Functional Biomaterials for Controlling Stem Cell Differentiation

Ameya Phadke, Chien-Wen Chang and Shyni Varghese

Abstract Differentiation of stem cells has shown to be strongly influenced through several cues provided by reciprocal interactions with the extracellular microenvironment, consisting of soluble bioactive agents and the extracellular matrix. While the dynamic extracellular matrix is difficult to mimic in its entirety, recent research has successfully mimicked individual matrix-centric cues using synthetic polymeric systems to influence differentiation of stem cells into tissue-specific lineages. Material properties that have been shown to direct this differentiation include chemical functionality, mechanical properties, as well as tissue-mimetic modifications such as mineralization. Another aspect of the extracellular microenvironment that has been mimicked in the controlled differentiation of stem cells is the presence of specific bioactive agents. Material-based delivery of these agents allows for the spatiotemporal variation in their presentation to stem cells, allowing for precise control over their terminally differentiated phenotype. Thus, the delivery of bioactive agents to cells via synthetic materials has also been an effective method to influence stem cell differentiation to various tissue-specific lineages. In this chapter, we discuss the use of synthetic materials to direct stem cell differentiation through both, capitulation of matrix-specific biochemical, mechanical and physical cues, as well as the controlled delivery of specific bioactive agents.

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1 Introduction

1.1 Emergence of Stem Cell Engineering in Regenerative Medicine

Stem cells are proving to be an extremely invaluable tool in understanding developmental processes, disease progression, epigenetics, pathophysiology, drug screening and cell based therapies. Among these, cell therapies represent the most challenging yet potentially most fruitful applications for stem cells. Upon differentiation into a suitable phenotype, stem cells can be introduced at a damaged site in a tissue in order to facilitate its regeneration, halting any further tissue damage and even possibly reversing it. Approaches combining the use of stem cells and appropriate materials have thus shown great promise in treating several conditions emerging from the degeneration of tissues. When utilizing such a strategy however, it is important to understand the interaction between stem cells and materials and the effect of these interactions on the efficacy of the desired therapy in regenerating the desired tissue. A comprehensive understanding of these interactions allows for the effective design and development of materials capable of influencing stem cell adhesion as well as the lineage into which these cells differentiate. This requires a multidisciplinary approach integrating concepts in material science, chemistry, cell biology and physiology. In this chapter, we present such an approach capable of aiding in the design of suitable materials and subsequently efficient regenerative therapies.

1.2 Stem Cell Sources

Multipotent and pluripotent cells capable of differentiating into several lineages have been obtained from a variety of sources and are often classified based on the source from which they are obtained. Embryonic stem cells (ESCs) are obtained from embryonic sources and were first isolated through the in vitro fertilization of preimplantation blastocysts [1]. They are characterized by their high telomerase activity and pluripotent differentiation potential. Mesenchymal stem cells are multipotent progenitor cells and are typically isolated from bone marrow, although they have been isolated from a variety of adult tissues such as bone, cartilage, skin, fat and muscle [2]. They are characterized by a spindle-like morphology and have been shown to differentiate into adipocytes, chondrocytes and osteoblasts [3]. A more recent advance in sourcing stem cells has been the development of induced pluripotent stem cells (iPS) [4–7]. First reported by Takahashi and Yamanaka [6], these stem cells are obtained by genetic reprogramming of differentiated cells into a de-differentiated state resembling embryonic stem cells. These cells represent a promising method of obtaining autologous pluripotent stem cells sourced from adult tissues.

2 Stem Cell Expansion and Differentiation Using Biomaterials

2.1 Roles of ECM in Stem Cell Differentiation

The extracellular environment provides essential structural support and regulates signaling to cells [8]. Cells are organized in extracellular matrix (ECM), a hydrated extracellular environment specifically for supporting cell–cell and cell–ECM interactions. The interactions between cells and ECM are crucial in embryogenesis, tissue differentiation, wound healing and tumorigenesis [9]. ECM components regulate stem cell differentiation mainly by providing two-way biophysical and biochemical communications to the cells. The ECM is a 3D hydrophilic network comprising of fibrous structural proteins (collagens, fibronectin, laminins, elastin and vitronectin) and glycosaminoglycan (GAG) network. Among these structural components, collagen and elastin networks provide tissue with mechanical resistance to shear and tensile stress. Osmotic pressure created by the negatively charged GAGs results in the highly swollen viscous matrix, thereby providing compressive strength to the tissue. In addition to provide mechanical protection to cells, ECM contains various cell adhesion molecules to support cell attachment and proliferation. Figure 1 details cues influencing cell behavior in the extracellular environment.

ECM has a profound effect on stem cell differentiation. During the process of tissue development and morphogenesis, the dynamic remodeling of ECM components is required to direct differentiation of uncommitted progenitor cells into a specific lineage. It is generally believed that the interactions between ECM and cells initiate various signal transduction pathways [10] thereby regulating lineage of differentiation. This was shown by Datta et al. [11] in a study that demonstrated the ability of bonelike ECM to promote osteogenesis of human mesenchymal stem cells (hMSCs). An interesting study by Hoshiba et al. [12]

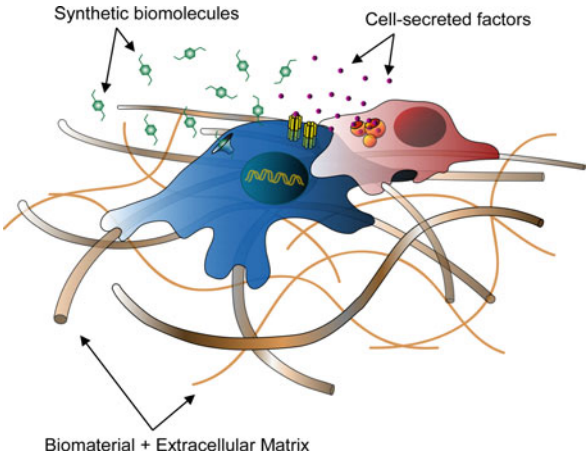

Fig. 1 Schematic demonstrating the reciprocal molecular interactions between the cells and their microenvironment comprising of extracellular matrix components, soluble factors and the surrounding cells

showed that enhanced osteogenesis of MSCs was observed when these cells were cultured in matrices produced by mesenchymal stem cells in the early stage of ostegenesis when compared to MSCs cultured on matrices on obtained from late-stage osteogenesis or from undifferentiated MSCs. This suggests that the ECM may show different structure and composition in different stages of differentiation.

Additionally, a number of studies have demonstrated the potential of using single ECM components to tailor materials to achieve desired stem cell lineage. Chung et al. [13] demonstrated the ability of hydrogels of hyaluronic acid (a component found abundantly within the ECM of cartilage) to promote chondrogenic differentiation of encapsulated hMSCs. Previous studies have demonstrated the capability of collagen gels to induce chondrogenic differentiation of MSCs [14, 15]. Brännvall et al. [16] reported the efficient neuronal differentiation of neural stem/progenitor cells (NS/PC) upon encapsulation in collagen–hyaluronan composite hydrogels, while Awad et al. [17] demonstrated that encapsulation within scaffolds of gelatin (a denatured form of collagen) promoted chondrogenic differentiation of human adipose derived stem cells. These examples illustrate the importance of utilizing ECM components in order to regulate the differentiation of stem cells into tissue specific cells. Although these naturally derived materials provide the necessary biological cues for cell–matrix interactions, they often suffer from batch-to-batch variations and challenges associated with modifications. In contrast, synthetic materials offer great control over structural and mechanical properties but lack biological cues. Hybrid scaffolds containing both naturally derived materials and synthetic materials often offer a "best of both worlds" approach and hence are extremely promising prospects as materials for cell culture matrices.

