECM that induces self-renewal and differentiation, and is therefore critical in promoting its proliferation during tissue recovery. In initial stages of muscle tissue regeneration, laminin promotes progenitor cells proliferation. Thereafter, remodeling of ECM by the cells induces its differentiation. While the precise ECM com-

position that caused the progenitor cells to differentiate is still not yet clearly identified, it is known that elastic tissues have an intermediate matrix stiffness of approximately 10 kPa. MSCs placed on artificial matrices made of materials with stiffness in this region showed myogenic differentiation. *In vivo*, myogenic progenitor cells differentiation might lead to a change in the physical properties of the ECM. These differences and limitations have to be taken into account when reconstructing a scaffold for muscle tissue regeneration.

A defining characteristic of muscle tissue is its elastic and load-bearing properties, and this is enabled by the presence of a highly elastic protein known to as elastin. The term "elastic protein" can be broadly used to refer to many structural proteins with diverse functions and mechanical properties. In the case of elastin, its elastic properties facilitate the tissue's ability to deform reversibly without a loss of energy. Therefore, elastic proteins like elastin have high resilience to external forces and can maintain its shape. It is also abundant in connective tissues, including the aorta−a large elastic blood vessel which originates from the heart that assist in pressure wave propagation and blood flow. Elastin can also be found elsewhere in the body, including the lungs, ligaments, skin, bladder, cartilage and the intervertebral disc above the sacroiliac wherein all of the above elasticity is necessary for its physiological function. In regenerative medicine, research has focused on elastin replacement for tissue regeneration [35] because damage tissue loses a lot of elastin that is not replaced in the wound healing process.

The behavior of myoblast progenitor cells is heavily regulated by the ECM condition. *Ex vivo* skeletal muscle ECM incubated with myoblast enhanced the latter's proliferation and differentiation. Patients with dystrophies like Duchenne muscular dystrophy were found to have mutations in genes that encode ECM molecules and its related factors, which further underlines the importance of an ideal ECM for muscle tissue generation. A more optimal ECM composition and the replacement of key proteins like elastin will aid myoblast maintenance and regeneration on tissue-engineered construct for elastic tissues.

ECM of soft tissue

The ECM of soft neuronal tissues (e.g., brain, spinal cord) affects neuronal functions such as nerve tissue development and axon plasticity. The ECM surrounding neuron cells regulate neuron progenitor's maturation at the end of the embryonic stage, and also neuron plasticity for neuron axon maturation [37]. ECM signaling is mediated by fibrous protein in the ECM and one such protein is laminin, a basal membrane protein that mediates neuron cell's adhesion to the ECM. It is secreted by Schwann cell and serves as a guiding cue for neurite outgrowth in nervous tissue development. Other major fibrous proteins in the ECM of brain tissues are collagen, fibronectin and vitronectin. These proteins make up a big portion of the brain volume by filling up the interstitial spaces within the tissue. Particularly, fibronectin plays a special role in neuron cell migration and outgrowth during the development of peripheral and central neurons. Despite a lack of a clear mechanism, fibronectin and laminin were shown to be able to treat spinal cord injury because these adhesion protein improve the rate of nerve regeneration [38]. One way to introduce fibronectin for tissue recovery and regeneration is by a hydrogel system at the site of nerve injury or degeneration [39].

Aside from fibrous proteins, the ECM of the brain tissue also contains lectican−a family of proteoglycans that consists four members of chondroitin sulfate proteoglycan: versican, aggrecan, neurocan, and brevican [40]. These proteoglycans are also capable of regulating cell adhesion to ECM, activity of nerve growth factor, and matrix stiffness of ECM assembly. They form an essential group of proteoglycan that provides a link between hyaluronic acid and the ECM assembly in brain tissue. This assembly is important for neuron cell growth, migration and other activities. To do so, it binds to neurons through laminin−a fibrous protein found in the brain and secreted by Schwann cell [41,42]. Schwann cell forms myelin sheath, an ECM-like material that protects neurons and aids their maintenance and regeneration [43]. Clearly, the ECM assembly constitutes the physical and biological properties of the brain tissue and biomaterial scaffold used for nerve tissue repair will have to replicate such an *in vivo* condition in order to effectively promote nerve regeneration.

