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Chapter 26

Stem Cells in Tissue Engineering

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1. Introduction

With the increasing number of patients suffering from damaged or diseased organs and the shortage of organ donors, the need for methods to construct human tissues outside the body has arisen. Tissue engineering is a newly emerging biomedical technology and methodology which combines the disciplines of both the materials and life sciences to replace a diseased or damaged tissue or organ with a living, functional engineered substitute [1, 2]. The so-called triad in tissue engineering encompasses three basic components called scaffold, cell and signaling biomolecule.

Whatever the approach being used in tissue engineering, the critical issues to optimize any tissue engineering strategy toward producing a functional equivalent tissue are the source of the cells and substrate biomaterial to deliver the cells in particular anatomical sites where a regenerative process is required. Due to their unique properties, stem cells and polymeric biomaterials are key design options. Briefly, stem cells have the ability to self-renew and commit to specific cell lineages in response to appropriate stimuli, providing excellent regenerative potential that will most likely lead to functionality of the engineered tissue. Polymeric materials are biocompatible, degradable, and flexible in processing and property design. A major focus of tissue engineering, therefore, is to utilize functional polymers with appropriate characteristics, as a means of controlling stem cell function. Based on their differentiation potential, stem cells used for tissue engineering can be divided into two categories: pluripotent stem cells and multipotent stem cells. Pluripotent stem cells include embryonic stem cells (ESCs) as well as induced pluripotent stem cells (iPSCs). Because ESCs are isolated from the inner cell mass of the blastocyst during embryological development, their use in tissue engineering is controversial and more limited while more attention has been paid to adult stem cells, which are multipotent and have a larger capacity to differenti-

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ate into a limited number of cell types [3]. Adult stem cells can be found in many adult tissue types including bone marrow, peripheral blood, adipose tissues, nervous tissues, muscles, dermis, etc. For instance, mesenchymal stem cells (MSCs) which reside in the bone marrow can differentiate into bone (osteoblasts) [4], muscle (myoblasts) [5], fat (adipocytes) [6] and cartilage (chrondocytes) [3] cells, while neural stem cells (NSCs) either give rise to support cells in the nervous system of vertebrates (astrocytes and oligodendrocytes) or neurons [7]. *In vivo*, differentiation and self-renewal of stem cells are dominated by signals from their surrounding microenvironment [8]. This microenvironment or "niche" is composed of other cell types as well as numerous chemical, mechanical and topographical cues at micro- and nano-scales, which are believed to serve as signaling mechanisms to determine cell-specific recruitment, migration, proliferation, differentiation as well as the production of numerous proteins required for hierarchical tissue organization [9].

In vivo, the cells are surrounded by a biological matrix comprising of tissue-specific combinations of insoluble proteins (e.g. collagens, laminins, and fibronectins), glycosaminoglycans (e.g. hyaluronan) and inorganic hydroxyapatite crystals (in bone) that are collectively referred to as the extracellular matrix (ECM). The varied composition of the ECM components not only contains a reservoir of cell-signaling motifs (ligands) and growth factors that guide cellular anchorage and behavior, but also provides physical architecture and mechanical strength to the tissue. The spatial distribution and concentration of ECM ligands, together with the tissue-specific topography and mechanical properties (in addition to signals from adjacent cells—juxtacrine signalling—and the surrounding fluid), provide signaling gradients that direct cell migration and cellular production of ECM constituents. In this dynamic environment, the bidirectional flow of information between the ECM and the cells mediates gene expression, ECM remodeling and ultimately tissue/organ function.

Native ECM exhibits macro- to nano-scale patterns of chemistry and topography [10]. Tissue stiffness is also known to vary depending on the organ type, disease state and aging process [11-13]. In tissue culture, stem cell differentiation has traditionally been controlled by the addition of soluble factors to the growth media [14]. However, most stem cell differentiation protocols yield heterogeneous cell types [15, 16]. Moreover, cells encounter very different, unfamiliar surfaces and environments when cultured *in vitro* or when materials are implanted into the body. Therefore, it is desirable to use more biomimetic *in vitro* culture conditions to regulate stem cell fate so as to advance clinical translation of stem cells through better expansion techniques and scaffolding for the regeneration of many tissues. Recent advances have facilitated further the creation of substrates with precise micro- and nano-cues, variable stiffness and chemical composition to better mimic the *in vivo* microenvironment [2, 17, and 18]. By employing various novel approaches, tissue engineers aim to incorporate topographical, mechanical and chemical cues into biomaterials to control stem cell fate decisions [2, 18, and 19].

This chapter will present various biomaterial designing considerations and strategies for stem cell-based tissue engineering for development as carriers for stem cells facilitating the *in vivo* use of stem cells in tissue engineering. This part first presents some biomimetic approaches to designing novel polymeric biomaterials with appropriate physical,

chemical, mechanical, and biological cues mimicking the natural stem cell niche in order to direct the desired stem cell behavior to facilitate the regeneration of desired tissues with particular emphasis on using adult stem cells including MSCs and NSCs. The next part will introduce some new trends emerging in the field of tissue engineering in terms of both cellular biology and biomaterial point of view in order to improve the overall efficiency of tissue regeneration for effectively controlling the cell fate and translating the stem cell research into much needed clinical applications in a not-too-distant future. The topics discussed in the latter part include 2D polysaccharide-based hydrogel scaffolds designed in the authors' studies for muscle tissue engineering applications. Hydrogel scaffolds made of natural polymers with proper handling for surgery and mechanical properties similar to muscle tissue, which could promote the desired muscle-derived stem cell behavior on the surface were developed in this study.

2. Biomimetic microenvironment design strategies

Damaged tissues often lose deeper layers which contain stem cell niches. In such cases, biomaterials could be useful tools for reestablishing the niches' functionality [20]. Artificial niches would need to incorporate appropriate 'homing' signals able to either localize endogenous stem cells or direct the desired incorporated exogenous stem cell behavior by means of developing various microenvironment design parameters including the dynamic control of soluble and surface-bound cytokines, ECM, cell-cell interactions, mechanical forces and physicochemical cues [21, 22].

The use of biomaterials as scaffolds is a fundamental component of tissue engineering since these materials serve as templates for tissue formation and are engineered depending on the tissue of interest. These scaffolds provide structural and mechanical support for the cells as well as present cues inducing tissue repair. The structure, morphology, degradation and presentation of bioactive sites are all important parameters in material design for these applications and may signal the differentiation of stem cells. Beside all the parameters related to the biomaterials scaffold, there are some other factors such as chemical cues (e.g. soluble reagents in terms of both concentration and their gradient, medium pH), mechanical cues (e.g. fluid shear stress) and other types of cues (electric and magnetic field) which are believed to have significant effect on stem cell behavior. These factors are reviewed extensively elsewhere [23, 24].

Figure 1 summarizes the biomimetic microenvironment design strategies for controlling stem cell behavior including chemical/biochemical (e.g. growth/differentiation factor presentation, density and gradient), structural, mechanical and some other types of cues.

Engineering these design parameters will effectively yield materials that create an architecture resembling the native environment for stem cells, and have controlled mechanical properties enabling adhesion and thus enhancing contractility in the cellular cytoskeleton, and present ligands directing intracellular signaling and gene expression. This section provides

an overview of biomimetic microenvironment design strategies to direct the stem cell behavior for tissue engineering applications.