2.2 Mimicking ECM with Synthetic Biomaterials

2.2.1 Mimicking the Biophysical and Biochemical Properties of ECM

The initial goal of studies involving synthetic biomaterial based scaffolds was to provide a 3D architectural/structural support to cells [18]. Of late, there has been an emphasis on the development of synthetic biomaterials eliciting various interactions observed between cells and ECM in native tissues by mimicking several well studied extracellular biochemical cues and biophysical cues. By utilizing several inherent properties of matrix materials, cell–matrix interactions can be harnessed to modulate stem cell differentiation. These properties include: matrix functional groups, mechanical properties, matrix degradability, surface geometry and microarchitecture [11, 19–27]. Additionally, scaffolds may also be modified through processes such as mineralization in order to stimulate differentiation into osteogenic lineage. A detailed discussion on the role of biomineralized scaffolds on osteogenic differentiation of stem cells is presented in Sect. 2.2.3.

Functionalization of Synthetic Substrates with ECM Derived Ligands

In addition to incorporating entire ECM components, biomaterials can be functionalized using specific ligands representing the ECM binding sites to modulate cell attachment, proliferation and differentiation [28]. This functionalization can be achieved by a variety of methods such as blending [29], copolymerization [30], and immobilization using techniques such as *N*-hydroxysuccinamide (NHS) chemistry [31].

Several studies have made use of well studied cell-binding peptide sequences such as RGD, YISR and IKVAV to improve cell adhesion to synthetic biomaterials [32–34]. However, it is important to consider that orientation of these ligands within the scaffold material may affect their ability to promote cell adhesion [35]. It is interesting to note that these RGD based peptide ligands promote cell adhesion and migration more effectively when clustered in scaffolds rather than when sparsely dispersed within the scaffold [36].

Modification of polymers with ECM derived ligands has also been reported to affect differentiation of stem cells. Silva et al. [37] demonstrated the differentiation of neural precursor cells into neurons and astrocytes by incorporating IKVAV moieties in self assembled ampiphilic nanofibrous matrices. A recent study indicated that the presence of decorin moieties tethered to PEG hydrogels stimulated chondrogenesis of encapsulated hMSCs [38]. Hwang et al. [39] reported that encapsulation of human embryonic stem cells within RGD modified polyethylene diacrylate (PEGDA) based hydrogels promoted increased chondrogenic differentiation, when compared to unmodified PEGDA hydrogels as well PEGDA hydrogels incorporating ECM molecules such as hyaluronic acid, collagen type I and collagen type II. This was attributed to reports from other studies indicating that RGD binding to integrin $\alpha v\beta_1$ stimulated release of TGF-β1, thereby stimulating chondrogenic differentiation.

Interestingly, several studies have reported enhancement of osteogenic differentiation by RGD incorporation into biomaterial scaffolds [40, 41]. Shin et al. [42] report that incorporation of RGD peptide into oligo-poly(ethylene glycol) fumerate stimulated osteogenic differentiation of rat bone marrow stromal cells even in the absence of β-glycerolphosphate and dexamethasone (DEX), typically used as supplements in medium to trigger osteogenic differentiation. It was suggested that the interaction between RGD peptide and surface integrins in these cells activated intracellular pathways triggering osteogenic differentiation in a manner similar to that seen when such cells are exposed to dexamethasone. This is supported by other studies indicating that selective activation of integrins can trigger osteogenic differentiation of progenitor cells [43, 44].

2.2.2 Effects of the Cell–Matrix Interface

Surface Chemistry and Interfacial Energy

The ability of cells to respond to differences in surface chemistry of synthetic biomaterials has been well demonstrated [45]. By altering the chemical structure

of the surface of synthetic materials, the binding of proteins to these surfaces and their orientation (and hence the binding of cells to the surfaces) can be affected. In other words, cellular response to biomaterials may be controlled by altering interaction of material with serum components. By tailoring biomaterial surfaces with specific surface properties providing specific extracellular microenvironments, desired degrees of attachment, proliferation and differentiation can be achieved [32, 46]. This is especially important in mimicking cell–matrix interactions to obtain materials with desired capacity to promote cell adhesion and tissue specific differentiation. Although recreating a synthetic mimic of the dynamic extracellular environment is fairly challenging, there have recently been rapid advances made in developing synthetic analogs incorporating various chemical functionalities typically observed within the ECM. For example, modification of polymeric surfaces with anionic groups causes formation of a negatively charged material; this is one potential method to obtain a highly water-swollen matrix with the ability to resist compression, thereby mimicking the role of glycosaminoglycans (GAGs) in load bearing tissues.

Plasma grafting has been explored as a potential method to alter surface chemistry of biomaterials. It is important to note that plasma grafting can be used to obtain highly localized modified domains (from several hundred angstroms to 10 mm) leaving the bulk properties of the materials unaffected. Mwale et al. [26] observed that altering surface chemistry through glow discharge plasma using ammonia affected the differentiation of hMSCs. This process, when applied to nylon 6-polyamide and biaxially oriented polypropylene, led to enrichment of the surface with nitrogen atoms, thereby promoting cell adhesion. Interestingly, application of this treatment to nylon 6-polyamide promoted osteogenic differentiation of MSCs while plasma-treated biaxially oriented polypropylene (BOPP) was found to suppress osteogenic differentiation. The authors attributed this suppression to the possibility that BOPP inhibited the formation of collagenous extracellular matrix by the seeded stem cells, thereby inhibiting further differentiation. Mwale et al. [47] also reported in another study, that doping of BOPP with nitrogen rich plasma polymerized ethylene (referred to as PPE:N) suppressed not only expression of collagen type X but also several osteogenic markers such as alkaline phosphatase, osteocalcin and bone sialoprotein in differentiating hMSCs. This indicates a potential application for this technique in promoting chondrogenic differentiation of MSCs while suppressing/delaying their endochondral ossification. Of interest is the fact that hMSCs for this study were sourced from patients aged 60–80 years undergoing treatment for osteoarthritis. Although these MSCs inherently expressed hypertrophic markers such as collagen type X and osteogenic markers under control conditions, culturing them on plasma treated BOPP down regulated these markers without affecting the markers which are characteristic of hMSC-derived chondrocytes. Plasma grafting was also used to great effect by Wan et al. [48] to alter the surface chemistry, surface energy and surface topology of poly(L-lactic acid) (PLLA) films, thereby increasing their ability to support cell retention.