ECM AS A CELL PROLIFERATION AND DIFFERENTIATION FACTOR

Cell proliferation factor of ECM

Cell proliferation is essential for tissue regeneration and repair [44]. Replication of stem cells and somatic cells are regulated by the normal cell cycle, and cell proliferation happens rapidly in physiological processes like embryogenesis and wound repair. The extent of cell proliferation is highly dependent on cell-cell and cell-matrix interaction. Adherent cells that fail to attach to cell surface could undergo apoptosis. The physical interaction of the cell with its ECM modulates the cell cycle and cell death through integrin-dependent cell signaling. Integrin-mediated signaling regulates progression through the G1 and M phase of cell-cycle. For example, kidney cells on a hard matrix show extracellular signal-regulated kinases inhibition and is arrested at G1 phase. On the other hand, when placed on a soft matrix, there is an increased in actin stress fibers formation and focal adhesion kinase (FAK) autophosphorylation. This leads to up-

regulation of ERK/Cyclin D1 and promotes G1 progression. In this case, regulating the activity of transcription factors like ERK is necessary to induce gene expression for cell proliferation [45].

Cell proliferation can also be directly promoted by actin tension. Increase in FAK activity induced by ECM can lead to active Rho/Rho kinase signaling and consequently high actin cytoskeleton tension. There are also various other pathways that transduce external forces into the cells. Signaling pathways can be mediated by secondary molecules combination (e.g., FAK-ERK, Paxillin-Src) and phosphorylation on the different position of these secondary molecules by specific mechanical cues and microenvironment conditions. For example, matrix condition in liver tissue specifically enhances ERK1/2 and protein kinase B (Akt/PKB) activity for hepatocyte growth factor signaling [46]. Evidently, the ECM has specific properties to act as a cell proliferation factor in different tissue. The ECM carries important signaling cue and biomaterial scaffold should carefully consider its physical property when used for tissue replacement or cell proliferation therapy.

Cell differentiation factor of ECM

Stem cell differentiation is another important process for stem cell therapy in regeneration medicine. It is very complex and is regulated by many soluble and insoluble factors. The stem cell niche within the *in vivo* microenvironment constitutes an insoluble factor made up of the various ECM components. The interaction of the cell with the ECM plays a major role in maintaining the stable expression of specific genes [47]. Even though studies have shown that the localized released of soluble factors affect cellular functions and decisions, equally important are the signaling cues from the ECM which consist a mixture of matrix proteins, proteoglycans and GAGs. Stem cells have the potency to differentiate into many distinct cell lineages. The stem cell niche provides the tissue-specific microenvironment that controls MSC differentiation and is made up of mainly ECM proteins and their associated growth factors. MSCs are multipotent stromal cells that derive cues from the environment in deciding their activity and fate. These cues are highly specific for differentiation into a variety of cell types. By providing the correct mechanical and topographic interface that mimics the native tissue environment, stem cells can be directed to differentiate into the desired tissue.

As previously discussed, the mechanical characteristics of the ECM can influence directional differentiation of MSCs. Matrix stiffness as determined by its composition and molecular structure, and the topography at the cell-matrix interface can determine a stem cell's fate. Evidence from studies which successfully manipulated different topographical surfaces to regulate progenitor/stem cell behavior clearly highlights the significance of the ECM as a regulatory platform for tissue engineering [48,49]. Unfortunately, only limited topographical patterns have been identified at present and there is potential for topographical patterns to be even more effective in controlling stem cells for differentiation. The mechanical property of the ECM is a multifaceted factor for stem cell differentiation. Therefore, the application of an appropriate bioactive scaffold is imperative for optimizing the secretion of cytokines and growth factors so that stem cells can differentiate into the desired mature cells [50].

