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ARTICLE

Biomaterial Strategies for Controlling Stem Cell Fate Via Morphogen Sequestration

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Protein sequestration plays an essential role in maintaining stem cell populations in the native stem cell niche. Both pluripotent and adult stem cells require the sustained presentation of numerous bioactive growth factors and other soluble cues to potentiate cell fate decisions and morphogenic events. Consequently, methods of natural protein sequestration employed by the stem cell niche present attractive strategies for developing novel protein delivery vehicles and engineering biomimetic stem cell microenvironments that enhance morphogen bioactivity. In this review, we will explore the role of protein sequestration in the native stem cell niche and how it has inspired the design of several classes of materials that exploit natural protein sequestration to effectively maintain stem cell populations and direct stem cell fate. We will also highlight several recent developments in protein sequestering biomaterials, in which material strategies to sequester complex mixtures of endogenously secreted proteins are also being investigated.

Introduction

Stem cells are functionally defined as cells that possess the ability to differentiate into a variety of more mature cell types, making them a promising cell source for many regenerative medicine applications. Current tissue engineering research has expanded beyond the initial premise of using stem cells as a means of directly replacing diseased and damaged cell populations, resulting in a broadened interest in their utility. The unique properties of stem cells have led to their more widespread use for other applications, such as *in vitro* screening platforms for pharmaceutical testing and models of predictive toxicology. Furthermore, recent research has also uncovered that complex mixtures of stem cell-secreted soluble factors can exert beneficial paracrine effects on other cell populations, thereby enabling transplanted stem cells to serve as reservoirs of secreted factors *in vivo* that can influence endogenous cell populations to repair and regenerate damaged tissues through a variety of mechanisms.

Because all of the aforementioned applications rely on either suitable delivery vehicles for stem cell transplantation *in vivo* or effective substrates for stem cell culture *in vitro*, extensive research has been undertaken to investigate the effects of biomaterial scaffolds on stem cell fate. Biomaterials can impact stem cell proliferation, migration, and differentiation through many physiochemical mechanisms, including mechanical properties, surface feature size and topography, cell adhesion/degradation sites, and presentation of soluble factors.[1] The natural stem cell niche, containing an assortment of matrix molecules and soluble factors, provides an instructive template from which biomaterials can be effectively designed. In particular, the stem cell microenvironment, with its myriad of cell-secreted factors, relies on effective protein sequestration and presentation to potentiate a number of biological processes, including the creation of stable morphogen gradients to direct cell movement and morphogenesis and the local enhancement of growth factor bioactivity and potency. Given the importance of stem cell-secreted factors in directing cell fate, a variety of natural and synthetic materials have been fabricated to mimic the substrates found in the native stem cell niche and finely tune the spatial and temporal presentation of numerous morphogens. Thus, protein sequestration and presentation in engineered stem cell microenvironments similarly play an essential role in stem cell culture. This review will first explore the numerous cell-secreted morphogens that influence stem cell maintenance and differentiation and then highlight emerging material strategies to mimic morphogen sequestration and presentation in the native stem cell niche. Finally, the use of endogenous protein sequestration strategies to exert control over stem cell fate in engineered microenvironments will be discussed.

Stem Cell-Secreted Morphogens

Pluripotent Stem Cells

Stem cell potency defines the ability of a stem cell population to differentiate into other cell phenotypes, and ranges from pluripotent stem cells, which can differentiate into all cells of an organism, to multipotent stem cells, which exhibit a more limited capacity for differentiation.

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Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of a pre-implantation blastocyst that can differentiate into cells of all three germ layers, much like that of a developing embryo.[2, 3] Embryogenesis is a tightly regulated process, consisting of numerous cell-secreted biomolecular cues that orchestrate the complex and overlapping series of cell proliferation, differentiation, migration, and organization events that ultimately give rise to the functioning tissues and organs of a complete organism. In addition to differentiating down the ectoderm, mesoderm, and endoderm lineages, human ESCs can also be used to derive the trophoblast,[4] which contributes to extra-embryonic tissue formation, including the placenta. Considering the numerous cell fate decisions that ESCs undergo, directing ESC differentiation towards particular cell phenotypes requires the expression of many morphogens in concert and is highly dependent on cell state and the coordinated presence of other signals. As such, ESCs retain the potential to secrete a variety of potent morphogens responsible for maintaining pluripotency or promoting differentiation, and thus contribute to both embryonic and mature tissue development.