Jiao and Cui [49] summarized several well-studied methods to modify surface chemistry of polyester biomaterials thereby improving their ability to support cell

growth. Hydrolytic degradation is often used in polyesters, thereby cleaving surface ester bonds and leading to formation of carboxyl and hydroxyl residues at the surface. This serves to increase the hydrophilicity and decrease the interfacial surface energy of the material, thereby altering its cellular response. Croll et al. [50] proposed two methods i.e. base hydrolysis and aminolysis to lead to formation of carboxyl or primary and secondary amines respectively on the surface of poly(L-lactic-co-glycolic acid) (PLGA), while minimizing collateral surface degradation.

Surfaces may also be modified through anchoring of monomers such as vinyl acetate, acrylic acid and acrylamide onto PLLA films by means of photo-induced grafting. Additionally, functional groups such as carboxyl, hydroxyl and amide groups were incorporated by means of photo-grafting of hydroxyethyl methacrylate, methacrylic acid and acrylamide, respectively, onto the surface. Such a modification was shown to improve the ability of the biomaterial (film) to support chondrocyte growth [51].

While there have been several well studied methods of modifying surface chemistry of polymers to improve cell adhesion, effects of chemistry of polymeric matrices on differentiation of multipotent cells have only recently been reported [20, 22]. Chastain et al. [52] used two different materials viz. PLGA and polycaprolactone (PCL) to modulate the preferential adsorption of ECM proteins from serum and demonstrated that depending upon the adsorbed protein the material showed differential effect on osteogenic differentiation of hMSCs. Photochemical modification of polystyrene surfaces with azodiphenyl derivatives of hydrophilic polymers such as polyacrylic acid (PAAc), polyacrylamide (PAAm) and polyethylene glycol (PEG) was shown by Guo et al. [22] to affect chondrogenic differentiation of hMSCs. While modification with PAAc and PAAm were found to improve cell adhesion, modification with PEG was found to inhibit the cell adhesion. Additionally, surfaces modified with PAAm showed more rapid adhesion of cells than PAAc. The authors attributed this difference to the electrostatic attraction between the positively charged surface formed from photochemical modification with PAAm and the negatively charged cells. Modification with PEG would form a neutral surface which would not exhibit this attractive force with cells and as a result, cells cultured on PEG modified surfaces showed aggregation into pellets, indicating dominance of cell–cell interactions over cell–material interactions. PEG-modified and PAAm-modified surfaces were found to promote chondrogenic differentiation of these cells upon culturing in chondrogenic medium. Curran et al. [53, 54] reported that chondrogenesis is promoted on glass slides by the presence of surface hydroxyl and carboxyl groups whereas surface amine and thiol groups were found to stimulate osteogenesis. Methylated and untreated glass surfaces were found to maintain undifferentiated phenotype of MSCs. Similar results were also reported by Lee et al. [55] with Chinese hamster ovary cells. This study reported that low density polyethylene sheets functionalized with amine groups promoted cell adhesion to the greatest degree among charged groups (carboxyl and amine), while hydrophilic neutral groups (such as OH) promoted cell adhesion to a greater degree than hydrophobic neutral groups (amide groups).

An effect of material chemistry on differentiation of cells under 2D and 3D culture conditions was recently reported by Benoit et al. [20]. Human mesenchymal stem cells were plated on PEG hydrogel surfaces functionalized with carboxyl, phosphate and *t*-butyl groups, under 2D culture conditions. Morphological observations indicated that cells cultured on carboxyl-modified PEG surfaces showed a rounded morphology similar to that seen in chondrocytes cultured in 2D conditions, cells cultured on phosphate modified PEG surfaces assumed a spread morphology similar to that observed in osteoblasts and cells cultured on *t*-butyl modified PEG surfaces showed adipocyte-like morphology (see Fig. 2), along with the presence of intracellular lipid droplets (not shown).

This was confirmed through FISH analysis (see Fig. 3) wherein cells cultured on surfaces modified with carboxyl, phosphate and *t*-butyl groups showed elevated expression of aggrecan (a chondrogenic marker), core binding factor α-1 (an osteogenic marker) and peroxisome proliferator-activated receptor γ (PPARγ) (an adipogenic marker) respectively when compared to a control, unmodified PEG surface.

It has been reported that lineage into which stem cells differentiate can be influenced by cell shape, spreading and matrix stiffness [21, 25] and that these can be influenced through material chemistry. To evaluate whether material chemistry affected cell lineage in a manner independent of the aforementioned properties, MSCs were encapsulated by Benoit et al. in three-dimensional scaffolds functionalized with tertiary butyl and phosphate groups, respectively; due to small

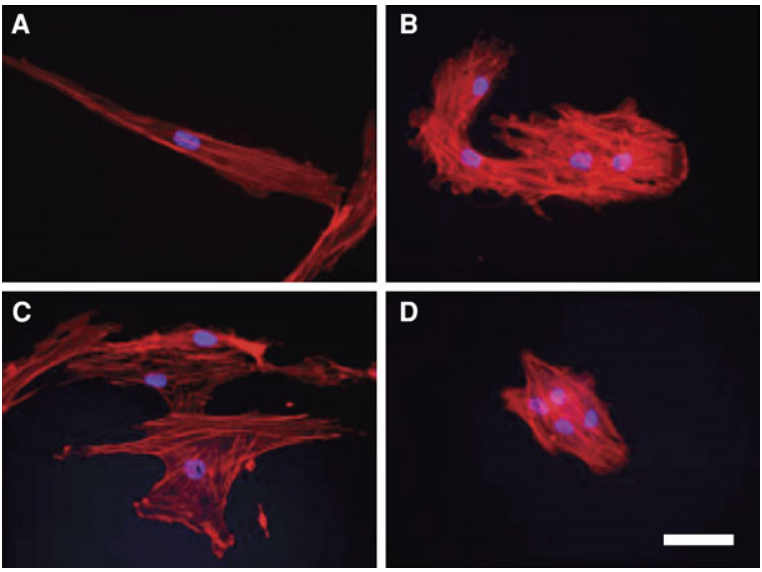

Fig. 2 Images from immunostaining (F-actin and nuclei) of hMSCs seeded on (**a**) unmodified PEG (**b**) carboxyl-modified PEG (**c**) phosphate-modified PEG and (**d**) *t*-butyl modified PEG. (Adapted from [20] with permission, copyright Nature Publishing Group, 2008)

