Integration of Biomaterials into 3D Stem Cell Microenvironments

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Abstract Stem cells receive physical and chemical cues capable of influencing their phenotype from inter-related elements of the microenvironment, such as cell–cell contacts, soluble molecule signals and physical interactions with the ECM. In contrast to conventional 2D culture systems, barriers to diffusion within 3D cultures limit the effectiveness of media manipulation as a method to direct cell behavior. Efforts to engineer stem cell microenvironments in 3D using biomaterials have generally been attempted by either scaffold seeding, cell encapsulation, or microcarrier/microparticle based approaches. These different methods have been applied not only for the propagation of pluri- and multipotent stem cells, but also to direct the differentiation of such stem cells into more differentiated phenotypes. This chapter discusses the unique benefits, as well as associated challenges of integrating biomaterials into 3D stem cell microenvironments.

1 Introduction

Stem cell fate is regulated by transcription factors that control gene and protein expression. The balance between self-renewal and differentiation of stem cells is determined by the sensitivity of gene regulation to changes in the extracellular microenvironment. A complex assembly of soluble factors, cell–cell and cell–extracellular matrix interactions constitute the molecular regulators of the microenvironment through which stem cell fate is determined [1]. Particularly in the case of pluripotent and multipotent stem cells, the microenvironment requires

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special consideration because of the vastly divergent array of cell lineages that can be adopted by such stem cells. In the developing embryo, morphogen gradients, cell–cell and cell–extracellular matrix signals are integrated to provide essential spatial and temporal information to direct cell differentiation, migration and tissue formation.

Pluripotent embryonic stem cells (ESCs) can differentiate into cells of all three germ lineages (ecto-, endo- and mesoderm); however, when the microenvironment is not strictly regulated, ESCs differentiate in a spontaneous, uncontrolled manner. Thus, much of the current focus of ESC research is to better understand environmental mechanisms regulating cell differentiation and to develop the essential technologies to direct differentiation more efficiently towards a homogeneous population of cells that could be used for regenerative therapies. Recently, the ability to produce "induced" pluripotent stem (iPS) cells from human somatic cell types has been reported, allowing for patient specific pluripotent cells to be developed, and thereby avoiding the potential immune rejection of allogenic ESCs [2, 3].

Multipotent adult stem cells, present in most tissues of mature organisms, serve to repair and regenerate tissues and thereby enable organisms to live beyond the lifespan of individual cells [4]. Multipotent stem cells are also responsive to signals in the microenvironment capable of directing self-renewal or differentiation. For example, in many tissues, contact, or a loss of contact with a specialized basement membrane is believed to influence the fate of stem cell daughter cells [4].

Stem cells are typically expanded in an environment intended to maintain "stemness" before being subjected to a different set of conditions designed to promote differentiation, preferably to a specific phenotype. Biochemical factors such as basic fibroblast growth factor (bFGF) for human ESCs [5, 6] or leukemia inhibitor factor (LIF) for mouse ESCs [7, 8] are routinely added to the culture medium to grow ESCs in an undifferentiated state. Withdrawal of these factors from the culture media allows differentiation to proceed. In contrast, specific factors have yet to be identified to stably maintain mesenchymal stem cells (MSCs) and extended passaging of MSCs in vitro generally results in genetic abnormalities and reduced differentiation potential.

The substrate on which stem cells are cultured also can be used to inhibit or promote differentiation. Feeder layers of cells can be used, such as mouse embryonic fibroblasts (MEFs) [5], to promote ESC self-renewal. In addition, ECM coatings, such as Matrigel^{TM}, a complex mixture of basement membrane components isolated from mouse tumor cells, or gelatin can be used as alternative surface coatings to feeder cell layers [9]. Matrigel^{TM} and MEFs contain or produce ECM and soluble factors which favor ESC self-renewal and growth while limiting cell differentiation. The introduction of xenogenic material, either through the substrate, or through animal serum, can lead to the expression of immunogenic animal proteins on human stem cells, limiting their clinical uses [10]. Thus, chemically defined media, which avoid the introduction of animal serum proteins, have been produced in recent years for the purpose of presenting the most basic biochemical factors required for stem cell growth and self-renewal [11]. Defined culture media and ECM substrates can also be advantageous in directed differentiation protocols in order to present defined

amounts of molecules and avoid presentation of conflicting signals which could contribute to heterogeneous differentiation.