Mass spectrometry and/or antibody-based assays have been used to identify secreted proteins in ESC cultures, demonstrating the presence of many potent biomolecules, including fibroblast growth factors (FGFs), transforming growth factors (TGF-βs), insulin-like growth factors (IGFs), insulin-like growth factor binding proteins (IGFBPs), bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), growth differentiation factors (GDFs), stromal cell-derived factor-1 (SDF-1), and stem cell factor (SCF).[5-9] The signaling pathways in which these growth factors are involved play key roles in defining ESC fate, and can promote or inhibit numerous cell processes depending on stage of differentiation. For example, early Wnt and Activin/Nodal/TGF-β signaling are required for ESC differentiation towards a primitive streak-like cell phenotype, while additional BMP signaling is required for specification towards the mesoderm germ layer in both human and mouse ESCs.[10, 11] In general, BMP is known to exert differential temporal effects on ESC differentiation, depending on ESC state and presence of other signaling molecules. Early BMP signaling can promote ESC differentiation to trophoblast or mesoderm, and eventually mesenchymal morphogenesis,[4, 12, 13] while inhibition of BMP signaling promotes pluripotency.[14] Later BMP signaling also influences the differentiation of primitive ESC-derived mesoderm towards cardiac phenotypes, as studies have demonstrated that both temporary inhibition and stage-specific activation of this pathway promote cardiomyocyte generation.[15-17] FGF and IGF signaling pathways play essential roles in supporting human ESC self-renewal and maintenance of pluripotency.[14, 18] This is contrary to what has been demonstrated in mouse ESCs, which require LIF/STAT3 and Wnt signaling to maintain pluripotency, and undergo differentiation in the presence of FGFs.[19]

Stable gradients of growth factors also serve important functions in ESC differentiation and embryogenesis. Gradients of VEGF in the developing embryo guide vessel development and promote endothelial cell differentiation,[20] while spatially regulated FGF signaling is involved in gross embryonic patterning.[21] Although this is not an exhaustive list of the mechanisms supporting ESC self-renewal, pluripotency, and directed differentiation, these examples demonstrate the importance of ESC-secreted morphogens in determining ESC fate, emphasize the conserved and divergent effects of individual morphogens on mouse and human ESC fate, and highlight the tight temporal and spatial control of morphogen presentation required to effectively maintain ESC pluripotency or activate differentiation and morphogenesis.

Similar to ESCs, induced pluripotent stem cells (iPSCs) are pluripotent cells that possess comparable abilities to self-renew and differentiate into cells of the three embryonic germ layers.[22, 23] iPSCs rely on many of the same signaling molecules to maintain pluripotency and direct differentiation. However, unlike ESCs, iPSCs are derived by reprogramming terminally differentiated, somatic cells with the overexpression of several key transcription factors.[22, 23] Although less information exists on the profiles of morphogens secreted by iPSCs in comparison to ESCs, recent studies that highlight potential benefits of iPSC-secreted morphogens on other cell types may lead to further investigation in this area.[24, 25]

Adult Stem Cells

In contrast to pluripotent stem cells, adult stem cells, such as mesenchymal, hematopoietic, and neural stem cells, are multipotent stem cell populations that typically only differentiate into tissue-specific cell types. Specifically, mesenchymal stem cells (MSCs) differentiate into cells that give rise to musculoskeletal tissues, such as bone, cartilage, and fat,[26] hematopoietic stem cells (HSCs) continuously replenish all blood cell types,[27] and neural stem cells (NSCs) generate the neurons, astrocytes, and oligodendrocytes of the nervous system.[28] Unlike ESCs, which exert paracrine effects on phenotypically similar cells to initially form diverse tissues of an organism, adult stem cell populations normally exert paracrine effects on other distinct cells within their respective mature tissue compartments, resulting in varying profiles of secreted morphogens that aid in maintaining both stem and somatic cell populations.