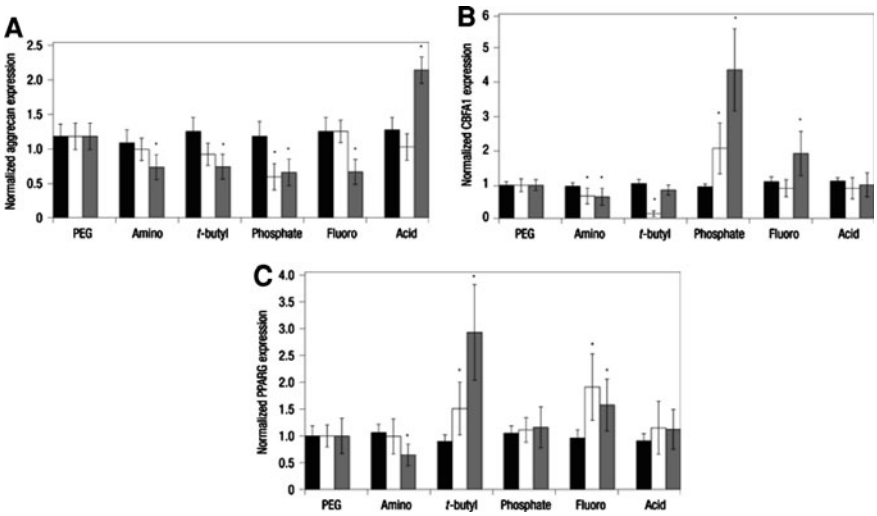

Fig. 3 Gene expression profiles of (**a**) aggrecan (**b**) CBFα-1 (**c**) PPARγ for hMSCs seeded on surfaces modified with various functional groups, normalized to expression on control surface of unmodified PEG. *Black bars* represent expression after 4 days, *white bars* represent expression after 7 days and *gray bars* represent expression after 14 days (adapted from [20] with permission, copyright Nature publishing group, 2008)

mesh size, encapsulated cells were restricted to a rounded morphology independent of material chemistry. Additionally, sufficiently small concentrations of the functional groups were used so as not to affect the bulk mechanical properties of the polymeric scaffold material to a significant degree. In this manner, effects of cell–material interaction on differentiation were effectively decoupled from effects on differentiation of cell morphology and matrix elasticity. Immunoblotting revealed that cells encapsulated in *t*-butyl-modified-PEG showed an upregulation of the adipogenic marker PPARγ after 14 days which remained constant at 21 days suggesting adipogenic differentiation. Cells encapsulated in phosphate-modified PEG showed an upregulation of the osteogenic marker CBFα-1 after 14 days and showed increased levels of expression after 21 days suggesting a differentiation into osteogenic phenotype. The authors proposed that these difference in matrix functionality triggered differentiation down different pathways through two potential mechanisms. Firstly, through interaction with surface receptors, it is possible that different functional groups triggered different intracellular signaling pathways promoting differentiation into varying lineages. Another mechanism is the selective sequestration of cell secreted factors by the functional groups; the sequestered factors may then influence the differentiation of the cells down a particular pathway.

It is also important to consider the effect of scaffold material chemistry on the adsorption of serum components such as fibronectin and their resulting interaction with cellular receptors such as integrins. Recently, chemistry of matrix materials has been used to vary the quantity and conformation of adsorbed fibronectin which

in turn was shown to influence the adhesion and differentiation of cells [24, 56]. Michael et al. [57] demonstrated an effect of varying surface chemistry of materials on the conformation of fibronectin in a coated layer; alterations in surface energy by varying surface functional groups (surface chemistry) affected the quantity of adsorbed fibronectin; neutral hydrophilic (OH) and hydrophobic (CH_3) groups promoted fibronectin binding to a greater extent than charged functional groups (-NH_2 and -COOH). Additionally, it was shown that surfaces modified with methyl, carboxyl, hydroxyl and amine groups respectively produced markedly different conformational changes in fibronectin coated on these surfaces, thereby allowing variation in exposure of sites capable of binding to specific integrins. This conformational variation was also shown to affect osteogenic differentiation in self assembled monolayers [56]. It was observed that surfaces containing hydroxyl and amine functionalities respectively promoted osteogenic differentiation to a greater degree than surfaces functionalized with carboxyl and methyl groups, respectively. Through immunological studies, it was determined that based on the surface chemistry (and hence conformation of fibronectin in the coated layer), different integrin binding sites (matrix ligands) were exposed and different cell surface integrins were activated. Based on the surface chemistry of the material, activity of binding sites for integrins α5β1 and αvβ3 were reported. It was observed that surfaces modified with carboxyl, amine and hydroxyl groups respectively promoted binding of integrin α5β1 to fibronectin; carboxyl modified surface additionally showed binding of integrin αvβ3 to fibronectin. Interestingly, mineralization (indicating osteogenic differentiation) was observed only on amine and hydroxyl modified surfaces. Treatment of carboxyl modified surfaces with β3 blocking antibody promoted mineralization on this surface; additionally treatment of amine modified surfaces with the same antibody led to an increase in mineralization. It is important to note that amine modified surfaces were expected to show greater β3 affinity than hydroxyl modified surfaces. These observations led the authors to suggest that binding of integrin α5β1 promotes osteogenic differentiation while αvβ3 suppresses it. Moursi et al. [44] reported that activity of integrin α5β1 is essential for triggering of upregulation of factors representing osteoblast activity. It has also been shown that over expression of αvβ3 in MC3T3-E1 cells suppresses osteoblastic activity [58].

Changes in surface chemistry also affect material hydrophobicity/hydrophilicty, thereby affecting protein adsorption on biomaterial surfaces. This in turn can potentially affect interaction between cells and materials [45] and has been characterized as interfacial energy [59]. Surfaces with net positive or negative charges tend to be more hydrophilic than neutral surfaces. Several studies have reported the effect of surface energy on binding of ECM proteins such as fibronectin, vitronectin, albumin, globulin and fibrinogen to material surfaces [60, 61]. Previous studies showed improved cell adhesion to hydrophilic surfaces [62–64]. Changes in surface energy can lead to conformational changes in adsorbed fibronectin, influencing binding to cellular integrins. Binding to lineage-specific integrins may activate signaling pathways specific to a particular lineage and thereby influence the differentiation of stem cells.

Lieb et al. [24] reported the effect of surface energy on osteogenic differentiation of marrow stromal cells. This study focused on decreasing the hydrophobicity of poly (D,L-lactic acid) by preparing poly (D,L-lactic acid)–poly(ethylene glycol)–monomethyl ether (PLA–PEG–MmE) diblock copolymers. Interestingly, PLA–PEG–MmE diblock copolymers showed lower cell attachment and proliferation than PLA, PLGA and tissue culture polystyrene. This was attributed to the decreased adsorption of serum proteins in the case of PLA–PEG–MmE due to decreased hydrophobicity. However, upon long term culturing on these materials, it was observed that cells cultured on PLA–PEG–MmE showed significantly higher alkaline phosphatase activity and greater degree of mineralization (evaluated through Von Kossa silver staining) than PLA, PLGA and tissue culture polystyrene. These serve as biomarkers for osteogenic differentiation and suggest that a small decrease in the hydrophobicity of the material promoted differentiation into osteogenic lineage. The authors attributed this to specific conformational changes in adsorbed proteins due to changes in surface energy, thereby exposing binding sites specific to integrins active during osteoblast activity. Such conformational changes in ECM proteins have previously been shown to modulate osteogenic differentiation on synthetic materials [56]. It is also mentioned however, that the PEG–PLA diblock copolymers also did show rough surfaces, which previously have indeed been shown to promote osteogenic differentiation [65, 66]. Indeed a study by Dalby et al. [67] demonstrated the influence of nanoscale surface topology on the osteogenic differentiation of hMSCs. In sum, conformational changes in adsorbed proteins and the resulting selective activation of cell adhesion molecules seem to serve as the predominant mechanism through which surface energy and chemistry of biomaterials promotes differentiation of stem cells.