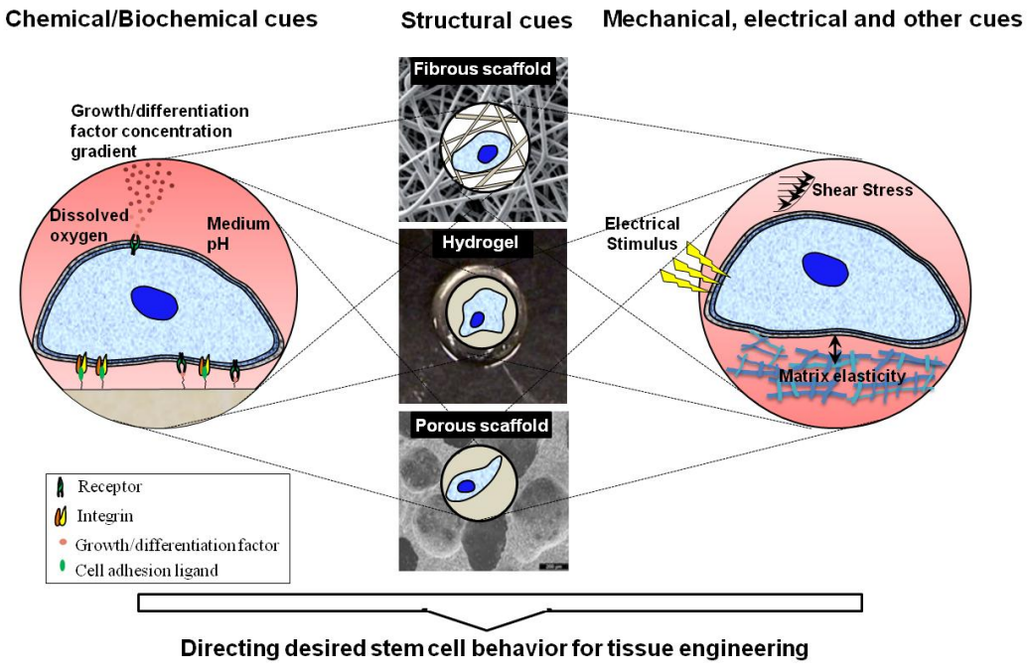


Figure 1. The biomimetic microenvironment design strategies for controlling stem cell fate

2.1. Chemical and biochemical cues

Biochemical cues are generally provided by soluble ligands, which may be either secreted by paracrinal cells or supplied by a capillary network in the human body. Insoluble ligands, which are adhesion proteins or molecules such as collagen, laminin and carbohydrates, are also present. Biochemical factors typically influence the cell microenvironment in a concentration or gradient-dependent manner.

Chemical and biochemical means are the first choice for stem cell differentiation. Small ions, growth factors, and cytokines can exert potent, long-range effects over stem cell microenvironments. Owing to their relative ease of study, *soluble biochemical cues* and their downstream signal transduction pathways are the best characterized determinants of stem cell fate and have been extensively used in *ex vivo* stem cell culture systems, as extensively discussed elsewhere [24-26]. Therefore, the following section will mainly focus upon the application of other types of *soluble* signals such as dissolved oxygen as well as insoluble chemical and biochemical cues (e.g., immobilized growth factor, extracellular matrix material, etc) to engineered niches.

In vivo, numerous growth factors and morphogens are immobilized by binding to the ECM through specific heparin-binding domains or by direct binding to ECM molecules such as collagen, or direct anchoring to cell membranes [27]. Immobilization of growth factors in

this manner can serve to increase local concentration of the protein by hindering diffusion and receptor-mediated endocytosis. For example, the morphogen Sonic hedgehog (Shh) is modified at its termini by lipids that link it to the cell membrane and thereby limit its mobility. Removing the lipids dilutes the factor to a lower concentration and thereby shrinks its effectiveness [28, 29]. Accordingly, mimicking the natural immobilization of cytokines is one approach utilized by engineers to concentrate factors in proximity to the cell surface in a manner that activates target signaling pathways effectively, and reduces, as well, the levels of growth factor necessary to elicit a potent cellular response.

An early study exploring this design concept focused on epidermal growth factor (EGF) [30] which is beneficial in repairing the damaged tissues, but is often difficult to deliver at sufficiently high concentrations to mediate downstream signaling events as it does not contain a matrix-binding domain and rapidly undergoes receptor-mediated endocytosis [31]. In a recent example involving human and porcine MSCs, amine-targeting chemistry was used to tether EGF to the surface of poly (methyl methacrylate)-graft-poly (ethylene oxide) comb polymers [32]. The tethered EGF led to sustained EGF receptor signaling and subsequent cellular responses including cell spreading and protection from apoptosis, whereas saturating levels of soluble EGF did not. Sakiyama-Elbert et al. incorporated heparin into biomaterial scaffolds to allow for immobilization of basic fibroblast growth factor (bFGF) [33]. bFGF was released either passively by diffusion, or actively via heparinases secreted by neighboring cells, thereby allowing for a controlled release and presentation of signal which was not possible with soluble growth factor delivery. The same delivery system has been used for differentiation of murine ESCs into mature neural cell types, including neurons and oligodendrocytes, indicating that biomaterials scaffolds functionalized with immobilized growth factors may be a potential strategy for generation of engineered tissue for treatment of spinal cord injury [34]. Finally, in a recent study, polymer substrates functionalized with the signaling domain of Shh supported enhanced osteogeneic differentiation of bone marrow-derived MSCs, as compared to cells cultured on the same surfaces with soluble Shh at the same concentration [35]. This example further demonstrates how growth factor or morphogen immobilization serves as an effective means to achieve sustained activation of downstream signaling pathways due in part to the finding that the local concentration in the scaffold was greater for immobilized growth factor than for soluble form.

There is a significant scope in the application of surface modifications, despite the use of protein biomolecules to provide more cues for cell adhesion, proliferation and differentiation. Arg-Gly-Asp (RGD) sequence and several natural proteins like collagen, laminin and fibronectin were shown to be essential for cell attachment to polymeric material surfaces devoid of any cell recognition sites [36, 37]. The immobilization of these proteins to polymers not only promotes cell adhesion and proliferation but also increases hydrophilicity of the polymers such as aliphatic polyesters. One such surface functionalization for biopolymer substrate surfaces is attachment of RGD peptides that is the most effective and often employed peptide sequence for stimulating cell adhesion on synthetic polymer surfaces. This peptide sequence can interact with integrin receptors at the focal adhesion points. Once the RGD sequence is recognized by the integrins, it will initiate an integrin-mediated cell attach-

ment pathway and activate signal transduction between the cell and ECM, thus influencing various cell behaviors on the substrate including proliferation, differentiation, survival and migration [38]. Roeker et al. showed that the composite materials modified by immobilizing poly-L-lysine and BMP-2 as bioactive ligands on the ceramic surface had promising potential to enhance the adhesion of hMSCs and directing cell differentiation into osteoblasts [39]. In another study, it was demonstrated that hMSCs encapsulated in poly (ethylene glycol) (PEG)/ RGD hydrogels undergo chondrogenic differentiation in the presence of TGF-β3. More importantly, this effect has been found to be RGD-dose dependent and there is an optimal concentration of RGD present in PEG hydrogels, which improves cell viability and promotes chondrogenesis [40].