1.1 Culture in Two or Three Dimensions

Traditionally, two-dimensional culture (2D) is used to maintain cells in an undifferentiated state whereas three-dimensional (3D) culture techniques are more commonly implemented in differentiation protocols. For example, ESCs are commonly differentiated through the formation of 3D multicellular aggregates, referred to as embryoid bodies (EBs) [12]. Spheroid culture provides a platform for scalable culture of cells because they can be grown in suspension and require a lower tissue culture surface area-to-volume ratio compared to cells in monolayer. The 3D spheroid microenvironment is complex and becomes more so as differentiation progresses. Differentiating cells deposit extracellular matrix and are subjected to homo- and heterotypic cell–cell interactions, as well as autocrine and paracrine factors.

Soluble factors added to the culture media are more accessible to cells grown in monolayer and the temporal resolution of these factors can be fairly well controlled by simply exchanging the culture media at specific times [13]. In contrast, 3D culture provides more physiological cues such as increased cell–cell interactions and the potential for increased cell–extracellular matrix interaction; however, diffusion limitations complicate 3D cultures of cells. Concentration gradients of soluble factors added to the media, which result from the diffusion properties of 3D cellular aggregates or constructs, can result in differences in the microenvironment depending on the spatial positioning of cells. Stem cells can be very sensitive to small perturbations in the biochemical composition of their surroundings and the effects of soluble factors often vary in a dose-dependent manner. This limited control of the 3D environment has necessitated the development of biomaterial technologies to engineer the microenvironment of 3D culture systems in order to further develop stem cell differentiation protocols.

1.2 Strategies for Biomaterial Control of the 3D Microenvironment

As introduced above, adhesive protein biomaterials (i.e. Matrigel and gelatin) have been used as culture substrates for stem cell maintenance culture in 2D. Biomaterials can also be integrated within 3D stem cell environments in order to control the increased complexity of the microenvironment. Strategies to control stem cell behavior using biomaterials have largely aimed to deconstruct elements of native biological complexity and integrate defined components into controlled systems to present molecular cues to stem cells.

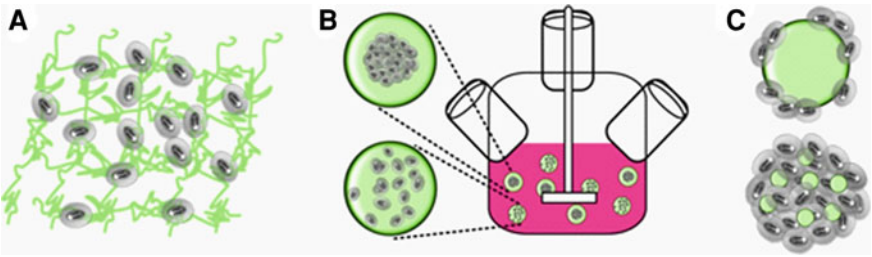

Fig. 1 Biomaterials can be incorporated within 3D stem cell microenvironments to direct cell behavior. Cells can be cultured on or within polymeric scaffolds (**a**) which provide physiochemical cues. Encapsulation of cell aggregates or single cells (**b**) can be used to increase the surface area to volume ratio for scalable culture and to provide an artificial matrix. "Inside–out" approaches to direct the microenvironment include culture of cells on microcarriers (**c**, *top*) and the incorporation of microparticles within stem cell aggregates (**c**, *bottom*)

Parameters such as hydrophobicity, porosity, degradation kinetics and surface coating can be engineered to create the desired material properties. The ability to engineer biomaterial surface properties can be utilized to present insoluble factors to mimic cell–cell or extracellular matrix interactions. Stem cells and biomaterials can be combined as scaffolds in the classic tissue engineering paradigm, wherein a spongy or fibrous scaffold provides mechanical support for attachment and migration guidance initially and then degrades as the cells produce their own natural scaffolding. In addition to scaffolds, which have been used extensively with other cell types, single cell suspensions or EBs are often completely encapsulated in either a natural ECM matrix or in a polymer designed to provide differentiation cues. Encapsulation typically occurs in the form of small spherical beads (hundreds of microns in diameter) grown in suspension culture whereas scaffolds are much larger and are grown in static or perfused cultures. Another approach is to integrate biomaterials within stem cell spheroids, either using microcarriers or microparticles, to control the microenvironment from the "inside–out." Strategies utilizing scaffolds, encapsulation and microcarrier/microparticles for control of the stem cell microenvironment are depicted in Fig. 1 and are discussed in further detail below.