Mesenchymal stem cells (MSCs), usually derived from the bone marrow compartment or adipose tissue, are known to secrete a diverse profile of soluble factors that can stimulate and modulate a variety of cell populations, contributing to basic cell growth, differentiation, and migration, as well as coordinated angiogenesis and immunomodulation.[29-33] While many studies have been undertaken to pinpoint specific soluble factors involved in defined MSC-mediated paracrine actions, several others have aimed to provide a more global characterization of MSC-secreted factors by using antibody-based and mass spectrometry techniques, similar to those used for ESC morphogen identification.[34-37] Taken together, these studies provide evidence for a number of soluble factors secreted by MSCs, including many similar morphogens to those secreted by ESCs (FGFs, TGF-βs, IGFs, IGFBPs, VEGF, SDF-1, SCF),[33, 35, 38-40] as well as other additional morphogens (platelet derived growth factor (PDGF)),[41, 42] and immune regulators, such as prostaglandin E2 (PGE2) and various interleukins.[29, 34, 41, 43] Importantly, MSCs display distinct capacities for secreting specific morphogens compared to ESCs, resulting in differences in the relative expression levels of overlapping morphogens. MSCs can also exhibit increased secretion of several pro-angiogenic factors, including VEGF and angiopoietin-1 (Ang-1), particularly when MSCs are cultured in low oxygen tension (i.e. hypoxic) conditions.[32, 39]

Hematopoietic stem cell (HSC) populations responsible for regulating hematopoiesis have also demonstrated the ability to secrete soluble factors contributing to paracrine effects.[44] HSCs occupy the bone marrow compartment of the body, along with bone marrow-derived MSCs and a number of other stromal cell populations. Consequently, the regulation of HSC fate is highly dependent on the exchange of soluble and matrix-bound biomolecules between various cell types in this stem cell niche.[45, 46] Many of the morphogens secreted by MSCs can impact HSC fate, including SCF, which supports HSC proliferation and multipotency, and SDF-1, which influences HSC migration and homing.[33] HSCs themselves also secrete SDF-1 to promote cell survival and maintain the cell population within the bone marrow niche.[47] More extensive proteomic analyses of HSC-secreted factors have demonstrated the presence of a number of other morphogens in HSC conditioned media that may contribute to regulating HSC fate and hematopoiesis, including VEGF, FGF-2, IGF-1, and several TGF-β proteins and

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interleukins.[44]

Similar to MSCs and HSCs, neural stem cells (NSCs) reside in specialized niche environments within the subventricular and subgranular zones of the adult brain.[48] The NSC niches are analogous to the bone marrow compartment for MSCs and HSCs, in that they also contain a variety of matrix molecules and soluble factors that maintain NSC proliferation and differentiation abilities. Morphogens such as VEGF, IGFs, FGFs, BMPs, SDF-1, and epidermal growth factor (EGF) have been found in neurogenic niches and are thought to play critical roles in NSC maintenance, specifically acting as survival and proliferation signals.[48, 49] Additionally, Lu, et al. demonstrated that NSCs secrete several neurotrophic factors, including brain-derived neurotophic factor (BDNF), nerve growth factor (NGF), and glial cell line-derived neurotrophic factor (GDNF), which activate neural development during embryogenesis and confer NSCs with the ability to induce neurogenesis following injury.[50] Recently, NSC secretion of VEGF has also been observed in mature tissues, whereas previously, endogenous VEGF in neurogenic niches had been largely attributed to other supportive cell types in the niche.[51]

Multipotent adult stem cells secrete a plethora of morphogens that play specific roles in maintaining the mature tissues in which they reside. Unlike pluripotent stem cells, which are responsible for initially generating cells with widely varying phenotypes, adult stem cell populations are typically responsible for maintaining specific, lineage-committed, somatic cell populations. However, it is evident that the limited potency of adult stem cells does not similarly limit morphogen secretion, as both adult stem cells and pluripotent stem cells secrete a number of morphogens that can influence cell maintenance, proliferation, migration, and differentiation, including FGFs, BMPs, IGFs, SCF, VEGF, SDF-1, and TGF-βs. The profiles of morphogens secreted by these different cell populations often contain a number of corresponding biomolecules, despite the fact that the relative expression levels and combinations of morphogens can differ.