2.2.3 Mineralization of Matrix Materials

The term ‘biomineralization’ refers to the modification of materials by integration with a crystalline/semicrystalline inorganic phase resembling that seen in mineralized tissues, such as bone or tooth enamel. Presence of mineralized coating consisting of an apatite layer mimicking bone mineral has been shown to extensively promote osseointegration of implant materials while also promoting bone healing [68]. Bone mineral is similar to hydroxyapatite ($Ca_5(PO_4)_3OH$), although studies of Raman spectra of bone suggest the substitution of hydroxyl group by carbonate groups in the crystal lattice [69]. Materials such as bioactive glasses and calcium phosphate based ceramics have demonstrated the ability to form a layer of apatite resembling bone mineral upon incubation in simulated body fluid, a solution mimicking the ionic composition and pH of plasma [68, 70]. Of late, there has been an increased interest in generation of mineral–polymer composite materials; one reason for this is the similarity of these materials to the native structure of bone consisting of a composite of an organic phase associated with a crystalline, inorganic phase. The successful syntheses of hydrogel–apatite composites showing strong adhesion between the two phases indicate the vast potential of polymeric matrices in this field.

Mineralization of Polymeric Matrices

A variety of methods have been used to incorporate inorganic apatite phases into polymeric matrices and have been discussed in detail by Kretlow and Mikos [71]. A popular method is the incorporation of anionic polar groups into scaffold materials; upon soaking in simulated body fluid (SBF), these groups serve as potential initiators of apatite nucleation by binding to calcium. For example, incorporation of anionic functional groups was observed to induce mineralization of poly hydroxyl-2-ethyl methacrylate (pHEMA) scaffolds upon soaking in simulated body fluid [72]. Supplementing the simulated body fluid with fetal bovine serum/albumin has been demonstrated to promote mineralization of pHEMA without any chemical modification [73]. In another study, Song et al. [74] achieved mineralization of pHEMA scaffold materials utilizing a pH-mediated templating process from the thermal decomposition of urea. This mineralization process has also been successfully utilized to generate apatite–polymer composites with polycaprolactone [75], PLGA and PLLA [76].

Effect of Mineralization on Cell Adhesion, Proliferation and Differentiation

Several studies have indicated that incorporation of a mineral phase into polymeric scaffold material serves to enhance attachment, proliferation and osteogenic differentiation of multipotent cells. Koç et al. [77] observed that rat MSCs seeded onto mineralized PLGA foams showed excellent adhesion to the scaffold material and underwent osteogenic differentiation. This study suggested that presence of a mineralized layer not only promotes osteoinduction of seeded cells but also promotes cell adhesion as a result of increased surface roughness. A study by Osathanon et al. [78] compared osteoinductive capacity of two kinds of mineral–polymer composites involving electrospun fibrin scaffolds: one wherein varying quantities of nanoparticles of hydroxyapatite were incorporated directly into the polymeric phase and one in which fibrin scaffolds were incubated in a solution containing concentrations of calcium and phosphate ions resembling those seen physiologically, leading to the deposition of a mineral. Upon seeding with murine calvarial cells and incubation with osteogenic medium, both of these materials showed enhanced expression of osteogenic markers (BSP, OCN, COL 1, ALP, CBFA 1 and OSX) as compared to non-composite scaffolds consisting of fibrin alone. However, mineralized fibrin scaffolds showed higher expression of these markers at earlier time points as compared to scaffolds prepared by incorporation of nano-size hydroxyapatite as well as higher alkaline phosphatase activity and a greater increase in calcium content. Additionally, mineralized fibrin scaffolds showed greater calcium phosphate dissolution than fibrin/nanosize hydroxyapatite scaffolds, leading the authors to suggest a link between extracellular calcium and phosphate concentrations and osteogenic differentiation through resultant upregulation of osteogenic markers (see Fig. 4).

This is supported in a study by Dvorak et al. [79] in which murine and rat fetal calvarial cells exposed to higher extracellular ionized calcium levels led to

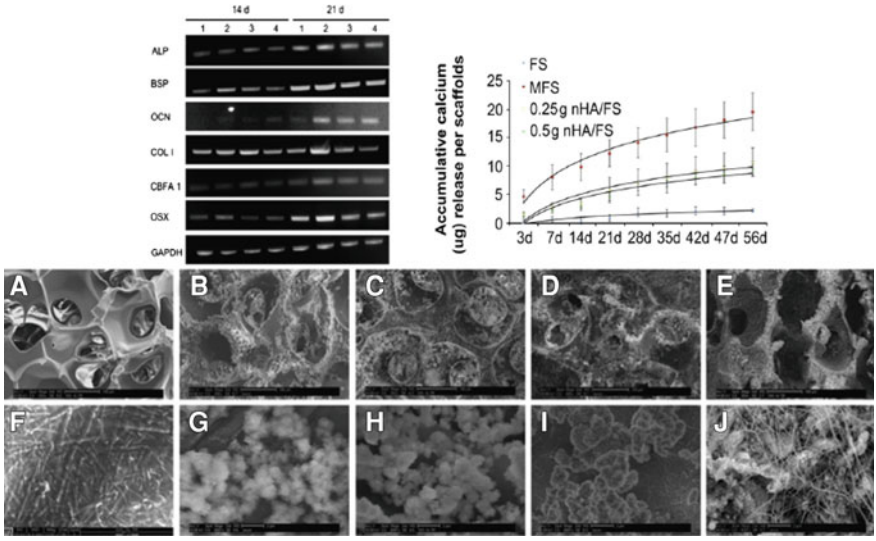

Fig. 4 *Top*, *left* gene expression for murine calvarial cells seeded on (*1*) fibrin scaffolds (*2*) mineralized fibrin scaffolds (*3*) fibrin scaffolds with 0.25 g nanosize hydroxyapatite and (*4*) fibrin scaffolds seeded with 0.5 g nanosize hydroxyapatite. *Top*, *right* dissolution profile for fibrin scaffolds (*FS*), mineralized fibrin scaffolds (*MFS*), fibrin scaffolds with 0.25 g nanosize hydroxyapatite (0.25 g *nHA*/*FS*) and fibrin scaffolds seeded with 0.5 g nanosize hydroxyapatite (0.5 g *nHA*/*FS*). *Bottom* Scanning electron micrographs of (**a**, **f**) FS in PBS for 14 days (**b–d**, **g–i**) *MFS* after soaking in simulated body fluid for (**b**, **g**) 24 h (**c**, **h**) 7 days (**d**, **i**) 14 days and (**e**, **j**) 0.5 ng HA/FS (adapted from [78] with permission, copyright Elsevier, 2008)

upregulation of core binding factor α-1, collagen type 1, osteopontin and osteocalcin, all of which are markers indicating osteogenic differentiation of progenitor cells. These findings imply that mineralized scaffolds may be able to promote osteogenic differentiation even in the absence of soluble factors often used to promote it. These mineralized scaffolds promoted osteogenic differentiation by exposing the murine calvarial cells to higher extracellular calcium levels in the microenvironment around the material due to dissolution of the inorganic mineralized phase. It is important however, to note that scaffolds undergoing excessively rapid dissolution may produce excessively high extracellular calcium concentration, thereby inhibiting osteogenisis [80].