In spite of the addition of differentiation factors in the culture media, the matrix materials which support the cells affect the differentiation of stem cells as well. Mauney et al. found that the matrix-denatured collagen type I is more capable in retaining the osteogenic differentiation potential *in vitro* and even bone-forming capacity *in vivo* of hMSCs than the conventional tissue culture plastic [41]. Mwale et al. discerned that bi-axially oriented polypropylene plasma treated in ammonia reduced upregulation of the expression of osteogenic marker genes, such as alkaline phosphatase (ALP), bone sialoprotein and osteocalcin significantly [42]. According to a report presented by Ager et al. [43], collagen I/III and PLLA porous scaffolds showed certain osteoinductive properties without Dex, ascorbic acid, and βGP (DAG) stimulation, verified by immunocytochemical staining against osteoblast-typical markers and completed by calcified matrix detection. Wang et al. demonstrated that ascorbic acid-functionalized poly (methyl methacrylate) can modulate the proliferation and osteogenic differentiation of early and late-passage bone marrow-derived hMSCs [44].

More recently, Xu et al. showed that hMSCs attached, and subsequently proliferated and differentiated toward the osteogenic lineage on the biomimetic bioglass-collagen-hyaluronic acid-phosphatidylserine (BG-COL-HYA-PS) composites to a significantly higher degree compared to those cells on the BG-COL, BG-COL-HYA composites, suggesting the BG-COL-HYA-PS composite porous scaffolds have high potential for bone tissue engineering [45]. In another study, it was shown that the incorporation of gelatin in the poly [(L-lactide)-co-(e-caprolactone)] (PLCL) nano-fibers stimulated the adhesion and osteogenic differentiation of hMSCs, suggesting that the chemical composition of the underlying scaffolds play a key role in regulating the osteogenic differentiation of hMSCs [46].

Regarding chondrogenic differentiation, investigating the effect of cartilage-tissue chondroitin-sulfate (CS) in a fibrin scaffold on the differentiation of adipose-derived adult stem cells into chondrocytes revealed the significant effect of CS on the differentiation efficiency. It can be concluded that the fibrin–CS matrices mimicking native cartilage extracellular matrix could act as a three-dimensional scaffold for cartilage tissue engineering and have the potential for promoting the differentiation of adipose-derived adult stem cells into chondrocytes [47].

Since the chemical properties of substrates (e.g., hydrophobicity) play an important role in the kinetics of protein adsorption and folding, which in turn influence cellular activities, direct the stem cells' fate can be controlled by chemical modification of the sub-

strate. Surface modification techniques such as plasma treatment, ion sputtering, oxidation and corona discharge affect the chemical and physical properties of the polymer surface without significantly changing the bulk material properties. For example, plasma processes makes it possible to change the chemical composition and properties of the polymer system such as hydrophobicity, surface energy, refractive index, hardness, chemical inertness and biocompatibility [48]. Plasma techniques can easily be used to induce the desired groups or chains onto the surface of a polymer [49, 50]. Appropriate selection of the plasma source facilitates the introduction of diverse functional groups on the polymer surface to improve biocompatibility or to allow subsequent covalent immobilization of various bioactive cues. For instance, plasma treatments with oxygen, ammonia, or air can generate carboxyl groups or amine groups on the polymer surface [51, 52]. A variety of ECM protein components such as gelatin, collagen, laminin, and fibronectin could be immobilized onto the plasma-treated surface to enhance cellular functions [53]. Curran et al. show that stem cell differentiation is guided by surface chemistry and energy, independent of inductive media [54]. Although all the surfaces tested maintained cell viability, silanized hydrophobic surfaces with CH_3 end groups (with low surface energy) maintain MSC phenotype, while increasing the surface energy by adding NH_2- or SH- terminal groups promotes osteogenesis. Further increase of surface energy by addition of OH or COOH moieties promotes chondrogenesis. However, there are reports indicating that both hydrophobicity and surface energy play a role in cell adhesion, but only in the short term until cells themselves modulate their extracellular environment [55, 56].

Probably one of the best known soluble reagents is dissolved oxygen. Typical oxygen concentrations *in vivo* vary from 12.5 to 5%, whilst the oxygen concentration in cell culture incubators is the same as that in the air, which is 20%. Several reports show that lowered oxygen concentrations (5%) increase stem cell proliferation [57-59]. Grayson et al. [60] have shown that even lower oxygen concentrations of about 2% increase MSC proliferation whilst maintaining an undifferentiated state, thus suggesting that hypoxic conditions are the characteristic of the niche environment. Some authors have observed an induction of adipose-like phenotype in MSCs in severe hypoxia (1%) [61], whilst others showed that adipogenesis is suppressed at 6% oxygen compared to 20% oxygen [62]. Lennon et al. reported that rat MSCs exposed to 5% oxygen during amplification show enhanced osteogenesis after implantation, compared with cells amplified in 20% which may probably be due to increased proliferation as suggested above [63]. Buckley et al. showed the beneficial response of chondrocyte cells to a low oxygen environment in the absence of TGF-β, suggesting that hypoxia can be used as an alternative to growth factor stimulation to engineer cartilage from culture-expanded chondrocyte [64].

2.2. Structural cues

Biomaterial scaffolds take on a variety of structures based on their material composition and processing for forming 3D environments. These materials consist of natural polymers such as collagen, hyaluronic acid, fibrin, alginate, or synthetic polymers such as polyethylene gly-

col (PEG), dextran, or polyvinyl alcohol and can be formed into hydrogels, fibrous structures, and microporous scaffolds [65,66]. Figure 1 illustrates examples of the structure of each of these scaffold types. The biomaterial structure controls how a cell interacts with the material and is important in stem cell fate decisions as the presentation of cues and cellular morphology are dependent on this structure.

Hydrogels are comprised of insoluble networks of cross-linked polymers with high water contents [67]. Hydrogels with the ability to encapsulate stem cells have been used for applications such as cartilage [68, 69] and cardiac [70, 71] tissue regeneration. In order to achieve tissue formation, stem cells must either be encapsulated within or recruited to the hydrogel. Some recently reported applications of hydrogel in tissue engineering are presented the following part.

Hydrogels such as those derived from alginate, collagen and hyaluronic acid have been found to be quite promising – they provide a homogeneous, structureless soft 3D environment which is probably ideal for stem cell proliferation and maintenance, as well as for differentiation into softer tissues such as neural or hepatic [72, 73]. Pranga et al. showed the promotion of oriented axonal regrowth in the injured spinal cord by alginate-based anisotropic capillary hydrogels [74]. In a recent study, Nguyen et al. demonstrated that a three-layer polyethylene glycol-based hydrogel creates native-like articular cartilage with spatially-varying mechanical and biochemical properties that can direct a single MSC population to differentiate into the superficial, transitional, or deep zones of articular cartilage. They concluded that spatially-varying biomaterial compositions within single 3D scaffolds can stimulate efficient regeneration of multi-layered complex tissues from a single stem cell population. The ability to generate such zone-specific tissue could eventually allow tissue-engineering of more native-like articular cartilage substitutes with spatially varying ECM composition and mechanical properties [75, 76]. Moreover, injectable hydrogels have been extensively explored as cell delivery systems with the advantage that cells and biomolecules can be readily integrated into the gelling matrix [77, 78]. The injectable nature of the hydrogels provides the attractive feature of facile and homogenous cell distribution within any defect size or shape prior to gelation. In addition, injectable hydrogels allow good physical integration into the defect and facilitating the use of minimally invasive approaches for material delivery [79, 80]. Tan et al. demonstrated the usefulness of the aminated hyaluronic acid-g-poly (N isopropylacrylamide) copolymer as an injectable hydrogel for adipose tissue engineering [81]. Recently, Tan et al. demonstrated that the thermo-sensitive alginate-based injectable hydrogel has attractive properties that make it suitable as cell or pharmaceutical delivery vehicles for a variety of tissue engineering applications [82].