2 Scaffolds

The application of polymeric scaffolds to support somatic cells was one of the original tenets of tissue engineering strategies [14]. Polymer scaffolds were originally designed primarily as carriers for cell transplantation which provided temporary structural support until cells could adequately synthesize their own matrix to replace the biodegradable synthetic material. However, advancements in the field of biomaterials have led to the development of more sophisticated scaffolds in various forms (e.g. porous, fibrous), capable of responding to

environmental changes (e.g. temperature, pH, electrical stimulation, proteases) and directly incorporating biomolecular cues to mediate cell attachment, proliferation and differentiation. Recently, pluripotent and multipotent stem cells have been seeded onto polymer scaffolds as a means to examine self-renewal and differentiation properties in 3D.

Scaffolds have been studied in combination with mesenchymal stem cells (MSCs) for a wide variety of applications including bone and cartilage regeneration. The ability of MSCs, derived from bone marrow or other tissues (e.g. adipose tissue), to differentiate into a variety of cell types, including osteo-, chondro-, and adipogenic lineages, has made MSCs the most common cell source for musculoskeletal tissue engineering strategies. A variety of synthetic and natural polymers have been utilized for both osteogenesis and chondrogenesis of MSCs, including nanofibrous electrospun poly(caprolactone) (PCL) [15–17], PLGA [18–22], and silk [23–26]. The ability of multiple scaffold types with a wide range of chemical and physical properties to support MSC proliferation and differentiation makes this a promising and active area of stem cell research.

Pluripotent stem cells can likewise be cultured on or within scaffolds, and much of the knowledge gained from previous studies with MSCs can be applied to ESC culture. As discussed above, culture of undifferentiated ESCs is typically performed in monolayer, with cells grown on either an inactivated MEF feeder layer or Matrigel. However, the use of synthetic scaffolds for self-renewal culture may circumvent the issues of xenogenic contact and scale-up feasibility associated with MEF and Matrigel substrates. The culture of hESCs on an artificial matrix composed of semi-interpenetrating polymer networks (sIPN) supported short-term maintenance of pluripotency [27]. The sIPN hydrogels were functionalized with the arginine-glycine-aspartic acid (RGD) peptide sequence, and RGD concentration, as well as the mechanical properties of the hydrogel, were varied independently to identify conditions which promoted self-renewal of hESCs. Artificial extracellular matrices for stem cell renewal can be used as a xeno-free alternative to defined media.

Additionally, ESCs have been cultured on nanofibrillar polyamide matrices known as Ultra-Web under self-renewal conditions [28]. ESCs grown on Ultra-Web displayed higher alkaline phosphatase activity, indicative of pluripotency, as well as enhanced proliferation, relative to gelatin-coated glass coverslips. Activation of Rac, a small GTPase, was also enhanced in cells cultured on Ultra-Web, as was activation of the PI3K pathway and Nanog expression. These data indicate that the 3D architecture on which cells are cultured may play an important role in cell fate determination and must be taken into account in future design of cell culture systems for ESC self-renewal.

Synthetic scaffolds have also been applied to differentiation approaches for ESCs. Porous scaffolds composed of PLGA/PLLA have been investigated as substrates for hESC adhesion and differentiation, with the intent of forming complex tissue architectures to be used in transplantation therapies [29]. The combination of seeding human ESCs on porous scaffolds and media supplementation with growth factors was found to induce differentiation into various

cell types that expressed markers of neural, chondrogenic and hepatic lineages. Cells remained viable on the scaffolds following implantation in severely immune compromised mice, and continued differentiation and reorganization was observed. Studies focusing specifically on neural differentiation of hESCs seeded onto PLGA scaffolds were performed, with the effect of various media supplements reported [30]. The addition of nerve growth factor and neurotrophin 3 to the scaffold cultures enhanced differentiation to nestin and βIII tubulin positive cells, indicative of neural progenitors and neurons, respectively. However, formation of functional, higher-ordered tissues will likely require greater sophistication in scaffold architecture and differentiation-cue presentation to ESCs.