OUTSIDE-IN SIGNALING PATHWAY BY ECM

ECM signaling pathway membrane receptor

Mechanotransduction is the process cells use to convert mechanical stimulus into biochemical activities, and is the primary way the ECM regulates important cellular activities [51,52]. Cell surface receptors such as integrin, discoidin domain receptor (DDR) and elastin-laminin (EL) receptor mediate the transmission of external stimulus into the cells (Fig. 2) [53-55]. Integrin is one of the most common transmembrane receptor found in all eukaryotes. It is a heterodimer made of alpha and beta subunit that function as binding sites for specific ligands. The combination of alpha and beta chain variant allows the integrin protein to have different binding specificity and modulate different cellular behaviors. The activity of integrin receptor is trigged by signals initiated from the extracellular environment [56], and it can be qualitative or quantitative depending on the nature of activation. The stimuli are qualitative when a previously inactive integrin heterodimer combination becomes activated upon encountering a new ECM component. An example is in wound healing where deposition of a fibronectin matrix results in upregulation and activation of $\alpha_5\beta_1$ integrins [57]. The stimuli can also be quantitative, in which changes in the magnitude of stresses and strains produced by existing ECM interactions initiates signal transduction pathways [58].

Osteogenic differentiation of MSCs is augmented on several ECM substrates (e.g., type I collagen, fibronectin, laminin, and vitronectin [59-61]) when cells attach to these ECM molecules via distinct integrin heterodimers. Out of these, fibronectin has the greatest capacity to drive osteogenic differentiation [60] possibly both 2D and in 3D environment [62] and this qualitative response could be attributed to fibronectin's affinity for $\alpha_5\beta_1$ integrin. However, $\alpha_5\beta_1$ is not the only integrin capable of doing so as $\alpha_5\beta_3$ and other β_1-containing integrins can also induce a similar effect [60,63]. This explains why type I collagen, laminin and vitronectin also support osteogenic differentiation even though they do not bind to for $\alpha_5\beta_1$ integrin. Nevertheless, fibronectin

preferentially promotes osteogenic differentiation as reflected by the rapid and sustained activation of $\alpha_5\beta_1$. This is further verified since fibronectin fragments that specifically activate $\alpha_5\beta_1$ produced greater osteogenic responses as compared to the other fibronectin-integrin receptor $\alpha_5\beta_3$ [62]. Quantitative activation of integrin-mediated signaling can also enhance osteogenic differentiation efficiency of MSCs through the application of external mechanical forces since β_1 subunit expression is upregulated for differentiation into osteoblast [64]. For the production of vascular grafts, shear stress upregulates $\alpha_5\beta_1$ expression in MSCs [65]. Conclusively, activation of integrins usually has qualitative and quantitative components.

DDR is a non-integrin receptor and a classical receptor tyrosine kinase for collagen matrix [66]. As of now, DDR1 and DDR2 are the two types of DDR genes reported. DDR is usually activated by insoluble factors and a few types of collagen ligands. Amongst the different collagen, DDR1 is activated by collagen type I to IV while DDR2 is only activated by collagen type I and III. DDR is expressed in many organs (e.g., brain, lung, skin, heart, liver, kidney, etc.) and in some solid cancer tissues (e.g., brain, lung, ovarian, etc.) [67]. Besides affecting major cellular activities, it is also an important factor of wound healing and scar formation. During wound repair, DDR regulate collagen deposition by acting as a receptor to collagen ligand. Activation of DDR2 results in the upregulation of matrix metalloproteinase (MMP). MMP-1 is involved in matrix remodeling, a part of the wound healing process, and together with collagen they both play a critical role in wound repair [68]. Without DDR, wound healing process might be impaired. DDR2 knockout mice have shown defective ability to synthesize collagen type I and cross-link molecules for dermal wound repair. Therefore, DDR needs to be sufficiently expressed in cells. In the hepatic cells DDR2 mediates MMP-2 release and growth activation of hepatic stellate cells for proliferation and basement membrane invasion [69], potentially preventing chronic hepatic fibrosis. Furthermore, overexpression of DDR could be a solution to scarless