Paracrine Effects of Stem Cell Morphogens

The diverse profiles of morphogens secreted by stem cell populations can influence the cell fate decisions of not only the stem cells that secrete the morphogens, but often other cell populations within and outside of their respective stem cell niches. Morphogens such as FGFs, BMPs, IGFs, SCF, VEGF, SDF-1, and TGF-βs have far-reaching effects on basic cellular processes required by many cell types. Consequently, recent research has delved further into the use of stem cells as reservoirs of soluble factors that can act through paracrine mechanisms to influence other cell populations.[41]

Delivery of ESC-conditioned media containing secreted factors can stimulate the survival, proliferation, and/or migration of a variety of differentiated cell types, including muscle satellite cells, neural progenitor cells, fibroblasts, endothelial cells, and hematopoietic progenitor cells.[8, 52, 53] Such effects on other cell types have been observed with conditioned media obtained from ESCs in various stages of differentiation, including undifferentiated ESCs, ESCs differentiated as adherent monolayer cells, and ESC differentiated as suspension aggregates (embryoid bodies). For example, we have demonstrated that conditioned media collected from spontaneously differentiating embryoid bodies after 6, 9, and 12 days of differentiation could induce the proliferation and migration of both mouse fibroblasts and human endothelial cells HUVECs. Interestingly, conditioned media collected at Day 12 of differentiation that contained higher levels of IGF-2 and VEGF-A induced greater endothelial cell proliferation and migration than conditioned media obtained from earlier time points.[53]

Similar studies have been conducted with MSC-conditioned media. Chen, et al. demonstrated that conditioned media collected from MSCs cultured under hypoxic conditions stimulated the proliferation of keratinocytes and human umbilical cord vein endothelial cells (HUVECs) and migration of keratinocytes, HUVECs, and macrophages.[39] MSC conditioned media has also been used to successfully maintain the self-renewal abilities and pluripotency of ESC cultures in the absence of feeder cells.[54] Furthermore, a number of other studies have utilized MSC conditioned media or MSC co-cultures to expand and maintain the multipotency of HSC populations *in vitro*, hence mimicking the native MSC/HSC relationship of the bone marrow niche.[55-57] Finally, other factors secreted by MSCs, such as PGE2 and TGF-β1, can exert immunomodulatory effects on lymphoid and myeloid cell populations. These factors are typically secreted by MSCs stimulated with pro-inflammatory cytokines and have been shown to suppress the activation of various immune cells, by reducing $CD4^+$ T cell, $CD8^+$ T cell, macrophage, and dendritic cell proliferation, B cell migration and differentiation, and T cell secretion of pro-inflammatory cytokines.[29, 58-63]

Promising paracrine effects of stem cell-secreted factors have not only been observed on cell populations *in vitro*, but have also resulted in tissue repair *in vivo* following stem cell transplantation. Despite the low persistence of transplanted stem cells within tissue injury sites, a number of studies have demonstrated improvements in tissue/organ function following stem cell transplantation in cardiac, neural, and bone defect environments.[17, 64, 65] Recent research has established that many of the beneficial effects of stem cell transplantation can be attributed to the ability of stem cells to recruit and direct endogenous cell populations through the secretion of various soluble factors at the tissue defect site, instead of the traditional notion that transplanted stem cells directly contributed to tissue repair through stable engraftment and differentiation.[66] This new focus on paracrine effects may represent a paradigm shift in the way researchers consider applying stem cells for regenerative medicine, placing emphasis on the viability of the cells themselves and duration, amount, and efficacy of soluble factors they secrete.