While such methods have been used to ascertain the effect of mineralization of matrices formed from polymers of natural origin, similar reasoning can be used to predict the effect of mineralization of synthetic matrices on cell lineage. A study by Yu et al. [81] reports the ability of surface mineralization of nanofibrous ε-polycaprolactone scaffolds to stimulate osteogenesis of seeded rat MSCs. Mineralized scaffolds were found to promote cell adhesion and proliferation to a greater extent than unmineralized scaffolds. Additionally, cells seeded on unmineralized scaffolds showed increased proliferation. It was proposed by the authors based on microscopic evidence that cells seeded on mineralized scaffolds

reached confluence before unmineralized scaffolds thereby inhibiting further proliferation; this would promote differentiation of these cells.

As evidenced in the above studies, mineralization of synthetic scaffolds is an effective technique to promote cell adhesion, stimulate differentiation into osteogenic lineage and improve ossteointegration of synthetic implant materials. In other words, this technique shows promise in application to cell based therapies for the efficient healing of mineralized tissues.

2.2.4 Mechanical Properties

In addition to the effects of extracellular biochemical cues, reciprocal mechanical interactions between cells and environment have significant impact on the differentiation of stem cells [21]. The effects of mechanical forces on cells due to the matrix can be observed from single cell level to the development of complex tissues. At the single cell level, adhesion of cell to a material with specific stiffness triggers signaling transduction cascades allowing translation of extracellular mechanical cues into intracellular events [82]. Dynamic interactions between cell and matrix control several cell behaviors such as spreading, migration and cell shape through binding with integrins, the chief mechanotransducers for cells [83]. For example, cell geometry (spreading) is a result of pre-stress between ECM and cellular microtubules [84]. Previous studies have demonstrated that cell spreading controls processes such as proliferation and apoptosis [85]. The ability of various types of cells to respond to mechanical differences in the extracellular environment has been described in detail in a review by Discher et al. [86]. A recent study by Engler et al. [21] showed that stem cell differentiation can be directed by varying elasticity of matrix. In this study, hMSCs were cultured on 2D polyacrylamide hydrogels with different elasticity prepared from using different amount of crosslinker. Neurogenesis, myogenesis and osteogenesis of hMSCs were observed on soft, medium and stiff hydrogel matrices, respectively. Khatiwala et al. [87] have evaluated the effect of ECM compliance on osteogenesis of progenitor cells and the signaling pathways involved. ECM compliance was found to affect activity of extracellular signaling kinase (ERK), with stiffer matrices promoting osteogenic differentiation. Additionally, a potential mechanism suggested by the authors was the downstream ERK–mitogen-activated protein kinase (MAPK) activation of the RhoA–Rho associated protein kinase (ROCK) signaling pathway.

2.3 Biomaterial Based Delivery of Soluble Factors for 3D Cell Culture

2.3.1 Incorporation of Bioactive Agents into Matrix Materials

In addition to the extracellular matrix, soluble bioactive agents (such as growth factors, hormones, proteins, small molecules, cytokines and chemokines) also play

many roles in regulating proliferation and differentiation of stem cells. Incorporation of bioactive agents into growth medium represents the simplest way to harness the beneficial effects of such factors on stem cell differentiation [88]. However, this approach suffers from certain limitations. For instance, hydrophobic agents show poor solubility in aqueous media. Hydrophilic bioactive agents can be readily dissolved but their stability is strongly dependent on environmental factors such as ionic strength, pH and enzymatic degradation/inactivation [89]. This approach has major limitations for 3D cell-laden systems of critical size due to limited degree of diffusion across cell-laden matrices. Heterogeneous differentiation may occur due to non uniform distribution of growth factors throughout the cell laden construct upon their delivery through incorporation into medium. Additionally, growth factor signaling during development involves precise concentration of these factors and their spatial and temporal gradients. Figure 5 represents the various methods by which bioactive agents can be delivered to progenitor cells via functional biomaterials.

A variety of biomaterial-based technologies have been developed of late to precisely control delivery of bioactive agents to stem cells in a spatiotemporal manner. One such approach involves the direct incorporation of bioactive agents within the biomaterial; however, this bolus delivery approach does not provide sustained delivery of the desired agents. Studies have shown that manipulation of material microstructure allows retention of growth factors within the scaffold thereby offering their sustained delivery to embedded cells. For instance, β-sheet microstructure was created in one study by treating lyophilized silk with an organic solvent. The steric hindrance effect arising from resultant β-sheet microstructure was thought to contribute to the sustained release of IGF-I from scaffolds constructed with these modified silk materials [90].

Another approach involves nano- and micro- carrier based delivery vehicles in which carriers containing the bioactive agents of interest are dispersed within the 3D scaffold seeded with stem cells to achieve localized controlled delivery of the agents. Such sustained release of soluble factors to differentiating stem cells inside

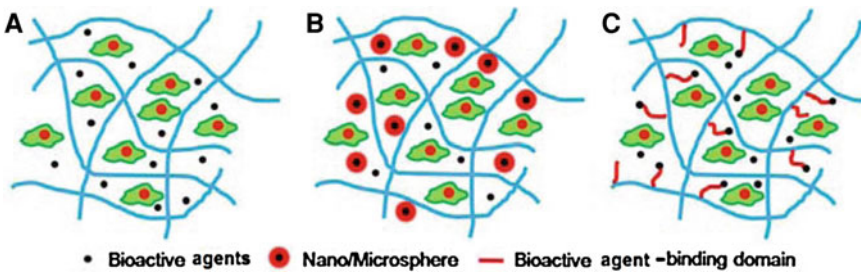

Fig. 5 Schematic drawing of various methods used to deliver bioactive molecules to cells within a biomaterial scaffold. **a** Dispersion of the agent within the scaffold material. **b** Encapsulation of bioactive agents in degradable nano/microspheres. **c** Use of interactions between bioactive agents and binding domains in the scaffold material

biomaterial matrices usually exhibits higher differentiation efficiency. Potential toxicity is also minimized by preventing the effects of administration of soluble factors at excessively high doses, referred to as 'dose dumping'. Thus, the incorporation of delivery vehicles carrying bioactive agents into tissue scaffolds allows for the engineering of stem cells in a 3D defined microenvironment [91]. The release profile of soluble factors from carriers within the scaffold depends on various properties of the biomaterial such as porosity [92–94], composition [92, 93], degradability and microstructure [90]. Generally, biomaterial chemistry determines the strength of interactions between soluble factors and the material [94]. Soluble factors not interacting appreciably with materials can diffuse freely and thus shorten the delivery time to the encapsulated cells. Due to the sensitivity of bioactive molecules it is important to use mild encapsulation conditions so as to avoid their deactivation and/or degradation, again restricting the choice of biomaterial [89, 95]. Commonly used delivery vehicle systems include biodegradable nano/microsphere (beads) [96] and polyelectrolyte complexes, formed by electrostatic interactions between the bioactive molecules and materials. A variety of biodegradable synthetic polymers such as PLA, PGA, PLGA, PEG–PLGA, PEG–PCL have been widely used to prepare drug/protein-loaded nano/microspheres [96]. In addition to synthetic biodegradable polymers, natural materials such as gelatin and chitosan can also be used to prepare protein-loaded nano/micro particles. The application of degradable particle allows the sustained protein release via particle degradation.