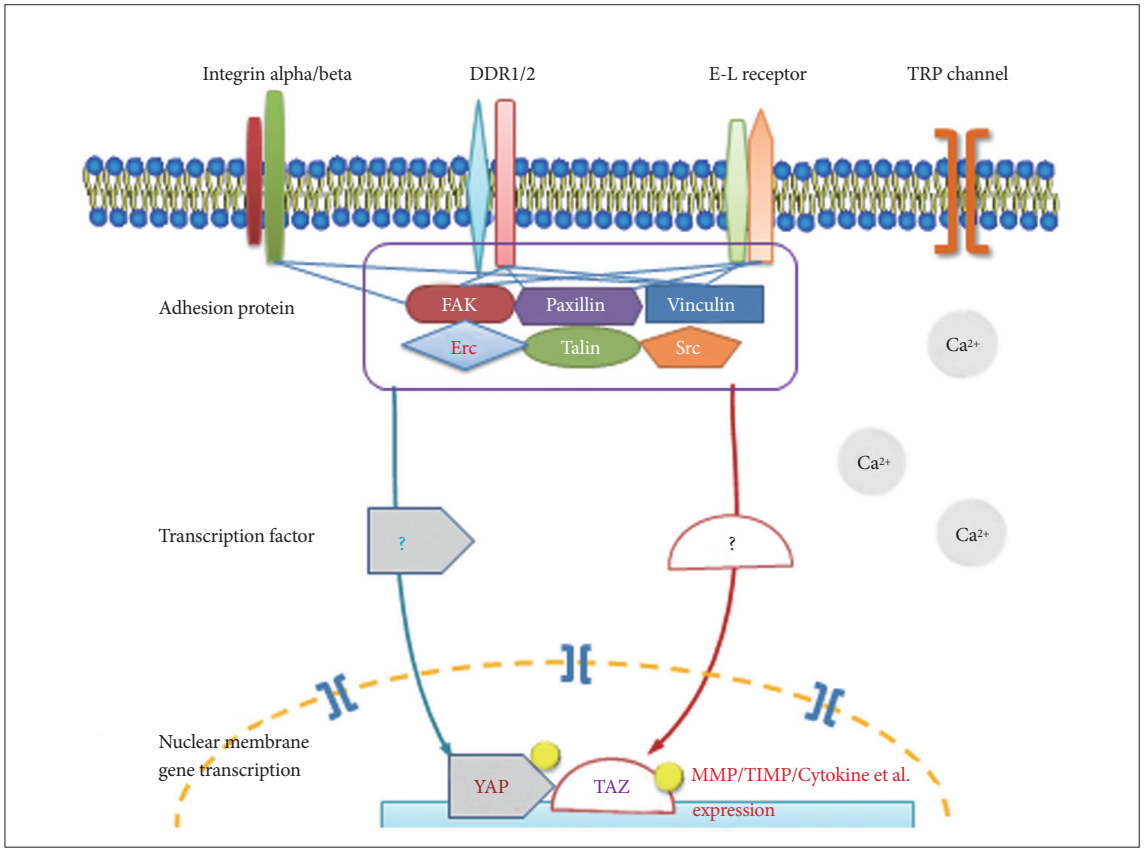

Figure 2. ECM receptor activation and its downstream intracellular signaling cascade. Extracellular mechanical signaling induces or disrupts focal adhesion and the spreading of cells on the ECM. This process is mediated by the perturbation of ECM receptors like integrin dimer receptor, DDR 1/2 and EL receptor. These receptors induce Ca^{2+} influx through transient receptor potential (TRP) channel, and the activation and clustering of intracellular molecules. Transcription factors of the downstream signaling cascade reach nuclear transcription factors like TAZ and YAP that promote specific gene expression in response to the physical status of the ECM. ECM: extracellular matrix, DDR: discoidin domain receptor, EL: elastin laminin, FAK: focal adhesion kinase, TAZ: transcriptional coactivator with PDZ-binding motif, YAP: yes-associated protein, MMP: matrix metalloproteinase, TIMP: tissue inhibitor of metalloproteinases.

wound repair. DDR expression is significantly higher in fetal tissue as compared to adult tissue [68], and is thought to provide better control of collagen production and matrix reorganization at the site of tissue repair.

The EL receptor is another non-integrin receptor that binds to laminin in the plasma membrane of tumor and smooth muscle cells. It has a binding site for elastin and laminin peptides. The EL receptor is the primary mediator of mechanotransduction from the ECM in lung cell and vascular smooth muscle [70]. It contributes to vascular tone regulation of the endothelium and the attachment and extraversion of circulating metastatic cells through it, as well as tumor neovascularization. Given these multiple functions of the EL receptor, it is likely to be pivotal in the pathophysiology of the cardiovascular system and other systems.