Although hydrogels provide a highly controlled 3D microenvironment for cells, the nature of this scaffold does not entirely mimic the structure of native ECM. Generally the cells encounter and respond to basement membrane topography in the *in vivo* environment mainly composed of networks of pores, ridges, and fibers made by ECM molecules such as collagen, fibronectin and laminin at length scales ranging from nano- to micro-scale [83]. It is therefore important to incorporate features at such length scales into the development of biomaterials suitable for stem cell therapies.

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One of the most widely used biomaterial structures for tissue engineering involves microporous scaffolds, which can form interconnected porous networks that allow for cellular infiltration and tissue formation. These scaffolds are often formed with leachable components around which the desired polymer forms a scaffold [84]. Upon removal of the leachable components, a 3D structure can be obtained with varying parameters such as pore size, porosity, and interconnectivity. Aronin et al. created poly-(*e*-caprolactone) scaffolds with varied pore sizes and interconnectivity to monitor osteogenesis of dura mater stem cells [85]. High porosity and adequate pore-size are key requisites to increase the surface area available for cell attachment and tissue in-growth in order to facilitate the uniform distribution of cells and the adequate transport of nutrients. Murphy et al. has investigated the effect of mean pore size on cell behavior in collagen–glycosaminoglycan scaffolds for bone tissue engineering application [86]. The results show that cell number was highest in scaffolds with the largest pore size of 325 μm. While the increased surface area provided by scaffolds with small pores may have a beneficial effect on initial cell adhesion but ultimately the improved cellular infiltration provided by scaffolds with larger pores outweighs this effect and suggests these scaffolds might be optimal for bone tissue repair. Kasten et al. also showed that porosity, distribution and size of the pores of beta-tricalcium phosphate ceramic scaffold can influence protein production and osteogenic differentiation of hMSCs [87]. Tayton et al. have compared the porous and non-porous versions of poly (DL-lactide) for potential clinical use as alternatives to allografts in impaction bone grafting [88]. The results showed that the skeletal stem cells differentiated along the osteoblastic lineage in porous samples compared to the non-porous versions. This feature may result from the fact that the 3D microarchitecture could distribute cellular binding sites in a variety of specific spatial locations rather than on only the single plane of rigid substrate, as in traditional two-dimensional 2D architecture of cell culture plastic or the surface of the non-porous polymers. Cells, therefore, may have cytoskeletal adaptor proteins on a 3D matrix in addition to proteins present in 2D focal adhesions [89, 90]. Such differences in cell adhesion on the porous and non-porous polymers may therefore lead to different signal transduction and subsequent alteration in cellular rearrangement.

Natural ECM consists of various protein fibrils and fibers interwoven within a hydrated network of glycosaminoglycan chains [91]. The nano-scale structure of the ECM offers a natural network of intricate nano-fibers to support cells and present an instructive background to guide their behavior [92-94]. Each nano-fiber provides the way for cells to form tissues as complex as bone, liver, heart, and kidney. Researchers try to fabricate fibers to mimic the natural ECM as a support for cell growth. The proliferation and osteogenic differentiation of MSCs was investigated in 3D non-woven fabrics prepared from polyethylene terephthalate (PET) microfiber by Takahashi et al. They showed that the attachment, proliferation and bone differentiation of MSCs were influenced by the fiber diameter and porosity of non-woven fabrics in the scaffolds [95]. Several reports have demonstrated that nano-fibers are more favorable than micro-fibers, suggesting that cell activities can further be regulated by the size of the fiber [96-98] in terms of the biological response of chondrocytes, NSCs and endothelial cells cultured on nanofibrous and microfibrous scaffolds. Although the mechanisms by which a nano-fibrous scaffold acts

as a selective substrate are not known yet, it is clear that the enhanced adsorption of cell adhesion matrix molecules enhances cell adhesion. Xin et al. also confirmed that PLGA nano-fibers accommodate the survival and proliferation of human MSCs. hMSCs, as well as hMSC-derived chondrogenic and osteogenic cells, apparently attach to PLGA nano-fibers, and yet assume different morphological features [99]. These results demonstrate the full support of multi-lineage differentiation of MSCs within nano-fibrous scaffolds and the feasibility of multi-phasic tissue engineering constructs using a single cell source, which is of particular relevance to the development of multi-phasic tissue constructs. However, there are very few in-depth studies on nano-fiber topographical effects on stem cell differentiation. Other nano-scaled topographical features such as steps, grooves, pillars and pits also modulate cell behavior, as reviewed elsewhere [100].

Currently, there are three techniques available for the synthesis of nano-fibers: electrospinning, self-assembly, and phase separation. In particular, electrospinning technique is the most widely studied technique which has attracted wide attention due to its applicability for a variety of synthetic and natural polymers, exhibiting the most promising results for tissue engineering applications. Electrospinning is a spinning method to generate submicron to nanometer scale fibers from polymer melts or solutions. It is a physical process to obtain fibers from a bulk polymer of interest under the applied electric field. The most commonly used polymers for nano-fiber fabrication using electrospinning are the aliphatic polyesters [101]. There are several reports describing the potential of nanofibers fabricated by electrospinning method for neural [102-104], bone [105-108] and cartilage [109, 110] tissue engineering which mimic the native tissue environment and support the cell adhesion, proliferation and differentiation.

Nano-fibers hold great promise as potential scaffolds owing to their high porosity and high surface area-to-volume ratio, which are favorable parameters for cell attachment, growth, and proliferation in addition to possessing favorable mechanical properties [111]. Furthermore the effect of nano-fibers for stem cells' differentiation is promising further applications of nano-fibers for tissue engineering. Stem cells can be induced to differentiate into different cell types by growth/differentiation factors in the media, and we can incorporate such biomolecules into the nano-fibers to direct differentiation to a desired cell type. The biomimetic morphology of nano-fibers with different patterns may also help to direct the stem cells' differentiation, which is particularly attractive given differentiation induction by some of medium supplements, although successful, is not physiologically relevant and offers the possibility for development of improved clinical prostheses with topographies that can directly modulate stem cell fate.

2.3. Mechanical cues

Importantly, the various tissues of the body exhibit a range of matrix stiffness, and such differences in substrate stiffness have long been known to influence cell fate decisions in differentiated cell types [112]. An emerging area of study in stem cell biology and engineering is investigation of the role of these mechanical cues in stem cell fate decisions. Because MSCs can differentiate *in vitro* into cell types from tissues ranging from muscle,

bone, and potentially brain, it can be hypothesized that the mechanical cues provided by the ECM are particularly instructive in lineage specification. The study carried out by Engler et al. revealed that matrix elasticity influences differentiation of hMSCs into osteogenic, myogenic, and neurogenic cells [113]. Softer gels (0.1–1 kPa) were neurogenic, the hardest (24–40 kPa) were osteogenic, and the gels with intermediate elastic moduli (8–17 kPa) were myogenic. In all three cases, the elastic modulus matches that of the corresponding native tissue. It has recently been found out that substrate stiffness collaborates with soluble medium conditions to regulate the proliferation and differentiation of adult NSCs [114]. Cells exhibit optimum proliferation (in FGF-2) and optimum neuronal differentiation (in retinoic acid) at an intermediate stiffness that is characteristic of brain tissue. Furthermore, under conditions that induce nonspecific cell differentiation, stiff substrates support the differentiation of GFAP-expressing astrocytes, whereas soft substrates preferentially support the differentiation of β-tubulin III expressing neurons. This research demonstrates how the mechanical and biochemical properties of an adult NSCs microenvironment can be tuned to regulate the self-renewal and differentiation of adult NSCs. In another study, Leipzig et al. demonstrated that an optimal stiffness exists for both proliferation (3.5 kPa) as well as differentiation of neural stem/progenitor cell to neurons (<1 kPa) [115].