2.3.2 Effects of Controlled Delivery of Bioactive Agents on Stem Cell Differentiation

Delivery of Bioactive Agents to Embryonic Stem Cells

Addition of soluble bioactive molecules directly into culture medium represents a convenient approach for directing ESCs differentiation (through embryoid body, EBD, formation) with modest efficiency [88]. Homogenous delivery of soluble bioactive agents into EBD is highly challenging, because of their 3D spheroid nature [97]. As a result, cell differentiation within embryonic bodies is usually heterogenous and disordered due to the inefficient intra-EBD transport of soluble factors. Sachlos et al. [97] have characterized the composition of the shell surrounding EBDs and have reported improving diffusive transport into the EBD interior through the disruption of this membrane. The shell was found to consist of ECM comprised of collagen type I, a squammous cellular layer with tight cell–cell adhesions associated with E-cadherin and a basement membrane, as indicated by the presence of collagen type IV lining. In the latter part of the study, this basement membrane was disrupted either by preventing its formation with noggin or degrading it using collagenase. This treatment was found to increase the diffusive transport of retinoic acid and subsequently promote the neuronal differentiation of the ESCs.

Several carrier-based methods such as peptide-transmembrane domain (PTD)-protein conjugates [98] and microparticles [99] have been developed to further increase the delivery efficiency. Similarly, Carpanedo et al. [100] have adapted in situ release of retinoic acid to the interior region of EBDs using degradable PLGA microspheres. The in-situ release of soluble factors within EBDs has showed promising results by promoting homogenous differentiation of ESCs within EBDs into phenotypes resembling those observed in early mouse streak embryos (see Fig. 6).

Other studies such as those by Newman et al. [101] and Nojehdehian et al. [102] have demonstrated the efficacy of utilizing degradable PLGA microspheres to deliver retinoic acid to pluripotent P19 embryonic carcinoma cells for differentiation into neuronal lineage. As stated by Newman et al. these microspheres served two purposes viz. the delivery of retinoic acid to the cells and as potential transplantation matrices to support cell attachment, growth and differentiation. Nojehdian et al. also demonstrated that the presence of a poly-L-lysine coating on the microspheres further enhanced neuronal differentiation of the embryonic carcinoma cells.

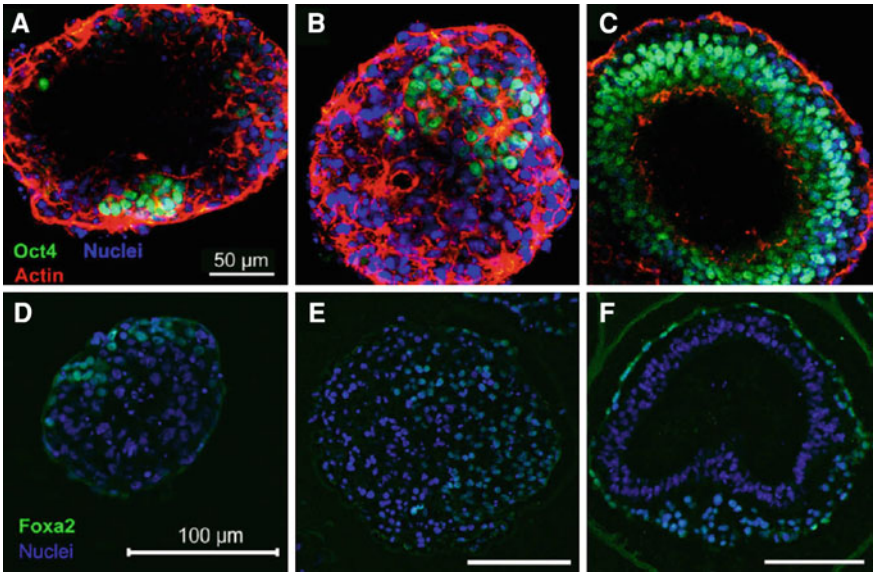

Fig. 6 Immunostaining of embryoid bodies. OCT4 staining was performed on day 10 untreated embryoid bodies (**a**), embryoid bodies with unloaded microspheres (**b**) and embryoid bodies with retinoic acid-loaded microspheres (**c**). Embryoid bodies with untreated and unloaded microspheres contained clusters of OCT4t cells, while OCT4t cells in embryoid bodies with retinoic acid-loaded microspheres were localized to the columnar cell layer. FOXA2, a marker of visceral endoderm, was also expressed in clusters of untreated (**d**) and unloaded MS EBs (**e**), but was localized to the outermost layer of cell in RA MS EBs (**f**). **a–c**, *bar* 50 mm; **d–f**, *bar* 100 mm. (Adapted from [100] with permission, Copyright Elsevier, 2009)

Tissue Specific Differentiation of Stem Cells Using Delivery of Bioactive Agents

Osteogenic differentiation of stem cells can be stimulated with various growth factors including bone morphogenetic proteins (BMPs), dexamethasone, bFGF and platelet rich plasma (PRP) derived growth factors. The sustained release of bioactive agents such as dexamethasone from microparticles has been shown to enhance osteoegenesis of stem cells in a 3D environment [103]. In this study, carboxymethylchitosan/poly(amidoamine) dendrimer loaded with dexamethasone was used to enhance the osteogenesis of rat bone marrow stromal cells (RBMSCs) growing on porous degradable hydroxyapatite and starch–polycaprolactone scaffolds, respectively [103]. Though biodegradability of the materials is a major consideration, it is extremely important to study biomaterials with inherent growth factor binding capability. In studies by Anseth et al. [104, 105], heparan and PEG were used to fabricate scaffolds for sustained growth factor delivery. Heparan, a highly sulfated glycoaminoglycan, contains binding domain for various growth factors such as bFGF and BMP-2. Their work showed that sustained release of bFGF (up to 5 weeks) from heparan domains in the scaffolds increased the expression of alkaline phosphatase (ALP) and other osteogenic markers such as collagen I and osteopontin at the transcriptional level. In another study by the same research group, Benoit et al. made use of fluvastatin release from PEG hydrogel scaffolds in order to activate release of BMP-2 from encapsulated hMSCs, thereby stimulating osteogenic differentiation of these cells [106]. In a study by Basmanav et al. [92], microspheres prepared by complexing poly(4-vinyl pyridine) (P(4)VN) and alginic acid loaded with the growth factors BMP-2 and BMP-7 showed enhanced osteogenic differentiation of bone marrow stem cells (BMSC) in porous scaffolds. In another study [94], the combination of degradable chitosan beads with degradable scaffolds (Porous nano-hydroxyapatite/collagen/poly(L-lactic acid)/chitosan microspheres (nHAC/PLLA/CMs)) supported sustained release of active BMP-2 derived synthetic peptides, the release rate of which was dependent on the degradation rate of both microspheres and scaffold. Enhanced osteogenesis of rabbit marrow mesenchymal stem cells was achieved using this system.