Scaffolds composed of biomimetic and natural polymers have been used in scaffold fabrication in order to present instructive microenvironments to pluripotent cells. ESCs cultured on the biomimetic material Cytomatrix formed 3D structures similar to EBs, but displayed enhanced ECM production as well as increased efficiency in differentiation to hematopoietic precursor cells [31]. Genes associated with ECM production as well as proliferation and differentiation were found to be enhanced relative to traditional EBs [32]. Incorporation of ESCs into porous alginate scaffolds resulted in efficient, homogeneous EB formation, with EBs spatially restricted within the pores. Agglomeration of EBs appeared to be inhibited, resulting in efficient cell proliferation and differentiation [33].

Fibrin scaffolds have also been investigated for use in directed ESC differentiation to neural cell types [34]. Cells from both dissociated and intact EBs were seeded within fibrin scaffolds, and conditions including cell density and fibrinogen and thrombin concentration were optimized for cell proliferation and differentiation. After 14 days, successful differentiation of ESCs into neurons and astrocytes was observed. In an independent study, cells were seeded onto fibrin scaffolds as well as PEGylated fibrin scaffolds, and proliferation and differentiation were assessed relative to traditional EBs as well as EBs grown in semi-solid methylcellulose [35]. Proliferation in both types of fibrin scaffolds was enhanced relative to EBs and methylcellulose EBs. Culture in non-PEGylated fibrin resulted in differentiation similar to that observed in traditional EBs, with down-regulation of OCT4 and expression of VE-Cadherin, while ESCs growth in PEGylated fibrin were more similar to methylcellulose controls.

Semi-interpenetrating polymer networks composed of the natural polymers collagen, fibronectin and laminin were examined as scaffolds for ESC differentiation [36]. Differentiation was found to be a function of both network composition and concentration, as high collagen concentration inhibited EB cavitation, fibronectin appeared to enhance endothelial differentiation, and laminin enhanced cardiomyogenesis. Use of the self-assembling nanofibrillar peptide scaffold PuraMatrix was investigated for osteogenic differentiation of ESCs [37]. EB-derived cells were seeded onto PuraMatrix scaffolds after 8 days of differentiation, and cells plated onto tissue culture polystyrene served as a 2D control. Both PuraMatrix and 2D substrates supported osteogenic differentiation,

although maintenance of OCT4 positive cells was more prevalent in 3D. Scaffolds can be used to control physiochemical elements of the microenvironment, however, their use for large scale production of homogeneous cells may be limited due to diffusional limitations of nutrients in large constructs lacking vasculature.

3 Encapsulation

Encapsulation of stem cells into hydrogels represents a scalable way to present factors locally to cells. Unlike scaffolds, large numbers of capsules can be cultured in suspension culture. Encapsulation of stem cells can affect diffusion, control aggregate size and prevent agglomeration as well as provide an instructive environment depending on the properties of the material chosen for encapsulation. From a bioprocessing perspective, encapsulation provides a method to grow anchorage dependent cells in suspension thereby increasing the surface area to volume ratio and scale up potential. MSCs are difficult to maintain as aggregates in suspension [38] and for this reason they are often studied using encapsulation. In addition to bioprocessing advantages, the creation of an artificial matrix aids in study of cellular response to specific elements of native ECM. Elements such as material stiffness or peptide density can be varied independent of other factors.

Single cell suspensions or cell spheroids can be encapsulated several ways depending on the material properties. Thermosetting hydrogels such as agarose can be used to encapsulate cells by emulsifying a mixture of agarose, cells and oil. Agarose capsules containing EBs are formed in the oil phase after emulsification and can be gelled by lowering the temperature below the gelation point of agarose [39]. Materials such as alginate are useful for encapsulation because gelation occurs in the presence of Ca^{2+} ions and does not require oil phase emulsion or temperature change that can lower cell viability. Precisely sized droplets of alginate and cells can be created and gelled in $CaCl_2$ baths and cells can be retrieved at later time points after transfer to a medium without Ca^{2+} [40–43]. Artificial polymers such as poly(NiPAAm-co-AAC) [44], poly(ethylene glycol) (PEG) [45], and PEG derivatives such as PEG diacrylate [46, 47] and oligo(poly(ethylene glycol)) fumerate [48] have been utilized as well.