The study conducted by Banerjee et al. [116] provided insights into the influence of the mechanical properties of 3D alginate hydrogel scaffolds on the proliferation and differentiation of NSCs, where varying the concentrations of alginate and calcium chloride provided facile control over the elastic modulus of the hydrogels. They demonstrated that the properties of the 3D scaffolds significantly impacted both the proliferation and the neuronal differentiation of encapsulated NSCs. In addition, they observed the greatest enhancement in expression of the neuronal marker β-tubulin III within hydrogels having an elastic modulus comparable to that of brain tissues. They noted that the optimal value of the elastic modulus might depend on the stem cell type and the lineage to which differentiation is being directed. Wang et al. reported an injectable hydrogel scaffold composed of gelatin-hydroxyphenylpropionic acid conjugate system with tunable stiffness for controlling the proliferation rate and differentiation of hMSCs in a 3D context in normal growth media. The rate of hMSC proliferation increased with the decrease in the stiffness of the hydrogel. Also, the neurogenesis of hMSCs was controlled by the hydrogel stiffness in a 3D context without the use of any additional biochemical signal. These cells which were cultured for 3 weeks in hydrogels with lower stiffness expressed much more neuronal protein markers compared to those cultured in stiffer hydrogels for the same period of time [117]. In another study, lower cross-linked matrix of hydrogel system comprising hyaluronic acid-tyramine conjugates enhanced chondrogenesis with increases in the percentage of cells with chondrocytic morphology, biosynthetic rates of glycosaminoglycan and type II collagen, and hyaline cartilage tissue formation. By increasing cross-linking degree and matrix stiffness, a shift in MSC differentiation toward fibrous phenotypes with the formation of fibrocartilage and fibrous tissues was observed [118]. In general, the ability to control stem cell fate – possibly without the use of chemical inducers – would be broadly useful for applications in regenerative medicine and tissue engineering [116].

Except mechanical properties of the matrix, the external mechanical stimulus can also induce stem cell differentiation. Bioreactors provide various active environments for stem cell growth under specific mechanical conditions. Flow perfusion culture of scaffold/cell constructs has been witnessed to enhance the osteoblastic differentiation of rat MSCs over static culture in the presence of osteogenic supplements such as Dex. Although Dex is known to be a powerful induction agent of osteogenic differentiation in MSCs, Holtorf et al. showed that the mechanical shear force caused by fluid flow in a flow perfusion bioreactor would be sufficient to induce osteoblast differentiation in the absence of Dex [119]. Flow perfusion also accelerates the proliferation and differentiation of rat MSCs seeded on non-woven PLLA microfibrous scaffolds toward the osteoblastic phenotype, and improves the distribution of the calcified extracellular matrix generated *in vitro* [120]. Li et al. reported that MSCs are also mechano-sensitive and that Ca^{2+} may play a role in the signaling pathway since MSCs subjected to oscillatory fluid flow exhibited increased intracellular Ca^{2+} mobilization [121]. More recently, studies have shown that *shear stress* can induce *differentiation* of *stem cells* toward both endothelial and bone-producing cell phenotypes. The current data supporting the role of shear stress in stem cell fate and potential mechanisms and signaling cascades for transducing shear stress into a biological signal are reviewed elsewhere [122].

In another study, it was shown that the cyclic compressive loading alone will induce chondrogenic differentiation as effectively as the TGF-β alone or TGF-β plus loading in short term culture. Regarding MSCs angiogenesis, DNA microarray experiments [123] showed that uniaxial strain increased smooth muscle cell (SMC) markers. But cyclic equiaxial strain downregulated SM α-actin and SM-22α in MSCs on collagen- or elastin-coated membranes after 1 day, and decreased α-actin in stress fibers. This result suggests that uniaxial strain, which better mimics the type of mechanical strain experienced by SMCs, may promote MSCs differentiation into SMCs if cell orientation can be controlled. Solvig Diederichs et al. applied singular and repetitive cyclic strain of short- and long-time strains [124]. Additionally, a gradually increasing strain scheme commencing with short-time strain and continuing elongated strain periods was applied. Adipose tissue–derived MSCs on planar silicone and a three-dimensionally structured collagen I mesh were exposed to these strain regimes. The results revealed that even short-time strain can enhance osteogenic differentiation. Elongation and repetition of strain, however, resulted in a decline of the observed short-time strain effects, which was interpreted as positively induced cellular adaptation to the mechanically active surroundings. With regard to cellular adaptation, the gradually increasing strain scheme was especially advantageous.

Taken together, these results suggest that the design of *ex vivo* stem cell culture systems should consider all types of mechanical cues in the microenvironment including matrix stiffness, compressive loading and shear stress as factors in guiding proper lineage specification.

2.4. Electrical stimulus and other cues

Several studies have recently shown the response of NSCs to electric fields. The studies reported by Matos et al. showed the response of murine NSCs encapsulated in alginate hydrogel beads to alternating current electric fields [125]. They found an enhanced propensity for

astrocyte differentiation over neuronal differentiation in the 1 Hz cultures. In another study, Park et al. discovered the enhanced neuronal differentiation of hNSCs on graphene, which had a good electrical coupling with the differentiated neurons for electrical stimulation [126]. The application of an electrical stimulus causes fibroblasts to change cell shape and reorient in the 3D collagen scaffold perpendicularly to the direction of electrical stimulus, while the same electrical stimulus applied to MSCs induces much less significant reorientation. A stimulus as strong as 10 V/cm is needed to induce a δV of 50 mV or greater, which would be sufficient to activate voltage-gated Ca^{2+} channels and regulate Ca^{2+}-dependent sub-cellular processes, including cytoskeletal reorganization that is likely to cause changes in the cell morphology and reorientation signaling pathways [127]. It needs to be identified as to whether the differentiation of stem cell following adhesion will change under electrical stimulus. Endothelial progenitor cells and muscle precursor cells can also be stimulated by electromagnetic fields to promote myocyte differentiation [128,129]. Interestingly, electrical stimulation (10–40 V, 5 ms, 0.5 Hz pulses) of human embryonic fibroblasts was found to cause loss of cell proliferation and cell number but also led to differentiation of fibroblasts into multinucleated myotube-like structures [130].

Ultrasound has also been shown to induce differentiation. In low-intensity ultrasound field studies, MSCs differentiate towards a chondrocytic phenotype [131]. In one study, Abramovitch-Gottlib L et al. have illustrated that the use of low level laser irradiation (~0.5 mW/cm^2) applied to a MSC/coralline construct stimulates the proliferation and differentiation of MSC into an osteoblastic phenotype during the initial culture period and significantly induced *in vitro* osteogenesis over time [132]. Thus, low level laser irradiation quickens the differentiation of MSC into an osteoblastic phenotype during bone formation processes in early culture periods.

Numerous recent papers have sprouted showing how even minor experimental modifications can change cell phenotype. Indeed, stem cells are so sensitive and unstable that even cell seeding density and seeding protocol have been observed to influence cell shape and gene expression [133].

3. Some novel trends emerging in the field of tissue engineering

In the following part we will introduce some novel trends emerging in the field of tissue engineering in terms of both cellular biology (cell reprogramming) and biomaterial (multifactorial design strategies) point of view in order to improve the overall efficiency of tissue regeneration.