Further investigation into the paracrine actions of transplanted stem cells *in vivo* has revealed improvements in heart function following myocardial infarction due to ESC-secreted VEGF, IGF-1, and interleukin-10 (IL-10),[67] axonal regeneration following spinal cord injury due to NSC-secreted NGF, BDNF, GDNF,[50] and bone healing induced by the delivery of MSC-conditioned media containing VEGF and IGF-1.[68] Perhaps the most extensive research regarding stem cell paracrine effects has been conducted with MSCs transplanted for cardiac regeneration following myocardial infarction.[43] Numerous studies have demonstrated improvement in cardiac function following MSC transplantation, prompting investigation into whether these effects are paracrine-mediated. Although the mechanisms by which MSCs exert cardioprotective effects are still somewhat unknown, both *in vitro* and *in vivo* studies have revealed a role of pro-angiogenic and pro-survival MSC-secreted factors, such as VEGF, SDF-1, and IGF-1.[38, 43, 69-71]

Protein Sequestration in Nature

The stem cell niche plays an essential role in maintaining stem cell potency and self-renewal. Both pluripotent and adult stem cell niches rely on the presence of extracellular matrix molecules and supporting cell types to produce, sequester, and present soluble signals necessary to regulate stem cell function (Fig. 2). Consequently, protein sequestration and presentation in native stem cell microenvironments are complex and dynamic processes

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that provide spatial and temporal control of soluble signals to stimulate and inhibit basic stem cell processes that maintain and replenish cell populations.

Role of Extracellular Matrix Molecules in Protein Sequestration

The extracellular matrix (ECM) contains a variety of molecules responsible for providing structural integrity, mechanical cues, cell adhesion sites, and protein binding domains within the stem cell microenvironment.[72] The ECM is comprised of numerous fibrillar proteins (e.g. collagens, elastin), glycoproteins (e.g. laminin, fibronectin, tenascin), and proteoglycans (e.g. heparan sulfate, chondroitin sulfate). Although fibrillar proteins and glycoproteins are thought to be primarily responsible for structural support and cell adhesion within the ECM, several of these proteins also have the ability to participate in the sequestration and presentation of cell-secreted morphogens.

Fibronectin, which has a well-known ability to promote growth factor binding through heparin binding domains and cell adhesion through integrin binding, can specifically interact with several morphogens, including VEGF, TGF-β1, IGFBPs, and hepatocyte growth factor (HGF).[73-77] Moreover, Martino, et al. demonstrated that when the fibronectin domains responsible for growth factor binding were purified, these fragments possessed a significantly increased affinity for VEGF, TGF-β1, PDGF, BMP-2, BMP-7, BDNF, HGF, and several IGFBPs and FGFs.[78] Similar results were also obtained when the growth factor binding domains of tenascin C were purified, conferring an increased affinity for PDGF, FGF-2, TGF-β1, and neurotrophin-3 (NT3).[79]

Unlike fibronectin, the collagen isoforms present in adult tissues do not contain dedicated growth factor binding sites, and their affinity for morphogens is typically lower than that of other ECM molecules.[80] However, collagens can indirectly contribute to morphogen binding through the adhesion of other ECM components that can bind morphogens, such as fibronectin, proteoglycans, and glycosaminoglycans.[80, 81] Furthermore, Type IIA collagen, which is expressed solely in chondrogenic tissues during development, contains an additional domain that exhibits specific affinity for BMP-2 and TGF-β1.[82] Consequently, Type IIA collagen may play a critical role in early fetal chondrogenesis by sequestering and spatially presenting growth factors necessary for cartilage development and subsequent endochondral ossification.

Role of Proteoglycans in Protein Sequestration

Proteoglycans are a particular class of ECM molecules that function to bind soluble factors and maintain their bioactivity within cellular microenvironments. They are typically composed of a core protein surrounded by several linear glycosaminoglycan (GAG) chains that provide the dense negative charges that influence protein binding. Interactions between GAGs and various proteins have been extensively studied in the past, revealing that GAG-protein binding is primarily electrostatic and relies heavily on negatively charged sulfate groups within the disaccharide chains.[83, 84] Heparin, heparan sulfate, and chondroitin sulfate are all highly sulfated GAGs, and thus have a strong affinity for number of potent, positively charged morphogens, known collectively as "heparin binding proteins." While heparan sulfate and chondroitin sulfate are somewhat less sulfated and almost exclusively found covalently attached to proteoglycans on cell membranes and in the ECM, heparin has the highest sulfation density of all naturally occurring GAGs and typically exists as free linear chains that are not bound to protein cores.[83]