Intracellular regulation factor of ECM

Role of focal adhesion protein

Mechanical forces on the ECM are transmitted via integrin and non-integrin receptor. Intracellular signaling of these transmembrane receptors is modulated by the assembly of focal adhesion, which serves as a biochemical signaling hub that binds to the cytoskeleton via focal adhesion proteins (e.g., FAK, paxillin, vinculin, talin) [5,9,71]. Focal adhesion proteins consist of key signaling proteins like protein kinase, G-protein and adaptor proteins that support the main protein in signal transduction (Fig. 2).

FAK plays a prominent role in early integrin-mediated signaling [72]. As previously discussed, ECM cue can lead to high levels of β_1 integrin expression. As a result, sites of integrin clustering are formed at the cell-matrix adhesion site that experienced high shear stress. These focal adhesions at sites of high integrin clustering and activation have a downstream effect, with FAK being essential for initiating ECM signal pathways [73]. FAK has two main domains: Focal Adhesion Targeting on the C-terminal and FERM on the N-terminal. Each domain has its specific mode of activation and its own protein composition and phosphorylation mechanism. This is best understood in the context of FAK engagement with integrins near the cell surface. Activation of FAK results in recruitment of each domain, which mediates signaling to several downstream pathways [74]. FAK-dependent activation of these pathways is involved in a diverse array of cellular processes, including cell migration, growth factor signaling, and cell cycle progression for survival. In bone environment, FAK is essential in initiating signaling pathway that results in osteogenic differentiation in response to fluid flow shear stress [75] and ECM-induced signals [61]. It is also a key protein that participates in the intracellular signaling pathway that leads to neuronal differentiation of MSCs within 3D collagen matrix [76]. Collectively, these observations highlight the importance of mechanical forces in the activation of integrin/FAK-mediated mechanotransduction [77].

Focal adhesion is a central hub that coordinates the interaction between integrin, the cytoskeleton and the incoming biochemical signal. Focal adhesion dynamics differ depending on the molecular composition, on whether the surrounding microenvironment is 2D or 3D, and if there is the presence of any external force originating in the ECM [18]. Under the physical and biochemical influence of the surrounding matrices, FAK is phosphorylated in response to integrin engagement, and activates a cascade of complex intracellular signaling that controls cellular behavior. ERK is a downstream protein kinase from FAK and is activated by mechanical force via the RhoA pathway. In general, phosphorylation of ERK induces actin-cytoskeleton reorientation and downstream regulation of transcriptional control in the nucleus. This eventually causes tissue remodeling in several tissue types. In the periodontal ligament, the ERK pathway controls cell proliferation and differentiation [78]. The physiology of osteocyte is highly regulated by mechanical force from the ECM, especially when it activates the ERK activation pathway that prevents osteocyte apoptosis [79]. Hence, the physiological health of the skeletal bone is highly dependent on the physical state of the ECM.

Paxillin is a multi-domain 68 kDa adapter protein that is tyrosine-phosphorylated by FAK and Src [80] upon binding with integrin. Co-activity of FAK and paxillin serve an important function for cell migration and tissue remodeling in the ECM of embryonic tissue. Not surprisingly, paxillin has a lot of binding domain for integrin and focal adhesion molecules like FAK and vinculin. The major role of paxillin is in cell migration, polarity, and survival. Paxillin induces Cdc42 activity and controls cell polarity during wound healing. Furthermore, paxillin can act as a physical linkage between the ECM and the actin-cytoskeleton via integrin. This connection is critical for the transduction of force generated during contraction of skeletal, smooth and cardiac muscles. In addition, paxillin is involved axon regeneration in neurons. The growth cone of regrown neuron cells contains highly dynamic focal adhesion assembles like paxillin for neurite outgrowth and nerve regeneration [81].