3.1. Cell reprogramming

Though all somatic cells of the human body have the same genome structure, differences in chromatin organization and expression pattern of genes lead to the formation of various types of cells with different physiology, function and morphology [134,135]. Therefore, one could speculate that by changing chromatin structure and pattern of gene expression, all

cells can be converted to other cell types [136]. The first cell reprogramming report has been presented in an earlier report [137] in which fibroblast cells converted into myocyte through the overexpression of MyoD gene. In a later study, the nucleus of the fibroblast cell has been transferred to the enucleated oocytes which finally led to the birth of Dolly sheep [135]. Yamanaka (2006) shed some light on the biology underlying cell differentiation and cell fate by converting the mouse fibroblast to iPS cells in his study; one year later, Yamanaka and Thompson [138-140] reported the generation of human iPS cells from fibroblast cells.

The possibility of directing lineage specific reprogramming of cells opens a window to a vast range of new possibilities in tissue engineering and regenerative medicines [141]. Herein, generation of iPS cell lines is an important issue in the way to derive pluripotent cells from somatic cells. Instability of the genome, high cost of culture, lack of an efficient protocol for differentiation as well as the presence of tumorigenic potential upon transplantation are among the main reasons for the slow progress of its clinical application [142].

Differentiation of stem cells into different types of tissue or organ is still a major limiting factor in the area of tissue engineering mainly due to the complexity and multicellular structure of the tissues and organs. To overcome such a limitation, it is highly demanded to have different types of cells for tissue engineering which is considered to be as important as mimicking the physiological condition *in vivo*. Self-renewing and pluripotency are unique properties of pluripotent stem cells that make the embryonic developmental process possible for the complex and integrated tissue-engineered systems. Accordingly, to make complex and integrated tissues, intrinsic developmental programs of inner cell mass of blastocysts such as those of post gastrulation events can be followed. Eiraku et al. [143] in a recent study managed to recreate the 3D structure of an organ for the first time in the world. They succeeded in growing a structure like the optic cup with the six cell types present in normal retina tissue. They mimicked aggregation and self-induction of mESCs as embryoid body and neurosphere formation to make optic cup that can be the source of retinal neurons like embryonic process of eye formation. For this, they used genetic engineered mES with tissue specific reporter RX-venues DNA construct for capturing the early stages of optic cup-cell mass formation and their separation for more maturation. Scientists hope to begin applying the same technologies used for retinal tissue to make 3D structure of other organs such as the brain, lung and kidney. However, despite advances like these, it is quick to note that we can determine as to whether pluripotent stem cells can be used for regenerative therapy. The best idea is not always to uprise the cells to the tip of potency pyramid and then downrise it to a low level with differentiation, whereas one can directly convert one cell type to another [143]. It has been shown that the fibroblast cells can be converted to myocyte, neuron, hepatocyte, cardiomyocyte simply with direct reprogramming [137, 144-146]. This provides us good tools for having wide ranges of cells for regenerative medicines [147]. New approaches to cell reprogramming such as direct reprogramming of somatic cells to tissue-specific stem cells and conversion of fibroblast to neural stem cells have been proposed [148]. Providing three types of cells, namely astrocyte, oligodendrocyte and neuron, which are required in neural systems, is the advantage of cell reprogramming [148]. Another advantage of using direct reprogramming to tissue specific stem cells instead of reprogramming to full matured

cells is that all types of cells which are necessary for the regenerating of that specific tissue will be provided in the former approach. For instance, it has recently been well demonstrated that convection of fibroblast cells to NSC is more promising than the conversion of the same cells to the neuron [149]. Moreover, adult stem cell generation through direct reprogramming has more capacity for self-renewal, which can be expanded and stored for different clinical applications. Tissue specific adult stem cells are natural stem cells of any tissue and match the normal homing tissue [149] and can respond to niche messages under both stress and damage condition.

Human body is a complex system that works with many regulatory and check points in coordination with many flexible programs. Using direct reprogramming, progression in regenerative therapy will be possible if all demanding material such as adult stem cells, ES, iPS are well prepared in a suitable place and appropriate manner.

3.2. Multifactorial design strategies

In contrast to elements of living systems' ECM, the designed scaffolds are very poor in information, which make them suboptimal for many tissue engineering applications. These passive biomaterials are unlikely to guide cell migration and differentiation or controlled matrix deposition, a problem that becomes even more evident in complex tissues with more than one cell type. Furthermore, they also cannot induce tissue neo-formation while preventing other undesirable tissue repair processes such as scarring; they are also unable to promote functional tissue integrations, such as vascular and/or nervous connectivity, in the host. Finally, these passive scaffolds largely lack the capacity to induce cell differentiation, thus resulting in a major limitation for their use together with current stem cell-based therapies [150]. A promising strategy to overcome these limitations is to consider the *multi-factorial design strategies* by combining various external cues with one another for efficient and controlled formation of complex tissues.

3.2.1. Combining structural and biological cues for scaffold bioactivation

While combining the structural and biological cues, a bioactive scaffold can be constructed in which biological functionality has been integrated to provide an information-rich support material for tissue engineering. Bioactive scaffolds are designed to control cell and tissue responses, and to provide a more efficient integration with the host. Indeed, bioactive scaffolds can also be prepared from synthetic materials by physical adsorption or chemical immobilization of biomolecules or oligopeptides on the scaffold surface, or by physical entrapment of bioactive molecules alone or incorporated in a drug delivery system into the scaffold. These strategies can also be applied to enhance the bioactivity of scaffolds made from ECM-native materials.

Engineered tissues need not only to remedy a defect and to integrate into a host tissue, but they also need to meet the demands of a constantly changing tissue. It was hypothesized that those tissues capable of growing with time could be engineered by supplying growth stimulus signals to cells from the biomaterials used for cell transplantation [151]. Smart drug

delivery system is able to transmit multiple signals to the cells in a timely controlled release pattern. This release may be controlled through properties of the drug delivery system itself such as biodegradation-controlled release devices or stimulisensitive systems. Polymeric materials can be used as tissue-engineering *scaffolds and drug release* carriers, a strategy that has been mainly used for soluble signaling molecules such as growth factors. Cell recruitment and migration to the site of injury may be promoted through various signaling molecules. Many of these factors, e.g. TGF-βs, BMPs and IGF-1, are not only involved in cell attraction but also affect stem cell proliferation and differentiation [152-155].

Drug delivery strategies are designed to provide a platform for the localized delivery of the growth factors at the site of implantation. This is to protect the bioactivity of the molecule, to provide a controlled release pattern of the drug over a desired time frame, and *deliver angiogenic factors so as to promote angiogenesis*.

Two approaches have been mainly used for scaffold bioactivation: growth factors can be encapsulated in a selected drug delivery system such as a microsphere or nanoparticle formulation, and these can be incorporated into the scaffolds. Otherwise, growth factors can be incorporated directly into the scaffold itself [156-158]. For example, IGF-1 has been directly incorporated into porous 3D silk fibroin scaffolds [159]. Silk scaffolds incorporating IGF-1 were able to preserve growth factor bioactivity, and prompted chondrogenic stimuli to seeded MSCs *in vitro*. By definition, implantation of growth factor-loaded scaffolds results in the localized delivery of the signaling molecule. Still, a certain fraction of the incorporated drug can reach the lymphatics or the circulation, and then distribute to non-target tissues. Therefore, even for these localized therapies, potential adverse effects of growth factor need to be carefully monitored.