Encapsulation also provides a method to investigate interactions between cells and specific signaling sequences in an artificial ECM environment in which the ligand density can be precisely controlled. For example, alginate can be modified to present small peptide sequences such as arginine-glycine-aspartic acid (RGD) [43], found on ECM proteins such as fibronectin, fibrin and vitronectin. Increasing RGD density in alginate gels resulted in dose dependent decrease in encapsulated MSC response to TGF-B1 and dexamethasone, components of chondrogenic media. It was hypothesized that integrin mediated signaling may be responsible for inhibition of chondrogenesis in the cells and control of integrin signaling may be a

useful target for directed differentiation strategies. Mimicking ECM interactions using small peptide sequences can aid in understanding the mechanisms by which ECM components contribute to the cellular microenvironment.

In regards to ESCs, encapsulation originated as a method to control the homogeneity of differentiation culture. ESCs express high levels of E-cadherin, a homotypic cell–cell adhesion molecule, which has been shown to be primarily responsible for EB formation in suspension culture [39, 49]. High levels of E-cadherin also can lead to agglomeration which is particularly problematic in static cultures. Agglomeration leads to heterogeneity in EB culture and contributes to the heterogeneity of the resultant differentiated cell population. In addition, the size of the initial ESC aggregate has been implicated in the trajectory of subsequent differentiation, and therefore precise size control of EB formation is considered advantageous [50–52]. In addition, EBs formed using other methods can be later be encapsulated with one EB/capsule to prevent agglomeration and to shield EBs from shear forces experienced in a stirred bioreactor. ESCs can be encapsulated as a single-cell suspension and depending on the material used and the size of the capsule formed, the result can be single EBs or small individual clumps of cells.

ESCs can be encapsulated in natural polymers such as hyaluronic acid [53] or alginate [54] to maintain a pluripotent state useful for production of large amounts of cells. Cells can then either be retrieved from the gels or switched to differentiation conditions for further culture. Retrieval from hyaluronic acid encapsulation requires that the capsules be incubated in hyaluronidase, while alginate capsules can be depolymerized through the removal of divalent cations. Encapsulation can be further used to promote differentiation into hepatocytes [42], chondrocytes [55], cardiomyocytes [56], and definitive endoderm [57]. In some cases, encapsulation is used as a method to support differentiation of ESCs; however, directed differentiation techniques, such as the addition of soluble factors, can also be combined with encapsulation to promote specific directed differentiation.

Bioengineers can apply knowledge of biological processes which are known to occur naturally to direct stem cell behavior, such as the presentation the RGD peptide to promote adhesion, however, another strategy is to screen biomaterials with different surface chemistries to discover new non-physiological interactions which can be useful in directing cell differentiation. Using this strategy, the gene expression of stem cells on different surfaces can be analyzed on an array set-up with high-throughput analysis and materials that promote the desired differentiation can be further analyzed in 3D culture. PEG hydrogels functionalized with small side functional groups of varying charge and hydrophilicity illicit different gene expression profiles of encapsulated hMSCs [58].

4 Microcarriers and Microparticles

Encapsulation of ESCs and other stem cell types is a method to introduce biomaterial control of differentiation cues; however, this method is an “outside–in”

strategy. Biomaterial interaction with the microenvironment is directly imparted on cells of the surface of encapsulated aggregates whereas interior cells are not directly affected. Alternative strategies have similarly focused on incorporating biomaterials with cells cultured in suspension; however, they rely either on culture of cells on microcarriers or the incorporation of microparticles within stem cell aggregates.

4.1 Microcarriers

Microcarriers are spherical beads, normally 150–500 microns in diameter, and can be made of a variety of materials such as polystyrene, dextran and glass. Cells can be grown on the surface of microcarriers to increase the available growing surface area per unit volume and have been used to scale up culture of anchorage dependent cells. Microcarriers have been reported to support maintenance culture of human ESCs [59–61], mouse ESCs [49, 62, 63] and MSCs [64, 65] and importantly, population doubling times remain comparable to 2D culture standards. Dextran beads coated in collagen are most often used for ESC attachment. These cells can then be differentiated while adhered to the bead or they can be separated from the beads for use in other differentiation protocols. The choice of coating is important to the cell yield and in the case of polystyrene beads, cells can adhere without a coating through electrostatic interactions. Cell collection from uncoated polystyrene is difficult and results in decreased cell viability, whereas with gelatin coated dextran, trypsin can digest the collagen layer and cells can be recovered with high yield. Matrigel has also been successfully used as a dextran bead coating as it is known that Matrigel will support undifferentiated growth of ESCs [59, 61].