While many proteins interact with GAGs in a purely electrostatic manner, specific carbohydrate sequences have been identified within heparin and heparan sulfate that mediate binding with known domains on certain heparin binding proteins. Thus, the sulfation patterns displayed on different GAG species may play a significant role in determining protein binding affinity. For example, a specific pentasaccharide sequence is required for heparin binding to antithrombin III to inhibit coagulation, while a tetrasaccharide sequence is necessary for FGF-1 and FGF-2 binding.[85, 86] Likewise, specific protein sequences on FGF-1, FGF-2, and BMP-2 molecules have also been identified as being necessary for heparin binding.[87, 88] Thus, heparin interactions with antithrombin III, FGF-1, FGF-2, BMP-2, and several other proteins can be considered similar in specificity to protein binding to fibronectin and tenascin and are much stronger than protein interactions with fibrillar ECM molecules, such as collagens and laminin.

Although GAG interactions with FGFs and BMPs are the most extensively characterized, GAGs can also strongly and reversibly bind other morphogens influential in stem cell maintenance and developmental and repair processes, including VEGF, Wnt3a, TGF-βs, IGFs, and IGFBPs.[84, 89-91] GAG binding to proteins can increase protein half-lives in *in vitro* cell cultures and *in vivo* tissues, by protecting them from enzymatic degradation and denaturing environmental agents.[92-94] Furthermore, interactions between GAGs and various growth factors have been previously shown to either enhance or inhibit a wide range of biological effects, depending on the relative concentrations of GAG and growth factor and context of interaction. This has been thoroughly investigated in the context of BMP-2-mediated osteogenesis, in which heparin has been specifically shown to extend BMP-2 half-life and enhance BMP-2/BMP receptor dimerization in some cases,[92, 95] while interfering with BMP-2/BMP receptor binding and downstream signaling pathway in others.[96] In general, higher concentrations of heparin over shorter periods of time tend to inhibit BMP-2 activity, while lower concentrations of heparin over longer periods of time promote BMP-2 signaling.[96, 97] Moreover, although soluble heparin can sequester BMP-2 away from cells to interfere with BMP-2-mediated alkaline phosphatase (ALP) activity, BMP-2 bound to similar amounts of heparin-based materials, such as microparticles, can directly interact with cell surface receptors to promote ALP activity.[98]

Effect of Protein Sequestration and Presentation on Stem Cell Differentiation

Protein sequestration in tissues and stem cells niches can enhance or inhibit the ability of soluble factors to interact with various cell populations and immobilize biomolecules over long periods of time to provide spatial and temporal control of cell signaling beyond soluble morphogen half-lives. Protein sequestration in the ECM can also be used to create spatial gradients that are required for morphogenesis and development.[99, 100] For example, VEGF-A plays an essential role in vasculogenesis and blood vessel branching during embryogenesis. While the VEGF165 and VEGF189 isoforms contain well-described heparin binding domains and are matrix-bound, VEGF120 lacks these binding domains and is soluble. Ruhrberg, et al. demonstrated that mice embryos deficient in heparin binding VEGF isoforms did not develop the steep VEGF concentration gradients necessary to direct endothelial cell differentiation and sprouting and thus failed to achieve a normal level of vascular branching complexity (Fig. 1).[99] Instead, local VEGF120 concentrations rapidly declined, and VEGF120 was found dispersed throughout the developing tissues, leading to aberrant vascular development. Thus, VEGF binding to heparan sulfate proteoglycans (HSPGs) located on cell membranes and in the ECM of the developing embryo is necessary to create growth factor reservoirs and spatially distribute