Silk fibroin nano-fibrous scaffolds containing BMP-2 and/or nanoparticles of hydroxyapatite which were prepared via electrospinning were selected as matrix for *in vitro* bone formation from human bone marrow derived hMSCs. Li et al. [160] reported that silk fibroin nano-fibrous scaffolds with BMP-2 supported higher calcium deposition and enhanced transcript levels of bone-specific markers in comparison with controls without BMP-2, suggesting that nano-fibrous electrospun silk scaffolds can be an efficient delivery system for BMP-2. The mild aqueous process required for electrospinning, offers an important option for delivery of labile cytokines and other biomolecules. Lee et al. reported that calcium phosphate cement (CPC(combined with alginate solution to form a porous scaffold showed the capability to safely load biological proteins (BSA and lysozyme) during preparation and to release them *in vitro* for over a month [161]. CPC–alginate scaffolds can further be developed into tissue engineered constructs which deliver biological molecules for bone regeneration stimulation.

In case of building biofunctionality into electrospun nano-fibers for neural tissue engineering, the challenge to produce nano-fibers with more bioactive surfaces, significantly improving specific targeting of cell substrate interactions and consequently creating a more biomimetic microenvironment for implanted cells remains. There are several methods, such as polymer blending and surface biofunctionalization, for improvement of nano-fibrous scaffolds bioactivity for nerve tissue engineering which are reviewed elsewhere [162]. It is possible to fabricate electrospun scaffolds from blends of synthetic and natural polymers,

which will then have improved cell substrate interactions. The orientation of neurites from chick embryonic dorsal root ganglia is enhanced on aligned blended polycaprolactone/collagen (PCL/collagen) (72:25) nano-fibers compared with that on aligned, pure PCL [163]. The migration and proliferation of Schwann cells is also significantly improved on aligned PCL/collagen nano-fibers, indicating more specific biomolecular interactions between cells and the collagen polymers on the nano-fiber surface [164].

Instead of direct electrospinning the naturally derived polymers such as collagen together with synthetic polymers to provide biomemitic nano-fibrous scaffolds, one can immobilize some specific peptide motifs derived from ECM protein, which have been discerned to play an important role in tissue regeneration to the synthetic nano-fiber surface, which provides an alternative method to render the fibers bioactive. For instance, immobilization of molecules, such as specific peptide motifs derived from fibronectin and collagen VI, to the synthetic nano-fiber surface provides an alternative method to render the fibers bioactive. Therefore, surface immobilization of these small molecules that are neuroactive can provide a great advantage for neural tissue engineering. In addition, immobilized growth factors such as brain-derived neurotrophic factor [165] and basic fibroblast growth factor [166] can also promote cell survival and neurite outgrowth.

3.2.2. Combining structural and mechanical cues for engineering large-scale and/or complex tissues

The successful replacement of large-scale defects using tissue-engineering approaches will likely require composite biomaterial scaffolds that have biomimetic structural and mechanical properties and can provide cell-instructive cues to control the growth and differentiation of embedded stem or progenitor cells.

The depth-dependent composition and structure of articular cartilage gives rise to its complex, non-homogeneous mechanical properties. Articular cartilage is generally composed of chondrocytes and a dense ECM, which mainly includes type II collagen and proteoglycans [167]. Articular cartilage is structurally comprised of four different layers that can be distinguished from one another by collagen fiber alignment and proteoglycan composition. The depth-dependent alignment of collagen leads to important tensile and shear properties, whereas the depth-dependent proteoglycan content contributes more to the compressive properties of each zone [168, 169]. Nguyen et al. demonstrated in a recent study that layer-by-layer organization of specific biomaterial compositions creates 3D niches that allow a single MSC population to differentiate into zone-specific chondrocytes and organize into a complex tissue structure [75]. The results indicated that a three-layer polyethylene glycol (PEG)-based hydrogel with chondroitin sulfate (CS) and matrix metalloproteinase-sensitive peptides (MMP-pep) incorporated into the top layer (superficial zone, PEG:CS:MMP-pep), CS incorporated into the middle layer (transitional zone, PEG:CS) and hyaluronic acid incorporated into the bottom layer (deep zone, PEG:HA) which ultimately created native-like articular cartilage with spatially-varying mechanical and biochemical properties. They concluded that spatially-varying biomaterial compositions within single 3D scaffolds can stimulate efficient regeneration of multi-layered complex tissues from a single stem cell population.

In another study, the potency of scaffold stiffness and topology in driving cardiac stem cell differentiation in a 3D culture context was confirmed by Forte et al. [170]. Cardiac stem cells adopted the cardiomyocytic phenotype only when cultured in strictly controlled conditions characterized by a critical combination of chemical, biochemical, structural and mechanical factors, and emulation of the inner myocardial environment. In these studies, the mimicry of myocardial environment was achieved by fine-tuning the array of growth factors dissolved in the culture medium and the chemistry, topology and stiffness of three-dimensional supports on which stem cells were seeded. Scaffold stiffness was modulated in this study by changing the topology of the structure using a rapid prototyping technique. The optimal stiffness to induce cardiomyocyte differentiation was around 300 kPa on the scaffolds with square pores of about 150 μm.

4. 2D Polysaccharide-based hydrogel scaffolds for muscle tissue engineering

Hydrogels have been used for a variety of biomedical applications [171-175], and because of their viscoelastic characteristics [176], similarities with ECM, excellent biological performance, inherent cellular interaction capability [177], ability to allow transfer of gases and nutrients [177], and their amiability of fabrication into specific shapes, they have recently been explored as scaffolding materials for tissue engineering applications [178-180]. On the other hand, in the recent decade, researchers realized that the mechanical properties of the used hydrogel material had to be adapted to the elastic properties of the damaged tissue [181]. Hydrogels such as alginate, chitosan, collagen and hyaluronic acid, which are derived from natural polymers, have been proved to be quite promising for stem cell proliferation, maintenance and differentiation for tissue engineering applications.

The authors of this paper tried to prepare hydrogels made of natural polymers (chitosan (CS) and gelatin (G)) with proper handling for surgery, and with mechanical properties similar to those of muscle tissues as well as good cell adhesion properties. In the current study, we investigated the effect of CS and G concentration in blend scaffolds on mechanical properties of the CS-G hydrogel sheets as well as the seeded muscle-derived stem cells (MDSCs) and smooth muscle cells' (SMCs) behavior on the CS-G hydrogel sheets. MDSCs and SMCs were isolated, expanded in culture and characterized with respect to the expression of surface markers with flow cytometry analysis. After crosslinking of CS and G, the CS-G blend hydrogel sheets were prepared by a casting method and used for 2D cell culture.

While the elasticity of the CS-G hydrogel sheets increased by increasing the CS concentration, the gelatin concentration did not have any notable effect on the hydrogel mechanical properties.

The MDSCs attachment on the surface with elastic modulus of 25 kPa stiffness and proliferation on different CS-G hydrogel sheet surfaces having varying modulus of elasticity is shown in Figure 2. The cell observation result on day 1 showed that by increasing the

elasticity of hydrogel sheets, most of the cells on the hydrogel surfaces with high elasticity (E=100 kPa, CS=4.5% w/v) didn't fully expand on the hydrogel surface, while the cells on the hydrogel surfaces with low and intermediate elasticity (E=15 kPa, CS=1.5 % w/v; E=25 kPa, CS=3% w/v) had more spindle shape (data not presented). Gelatin concentration was fixed (18% w/v) for all the samples. The greatest proliferation of the cells was found on the hydrogels with intermediate elasticity (25 kPa) and the number of cells increased over time during the 7-day culture (Figure2). Hydrogel blends with lower or higher gelatin concentration showed significantly lower attached cell numbers (data not presented). Recent studies have illustrated the profound *dependence of cellular behavior* on *the stiffness* of 2D hydrogel sheets. Boontheekul et al. demonstrated that alginate gel with higher mechanical strength (increasing from 13 kPa to 45 kPa) increased myoblast adhesion, proliferation, and differentiation in a 2D cell culture model [182]. They also showed that primary mouse myoblasts were more highly responsive to this cue than the C2C12 myoblast cell line.