In contrast to scaffold-based approaches, the material properties of microcarriers have not been extensively studied in regards to directed differentiation or microenvironmental control. Microcarrier materials are evaluated on their ability to expand large amounts of undifferentiated cells. This is in part due to the fact that microcarrier culture is analogous to 2D culture where media manipulation using growth factors or small molecules is a potent regulator of cell behavior. Limitations remain with microcarrier culture including agglomeration and low cell viability after collection procedures. Stirred suspension bioreactors are commonly utilized to agitate the culture and prevent agglomeration and a balance must be reached between shear forces experienced by cells and agglomeration at lower agitation rates.

4.2 Microparticles

While microcarriers are used to scale up the culture monolayers of cells, smaller-sized particles, ranging from 250 nm to 10 μm in diameter, can be incorporated within larger cell spheroids to take advantage of increased cell–cell contacts and 3D

ECM contact. The incorporation of materials within stem cell aggregates is a relatively new approach of "inside–out" engineering that can be used to place cells on the interior of the aggregate in direct contact with the biomaterials. Biomaterial microparticles have been widely used in the field of drug delivery as vehicles for controlled release of encapsulated molecules, and their surface can be functionalized with cell specific adhesion ligands for cell-targeted delivery, especially useful in cancer therapies. In addition, microparticle surfaces can be modified to mimic cell–cell interactions, loaded with soluble morphogens for controlled release or can be used to introduce ECM proteins for control of matrix properties [66]. The surfaces of materials such as polystyrene and poly(2-hydroxyethyl methacrylate) have previously been modified in 2D cultures to present LIF to prevent differentiation or Jagged-1 to mimic cell–cell signaling [67, 68]. Microparticles can be combined with other techniques discussed previously to add further control of soluble factor release. Microparticles incorporated in scaffolds [69, 70] can continually release encapsulated factors throughout the construct and this could similarly be used in capsules of encapsulated cells.

Microparticles incorporated within progenitor cell spheroids was first used to improve post-transplantation cell viability of neural cells [71]. Fetal rat brain cells were mixed with poly(lactic-co-glycolic acid) (PLGA) microparticles which released nerve growth factor (NGF) to increase cell viability after transplantation. This concept has also been applied to synthetic microenvironments for ESCs for the purpose of directed differentiation [72, 73]. As introduced in the beginning of the chapter, ESC spheroids present barriers to diffusion and therefore cells in the interior are not completely accessible to molecules in the media as is the case with 2D culture. Cell–cell contacts and deposited ECM can limit the diffusion of even small molecules and the formation of gradients is likely to contribute to the heterogeneous nature of EB differentiation.

Incorporation of biomaterials within EBs circumvents the diffusion barriers to cells on the EB interior and microparticles can act as point sources continuously releasing morphogen within the EB. In this way, gradients of molecules can be minimized to create a more homogeneous environment for the cells within EBs. An example of microparticle-mediated control of the EB microenvironment is the incorporation of PLGA microparticles within mouse EBs [72]. The microparticles were loaded with retinoic acid (RA), a small, hydrophobic morphogen, and were designed to continually release RA throughout EB culture. The resulting EBs upregulated gene expression of genes characteristic of epiblast stage embryos and uniform cavitation was observed in large populations of EBs. This effect could not be matched through any soluble addition of RA to the EB medium, suggesting that the controlled release of the RA by the particles inside the EBs was needed to provide the appropriate microenvironment for epiblast-like EB formation. Evidence that this effect was widespread throughout the entire culture indicates that this strategy could be used to direct differentiation of ESCs in a scalable manner. Controlled release of morphogens is also desired to conserve growth factor for large scale experiments. Biomaterials can preserve the bioactivity of encapsulated growth factor by maintaining the molecule in a bound state and preventing degradation.

Soluble addition of growth factors must be replenished as determined by the half-life of the molecule in order to maintain the desired concentration for cell signaling.

5 Summary and Conclusions

Stem cell differentiation is controlled by a complex system of extracellular signals from the microenvironment. Control over the microenvironment in 3D culture systems is limited when using traditional cell culture methods and therefore engineered biomaterials have been integrated within 3D stem cell environments for directed differentiation. Scaffolds, hydrogel encapsulation, microparticles and microcarriers all are used to control cell–cell interactions, cell–ECM interactions and the presentation of soluble or immobilized factors to cells. These methods have been used to increase the efficiency and scalability of maintenance culture as well as for directed differentiation.

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