Vinculin is a 1066 amino acids cytoskeletal protein that has a molecular weight of 117 kDa. Its protein structure can be divided into the head and tail domain, connected by a flexible hinge region. Vinculin modulates paxillin interaction with FAK from physical cue [82]. Vinculin also regulates cell migration speed subjected to the ECM condition. Vinculin connects integrin with the actin cytoskeleton and is critical for controlling cytoskeleton mechanics from external and internal forces. In regenerative medicine, vinculin is one important factor for skeletal

muscle regeneration. Cells respond to injury of myofibers by synthesizing more vinculin to aid myotube regeneration. Vinculin expression is induced in liver regeneration [83], suggesting its physiological significance in aiding tissue regeneration [84].

Talin is a high molecular weight cytoskeletal protein at the region of cell-substratum and cell-cell contact [85]. It has a large C-terminal rod domain containing alpha helices and an N-terminal FERM domain containing three subdomains: F1, F2, and F3. F3 domain has a high affinity for integrin β3 cytoplasmic tail. Talin also shows the strong affinity with vinculin at focal adhesion point. As such, talin is essential for integrin activation and the formation of focal adhesions. In migrating cells, proteolysis of talin by calpain regulates cell adhesion turnover [86]. Calpain cleavage of its respective head and rod domain also promote higher talin binding affinity to the β integrin cytoplasmic tails as compared to intact talin [87]. Lastly, talin appears to be a key endpoint for multiple signaling pathways of mechanotransduction that leads to integrin activation [88].

Role of calcium signaling

Activity of mechanically-gated ion channels can convert mechanical cues into biochemical signals. Calcium ions are important in intracellular signaling as mechanical stimulation of ECM can activate Ca^{2+} ion channels and Ca^{2+}-dependent signaling pathways [7]. Application of shear stress at the cell membrane induce momentary opening of tension-induced calcium channels or transient receptor potential ion channel, resulting in an influx of Ca^{2+} ions. The influx of Ca^{2+} can promote ERK-mediated osteogenic differentiation [89] and also enhance proliferation of MSCs [90]. However, there is a likely threshold factor at which osteogenic differentiation pathways are induced at the expense of proliferation. During shear stress-induced osteogenic differentiation, Ca^{2+} signaling leads to downstream activation of AP-1 transcription factors which then upregulate important osteogenic genes including COL I, RUNX2 and OCN [91-93]. This eventually cause mineralized matrix formation. ERK activation in the context of Ca^{2+} signaling is mediated by calmodulin-dependent protein kinase and protein kinase C. MSCs exposed to shear stress have upregulated Ca^{2+} signaling. This augments nitric oxide production due to the activation of nitric oxide synthase, as also observed during osteogenic differentiation. Subsequently, nitric oxide enhances phosphorylation of ERK proteins that will then participate in osteogenic mechanotransduction.

Role of cytoskeleton modifications

When cells receive physical stimulus, mechanical tension from the ECM is transferred via cell surface receptors to the actin cytoskeleton where structural changes occur. Rho-associated protein kinase (ROCK) is an effector protein downstream of Rho signaling that regulates actin organization and has a key role in cytoskeleton modification. Shear stress from the ECM is known to promote structural changes in MSCs [94,95] and induce osteogenic differentiation [96] via Rho signaling. As a result, osteogenic differentiation is preferentially promoted over chondrogenic and adipogenic induction [97]. This observation was made in studies where oscillatory fluid flow was applied to MSCs over-expressing constituents of the RhoA signaling pathway. In wild-type MSCs, the externally applied stimulus enhanced expression of tri-lineage differentiation markers (Runx2, Sox9, and PPARγ) without any preferential enhancement of a particular pathway. However, when Rho signaling via ROCKII was augmented due to overexpression, osteogenic differentiation was enhanced. Rho-mediated contraction of the actin cytoskeleton appears to be critical for MSC differentiation into osteoblasts by further enhancing RUNX2 expression [97,98]. Physical signals by mechanical forces on the ECM can lead to a change in cytoskeleton tension and regulation of the intracellular cytoskeleton controls cell migration, cell polarity and cell morphology. These cellular processes affect cell shape and are known to influence morphogenesis during embryo development.