An innovative approach has recently been described by Gilbert et al. as well. Using a bioengineered substrate in conjunction with a highly automated single-cell tracking algorithm, the authors showed that substrate elasticity is a potent regulator of muscle stem cells' fate in culture. In fact, muscle stem cells cultured on soft hydrogel substrates that mimic the elasticity of muscle self-renew *in vitro*, and contribute extensively to muscle regeneration when subsequently transplanted into mice. This study has provided novel evidence showing that recapitulating physiological tissue rigidity allows the propagation of adult muscle stem cells [183].

In the current study, the authors investigated the behavior of MDSCs and SMCs cultured on the prepared hydrogel surfaces. The results indicate that increasing the hydrogel mechanical strength from E=15 kPa to E=25 kPa, increases MDSCs adhesion and proliferation. The authors further found that MDSCs were more responsive to mechanical properties of the hydrogel sheets compared to SMCs, due to their higher ability and relatively smaller size (Data not presented). In contrast, for engineering central nervous system tissue, Leipzig et al. demonstrated that gels with lower mechanical properties of methacrylamide chitosan hydrogel sheet (E ≤3.5 kPa) were more appropriate for neural stem progenitor cell differentiation and proliferation [115]. As mentioned above, mechanical properties of hydrogel can regulate the cell adhesion, proliferation, and differentiation. However, the response and sensitivity to this variable is highly dependent on the cell source. In the current work, MDSCs exhibited maximal proliferation on hydrogel surface with 25 kPa elasticity. The same hydrogel sheet showed also the best handling qualities for surgery, with elasticity in the range of elastic modulus for muscle tissues [184], showing its potential for being used in muscle tissue engineering applications.

The strategy applied in the current study provides an opportunity to independently control mechanical and bioadhesive properties of the hydrogels so as to probe stem cell behavior. By changing both material mechanical and biochemical properties of the hydrogel blend, we could find the optimum condition for MDSCs attachment and proliferation in contact with CS-G hydrogel sheets.

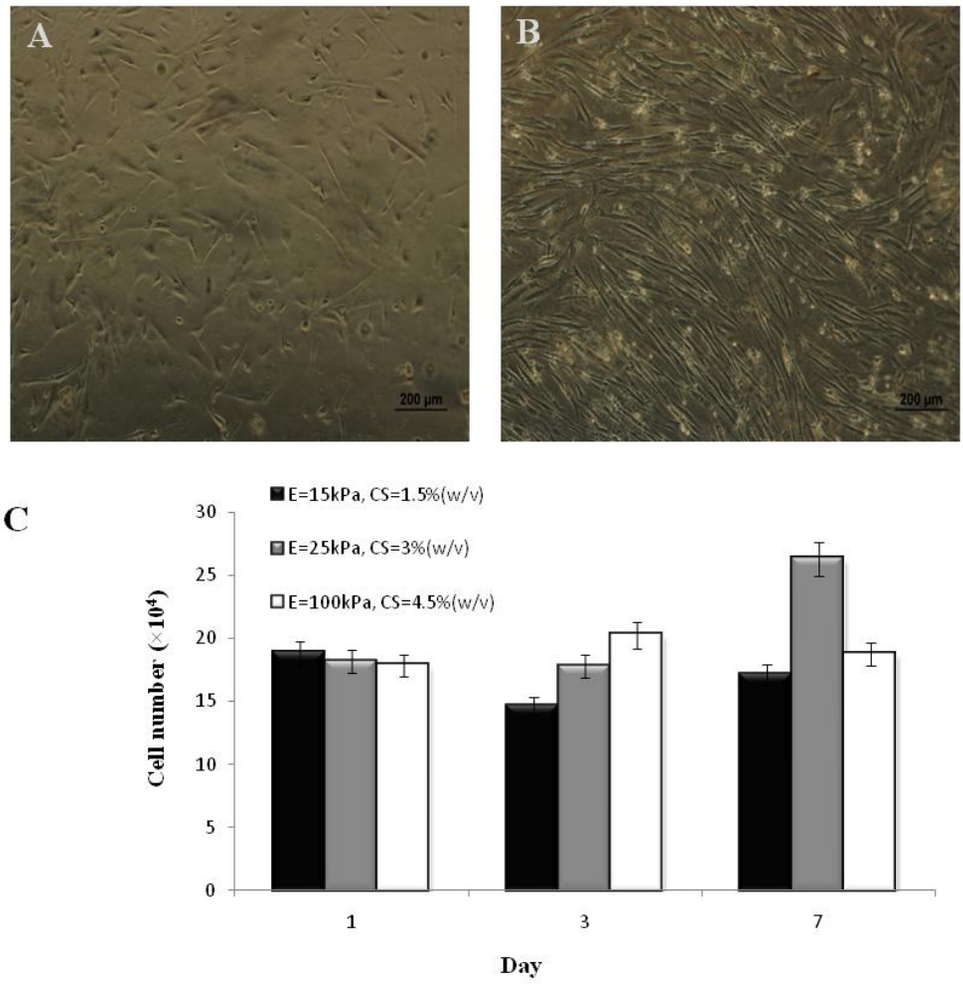


Figure 2. MDSCs adhesion and proliferation on CS-G hydrogel surfaces. Photomicrographs of MDSCs attachment on the surface with intermediate elasticity (25kPa, CS=3 % w/v) at: (A) day 1, (B) day 7 and (C) cell proliferation on CS-G hydrogel surfaces with different mechanical strength. CS-G hydrogel sheets prepared at different chitosan concentration (4.5, 3 & 1.5 % w/v) with constant gelatin (18 %w/v). MDSCs were seeded onto all hydrogel surfaces at the density of 7500cells/cm^2.

5. Conclusion and outlook for the future

In tissue engineering, directing the cells to differentiate at the right time, in the right place, and into the right phenotype, requires an environment providing the same factors that govern cellular processes *in vivo*. The current chapter described various biomaterials and external cues designing considerations mimicking the natural stem cell microenvironment in order to direct the desired stem cell fate, facilitating the regeneration of desired tissues. In addition we introduced our approach to designing a 2D polysaccharide-based hydrogel scaffolds as a potential and suitable biomaterial for muscle tissue engineering applications.

Overall, this chapter provides an overview of recent progresses made by application of novel engineering strategies that have been developed to emulate the stem cell niche for effectively controlling the cell fate and translating the stem cell research into much needed clinical applications in the not-too-distant future.

Future directions in tissue engineering will involve elucidation of molecular mechanisms by which all types of external cues influence stem cells' behavior, followed by translation of these scientific data to clinical applications. Further advances in controlling stem cell fate can be achieved by combining the above mentioned parameters in a more scalable and combinatorial manner to address the complexity of the natural stem cell niche. To this end, collaborative efforts between cell biologists and materials scientists are critical for answering the key biological questions and promoting interdisciplinary stem-cell researches in the direction of clinical relevance.

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