Similar to osteogenesis, bioactive agents are required to control and or enhance chondrogenic differentiation of stem cells. Chondrogenic differentiation of MSCs in vitro is often achieved by culturing them in a three-dimensional (3D) condition in the presence of TGF-β superfamily growth factors [107, 108]. The incorporation of these chondrogenic morphogens into biomaterial scaffolds is advantageous due the ability of this method to provide spatially and temporally moderated delivery of bioactive agents. In one study, a dual delivery system was designed by using double bead microspheres to deliver dexamethasone (DEXA) and dehydroepiandrosterone (DHEA) simultaneously to engineer inflammation-free tissue in the vicinity of the implant [96]. These microspheres contained a PLGA core which hydrolyzed in aqueous environment, releasing these two chondrogenic factors simultaneously. Up-regulation of collagen II, aggrecan, GAGs, cartilage oligomeric matrix protein (COMP) and down-regulation of collagen I were observed

from mMSCs. Another example of dual delivery systems is the delivery of TGF-β and dexamethasone from PLGA scaffolds [91]. In another study, increased chondrogenesis was observed upon release of IGF along with TGF-β release from silk scaffolds [90]. In a study by Park et al. [109] gelatin microparticles loaded with insulin-like growth factor-1 (IGF-1) and transforming growth factor β1 (TGF-β1) were incorporated into a degradable PEG fumarate hydrogel. These two growth factors were released from gelatin particles via simple diffusion. The degradation of PEG-fumarate hydrogels further enhances the delivery efficiency by increasing the mobility of encapsulated nano/microparticles which promoted chondrogenesis of rabbit MSCs. Researchers have also utilized the ability to manipulate the spatial- and temporal release of growth factors from biomaterial based delivery vehicles for engineering composite tissues. For instance, Wang et al. [110] have incorporated microspheres containing growth factors, BMP-2 and IGF-1, in a gradient fashion within the alginate scaffold to regulate osteochondral differentiation of MSCs.

2.4 In Vivo Applications

As seen above, the ability of functional materials to influence the terminal phenotype of various stem cells is quite well-illustrated. In addition to this, several studies have illustrated the ability of similar functional materials to recruit progenitor cells and promote the formation of new tissue in vivo, particularly cartilage and bone tissue. A study by Sharma et al. demonstrates in vivo chondrogensis of goat MSCs encapsulated in photopolymerized PEG hydrogels containing hyaluronic acid (HA) along with TGF-β, followed by the generation of cartilage-specific ECM (specifically collagen II and proteoglycan) upon subcutaneous injection into athymic nude mice [111]. Inclusion of HA and TGF-β in the hydrogels was found to promote proteoglycan synthesis; in the absence of HA, there was significant expression of collagen I, while no proteoglycan was produced in the absence of TGF-β. This synergetic effect of TGF-β and HA on promoting chondrogenic differentiation was attributed to the possibility of increased retention of TGF-β within the construct through restriction of its diffusion by inclusion of HA. The technique of cell encapsulation has also been used to create a tissue engineered mandibular condoyle [112, 113]. In these studies, chondrogenic and osteogenic cells derived from rat MSCs were encapsulated in PEG hydrogels; upon implantation, these constructs were found to contain stratified layers of both, osseous components such as mineral nodules and cartilaginous components such as glycosaminoglycans. Several in vivo studies in which functional materials are used to differentiate and/or deliver stem cells are summarized by Chai and Leong [114]. In one such study by Levenberg et al. [115], scaffolds consisting of poly(L-lactic acid) and poly(L-lactic-co-glycolic acid) were used in conjunction with various chemical cues such as retinoic acid, activin-A, TGF-β and Insulin Growth Factor 1 (IGF-1) respectively to commit them to germ layers to develop into tissues such as

liver, cartilage and nervous tissue. Upon implantation of these constructs in severe combined immunodeficient (SCID) mice, these constructs were found to maintain their viability for at least 2 weeks. Additionally, evidence of integrating with host vasculature was also observed. In another study, Cho et al. [116] demonstrated in vivo adipogenesis of adipocyte derived stem cells seeded in phospholipase/poly(glycolide) scaffolds upon implantation in athymic mice, although in this case, the scaffold served predominantly to provide mechanical support to the construct, cells and the resultant extracellular matrix.

Lee et al. [117] used thermosensitive hydrogels consisting of triblock copolymer of poly(ethylene glycol-b(DL-lactic acid-co-glycolic acid)-b-ethylene glycol) (PEG–PLGA–PEG) for the efficient engraftment of muscle derived stem cells (MDSCs) for the efficient healing of diabetic wounds. It was observed that MDSCs differentiated into fibroblasts, myofibroblasts and endothelial cells, thereby accelerating the healing and subsequent closure of the diabetic wound. In another study, Boldrin et al. [118] demonstrated the efficient delivery of human muscle precursor cells (hMPCs) by means of a degradable poly(glycolic acid) scaffold. Upon implantation in CD1 nude mice, these scaffolds showed a human nuclear antigen signal (utilized to evaluate the presence of human-origin cells as opposed to the host mouse cells) nearly threefold of that obtained by mere injection of hMPCs, indicating a much higher efficiency of delivery in the case of the cell-seeded polymer scaffolds. Kim et al. [119] have demonstrated the ability of committed adipose derived stem cells (ADSCs) in conjunction with injectable PLGA microspheres to form muscle tissue in vivo. ADSC-seeded PLGA microspheres were cultured in myogenic medium for 21 days and then injected subcutaneously into the necks of nude athymic mice. Mice injected with PLGA microspheres alone showed the formation of acellular matrix at the injection site while those injected with cell-laden microspheres showed the formation of muscle tissue morphologically resembling native muscle.

2.5 Future Perspectives

Both genomic [120] and proteomic [121] approaches have been intensively studied to elucidate the complex molecular regulation network behind stem cell differentiation. With these advances, more and more molecular markers for stem cell differentiation have been discovered. These differentiation markers in combination with high throughput assay-based methods have facilitated the discovery of biologically active small molecules and new materials for controlling stem cell engineering [122, 123]. The advances in this field could lead to the development of novel biomaterials with higher efficiency in controlling tissue specific differentiation of stem cells. While several advances have been made in developing materials capable of controlling stem cell differentiation, there are a multitude of areas which show great promise in revolutionizing stem cell based therapies. One such area is the synthesis of materials showing

anisotropy in their chemical and physical properties, especially across controlled gradients. Such materials would be especially useful in cell-based therapies for the repair of interfaces between different tissues such as the osteochondral interface. Controlled variations in material properties can allow for the simultaneous differentiation of common progenitor cells into multiple phenotypes on a single scaffold. Another potentially promising area is the development of self-healing materials capable of supporting cell adhesion and differentiation. Previous studies have investigated self healing using microvascular networks in materials [124]; self healing materials may also be used in conjunction with cells to mend damaged tissues, providing mechanical support at the damage site while simultaneously directing differentiation of cells to the appropriate phenotype. Native tissue cells are also subjected to dynamic mechanical cues and therefore developing advanced multi-functional scaffolds that can provide dynamic chemical and mechanical cues to encapsulated cells beyond being a structural support and/or static chemical and mechanical cues will have a profound influence on the field of biomaterials and stem cell engineering.

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