Role of nuclear relay proteins

External force triggers outside-in signaling and activates nuclear transcription factors that leads to specific gene expression. Nevertheless, the concept of nuclear relay proteins and its interaction with nuclear transcription factors in mechanotransduction is relatively new. One of the earliest study relating physical cue to transcription of genes was only published in 2011, and identified yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) as the bridge linking the start and end point of ECM signaling. These nuclear relay proteins regulate gene expression and protein synthesis for cell proliferation, differentiation and cancer malignant progression [99] by relaying mechanical signals exerted by ECM rigidity and cell shape. In addition, they help to maintain cell and tissue homeostasis by working together with other growth factor signaling. Intervention at the nuclear signaling level can change how cells typically respond to a particular type of substrate. However, the detailed biochemical mechanism of how rigidity mechanosensing lead to regulation of YAP/TAZ remains unclear and requires further investigation (Fig. 2).

INSIDE-OUT SIGNALING PATHWAYS ON ECM

Role of MMP and TIMP

ECM remodeling can be the result of cellular responses to

physical changes in the microenvironment. Cells can respond to outside-in biochemical and biophysical signals with an inside-out signaling pathway that leads to matrix degradation, synthesis and deposition of new ECM materials. This feedback to their physical environment is mediated through MMP, TIMP, polymer protein and growth factors [100]. There are 25 types of MMPs found in mammalian cells and while each MMP performs a different function, some of them have overlapping substrate specificities and can therefore cleave multiple similar substrates. Under the normal physiological condition, cells remain in a homeostatic condition with their ECM. When tissues are damaged, there is a disorganization of the ECM and scar formation is a common outcome of the wound healing process.

So far, TIMP are the most studied MMP inhibitors that are known to modulate MMP activities. There are four TIMP molecules characterized so far: TIMP-1 to TIMP-4. After the wound healing process, cells continue to secrete proteinase inhibitors like TIMP to maintain tissue homeostasis. TIMP also regulates cellular events like apoptosis and mediates cell survival signaling through FAK interaction with ECM. However, TIMP is frequently associated with MMP since the latter has an active role in proteolytic activity and matrix degradation while the former stops their activation and activity. Together, these molecules have an important role to play in tissue repair and remodeling [18]. For instance, there is a significant increase in MMP and TIMP activities during regeneration of hepatic tissues [101]. Their activity constitutes the inside-out signaling response on the ECM and regulates most cell and tissue physiology.

Inside-out signaling on ECM remodeling

Cellular responses via inside-out signaling is mediated by several mechanistic pathways and leads to various physiological consequences. For instance, secretion of MMP14 is facilitated by the ECM and is involved in skeletal muscle regeneration [102]. The ECM also activates focal adhesion-associated protein kinase (e.g., FAK, Src-family kinase, ERK) that are closely linked to MMP activities. MMP not only induce cell migration for recovery after injury, but also tumorigenesis and cancer metastasis. MMP controls cell migration by FAK-Src signaling and proteolytic remodeling of the ECM [103]. This constitutes an important guidance cue for cell migration through the ECM. *In vivo*, secretion of MMPs supports the 3D cell cluster formation of the tumor. In regenerative organs like the bone and liver, abundant secretion of various MMP were recorded in order for scarless repair, in contrast to other organs where tissue repair leads to scar formation. Remarkably, that they were also found to play an active role in embryo development of amphibians and mammals. As can be seen, MMP also has major roles in morphogenesis and cell migration on top of tissue maintenance by ECM remodeling.

An understanding of the inside-out signaling has strong relevance on the use of biomimetic scaffold because host cells interact with the biomaterial scaffold to change its microenvironment. Functionalization of the scaffold with MMP and TIMP can enhance its effect for regenerative medicine, and deliver a more biomimetic scaffold. Natural polymers (e.g., collagen) found in the extracellular environment with binding sites on MMP and TIMP depend on the activities of MMP and TIMP for modulation [104]. Scaffolds functionalized with coating of these ECM peptides and proteins have greater biological relevance as compared to non-functionalized synthetic scaffold. In the former example, MMP and TIMP can remodel the ECM constituted by such proteins and guide the formation of an ideal tissue structure similar to those found *in vivo*. Artificial mini-protein has been developed to target the catalytic sites of MMPs