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vasculogenic signals to promote normal morphogenic patterning. Similar mechanisms have also been uncovered for other key morphogenic pathways in the developing embryo; cell surface HSPGs that are spatially distributed create gradients of BMPs, FGFs, and Wnts during embryogenesis and function as co-factors during growth factor-receptor binding.[100] Furthermore, endogenous sulfatases that selectively remove sulfate groups from GAG chains have also demonstrated the ability to modulate Wnt and FGF binding to HSPGs, resulting in negative regulation of FGF signaling and increased Wnt signaling during different stages of development.[101, 102]

The influence of protein sequestration on growth factor signaling and subsequent cell fate extends far beyond the initial specification of tissues during embryogenesis. Many of the same GAG-dependent signaling pathways enacted during embryogenesis are relevant to *in vitro* ESC culture. The importance of heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs) in ESC maintenance and differentiation has been elucidated through the use of ESC lines deficient in GAG production. Izumikawa, et al. recently demonstrated that CSPGs are necessary to both maintain ESC pluripotency and initiate differentiation when ESCs are cultured as EBs.[103] Relatedly, Kraushaar, et al. used HSPG deficient cells ($EXT^{-/-}$) to establish the indispensible role of HSPGs in potentiating FGF and BMP signaling to drive ESC differentiation down the mesoderm lineage, and revealed that HSPGs and exogenous heparin can stabilize morphogens such as BMP-4 and extend their half-life in culture by several hours.[104] Other studies have similarly demonstrated the necessity of endogenous HSPGs in promoting Wnt and BMP signaling and subsequent hematopoietic differentiation of ESCs.[105]

The effects of endogenous GAGs on MSC differentiation via morphogen binding have also been investigated. The disruption of heparan sulfate and chondroitin sulfate in MSC cultures can increase BMP and Wnt signaling, resulting in amplified osteogenic differentiation,[106] or conversely, can promote BMP degradation and decrease BMP signaling, resulting in inhibition of osteogenic differentiation.[107] Heparan sulfate can also influence FGF signaling in MSC cultures by increasing levels of FGF secretion[106] and promoting FGF signaling by acting as a co-receptor for the FGF/FGF receptor complex.[108, 109]

Although the impact of protein sequestration by other ECM molecules on stem cell fate is less known, several studies have also investigated the roles of glycoproteins on stem cell regulation. Both fibronectin and tenascins demonstrate affinity for pro-angiogenic factors. Specifically, the cell adhesion and VEGF-binding domains of fibronectin can work in concert to promote endothelial cell differentiation and migration binding,[110] while tenascins can induce FGF-2 and VEGF-mediated endothelial cell sprouting.[111] Although the role of tenascins in stem cell regulation with respect to protein sequestration is still not well understood, the presence of tenascins in a variety of stem cell niches, including NSC and MSC/HSC niches, may indicate their ability to influence stem cell fate via binding and presentation of endogenous morphogens.

Effect of Protein Sequestration in Injury and Disease

Given the essential role that protein sequestration plays in both tissue development and homeostasis, it follows that disruptions in normal ECM patterning and subsequent morphogen distribution can result in a number of disease states. Significant changes in the activity of enzymes responsible for CSPG and HSPG synthesis, sulfation, and degradation have been observed in the muscles of rats following ischemic injury, resulting in increased GAG chain length and degree of sulfation.[112] The increased presence of GAGs in injured muscle may potentiate FGF-2 signaling to promote muscle satellite cell proliferation, and conversely, deficient GAG production in the muscle defect microenvironment may impair healing. Similarly, the presence of GAGs in the myocardium enhances the activity of several heparin binding proteins involved in tissue maintenance, including FGF-1, FGF-2, and VEGF. Huynh, et al. demonstrated that the ability of endogenous GAGs to regulate these cardioprotective growth factors was significantly reduced in older rats, perhaps contributing to age-related cardiac decline.[113]

Excess GAG biosynthesis can also be detrimental to the healing process. For example, CSPG deposition is a hallmark of spinal cord injury because it is abundantly expressed by macrophages in the ECM-rich glial scar that develops in the injury site.[114] Nervous tissue-specific CSPGs can bind several growth factors responsible for promoting neurite outgrowth and may inhibit the ability of these growth factors to interact with infiltrating cells.[115, 116] As a result, chondroitinases that have been used to degrade chondroitin sulfate chains in the glial scar have been effective in promoting axonal regeneration and functional spinal cord recovery.[117] Consequently, understanding the role of endogenous ECM-mediated protein sequestration in injured tissues may help to develop treatments to promote tissue repair.