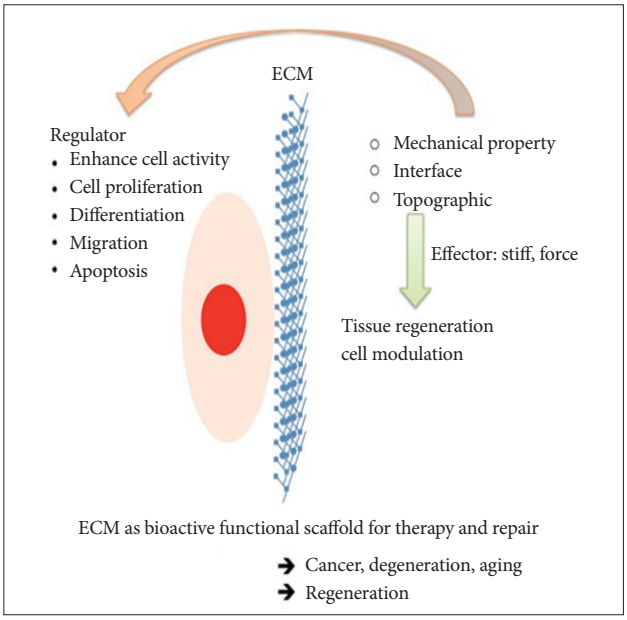

Figure 3. Summary of ECM modulation of bioactivity. Mechanical properties and the interfacial and topographic properties of the ECM are key signaling factors of cellular behaviors like proliferation, differentiation, migration and apoptosis. Effectors like matrix stiffness and mechanical forces applied through these factors affect cell modulation and tissue regeneration. A well-characterized ECM can serve as a design for a highly bioactive and functional scaffold used for regenerative medicine and applications relating to cancer, tissue degeneration and aging therapy in either a cell-seeded or cell-free scaffold. One approach is the use of additive or subtractive methods to control ECM components on extracted ECM. Furthermore, an elucidated understanding of the positive and negative ECM signaling *in vivo* will aid our ability to maintain and optimize the bioactivity of MSCs on a biomimetic scaffold. Based on these investigation, we are able to build a bioactive functional scaffold and this technology will become a powerful tool for enhancing stem cell activity in cell therapy and tissue engineering for regenerative medicine. ECM: extracellular matrix, MSCs: mesenchymal stem cells.

as functional motif-matrix. This technique extends the application of topographical effect to guide cell migration, proliferation and differentiation. It is therefore important to acknowledge not only that the ECM delivers inside-out signaling, for but also how this leads to ECM remodeling and other important cellular behaviors and processes.

CONCLUSION

The ECM is a complex and dynamic microenvironment consisting of many extracellular molecules secreted by the cells. It was traditionally understood to provide structural support for whole tissue, but the capabilities of the ECM are now known to be beyond this. As the starting point of mechanotransduction, we know that it regulates almost all of the important cellular behaviors like cell activity, proliferation, migration and apoptosis. At the tissue level, it has an effect on tissue regeneration and other tissue-related observations like carcinogenesis, degeneration and aging. In this review, we summarized the roles of the key players in the outside-in and inside-out signaling of ECM. A clear understanding of ECM signaling and regulation has many applications in tissue engineering, especially in optimizing a cell-free biomimetic scaffold for tissue therapy and regeneration (Fig. 3). A cell-free scaffold is preferentially preferred because the scaffold environment is more well-defined since it does not incorporate other cell types. It is more reliable, has a wider range of application and delivers a better outcome for tissue regeneration. On the other hand, a cell-seeded scaffold itself presents many challenges and ethical issues. Not only so, it is more cumbersome to prepare since cell preparation and optimization on the scaffold can take up significantly more time. Hence, many biomaterials are based on the concept of ECM-driven signaling in its scaffold design.

However, because of the complexity and the vast number of players in cellular regulation by the ECM, there are many tissue-specific pathways that remain unclear. This necessitates a better understanding of the ECM from the biological science perspective. Advancement in this area will ultimately benefit tissue engineering and regenerative medicine since a biomimetic scaffold is arguably superior to conventional scaffolds that do not consider the biochemical properties. The continuing discovery and understanding of the *in vivo* ECM will improve the quality and effectiveness of biomaterials for tissue engineering. However, a lot more needs to be done before we can truly replicate the extracellular microenvironment on an *in vitro* scaffold.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study has no any ethical issues and state for publication.

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