Materials that Mimic Natural Protein Sequestration

The natural capacity of the ECM to strongly and reversibly bind bioactive morphogens provides an attractive template for designing effective biomaterials. ECM-based, ECM-containing, and ECM-mimetic materials harness the protein sequestering qualities of the native ECM to provide biomimetic strategies for improving microenvironments for stem cell regulation and methods of morphogen delivery. Consequently, a variety of materials have been developed using this strategy (Fig. 2). This section will focus on the use of assorted ECM-like biomaterials for the delivery of exogenous growth factors to stem cell populations *in vitro* and *in vivo* to guide cell fate.

ECM-based Materials

Both fibrillar ECM proteins, such as collagens, and glycoproteins, such as fibronectin, have been used to create biomimetic materials for growth factor delivery and stem cell culture. Collagens, with their self-assembling network structure, biodegradability, and ability to interact with cells and biomolecules, have frequently been used in a variety of applications. Perhaps the most common clinical application of collagens is as a delivery vehicle for BMP-2 and BMP-7 in the context of bone healing;[118] porous sponges made of lyophilized Type I collagen networks are soaked in a concentrated BMP solution immediately prior to implantation, and growth factor binding occurs via electrostatic interactions between negatively charged residues on the collagen fibers and positively charged residues on BMP. When delivered *in vivo*, the porous network of the collagen sponge facilitates robust cell infiltration and degradation into biocompatible components, while the sequestered BMP is rapidly released to promote osteogenesis.

Collagen materials have also been used to direct stem cell fate *in vitro*. The use of collagen I and collagen II hydrogels for MSC culture has demonstrated enhanced chondrogenic differentiation, especially in the presence of the morphogen TGF-β1.[119] Gelatin, which is simply denatured collagen, has been previously used as a delivery vehicle for BMP-4, thrombopoietin (TPO), and the BMP-4 antagonist noggin in ESC culture.[120, 121] Presentation of BMP-4 via

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gelatin microparticles incorporated within ESC aggregates promoted mesoderm differentiation, while microparticle delivery of noggin enhanced ectoderm differentiation.[120] Robust mesoderm differentiation was achieved with microparticle delivery of BMP-4 concentrations that were 12-fold lower than that required for soluble BMP-4 delivery (Fig. 3), indicating that material-based protein sequestration may be a more efficient and effective method of locally presenting morphogen signals to stem cells.

As mentioned previously, collagens do not contain specific binding sites for proteins; thus, morphogen binding is primarily achieved through relatively weak, electrostatic interactions that may easily be displaced by stronger, competing interactions with other biomolecules. As a result, two common approaches to improve the growth factor retention capacity of collagens have been to covalently tether biomolecules of interest to its fibers or physically incorporate other ECM molecules with specific protein binding sequences, such as glycoproteins or GAGs, into its matrix. High retention of FGF-2, VEGF, and TGF-βs within collagen matrices has been achieved with covalent conjugation.[122-124] Tethering of VEGF165 to collagens using a homobifunctional polyethylene glycol (PEG) linker with reactive succinimidyl groups increased retention of VEGF within the collagen matrix without a significant reduction in VEGF bioactivity, thus enabling VEGF-induced proliferation of HUVECs.[122] Furthermore, similar chemical modification of TGF-β2 and addition to collagen substrates resulted in robust cell infiltration *in vivo*.[123] Most recently, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) and riboflavin/UV light-mediated cross-linking of FGF-2 and TGF-β1 to collagen microcarriers have both been successfully used to stimulate proliferation and chondrogenic differentiation in MSC cultures.[124]

Despite promising outcomes observed with the use of covalently tethered growth factors, chemical modification of many proteins can attenuate their overall bioactivity by disrupting active sites for signaling, heparin binding domains, charges contributing to electrostatic interactions, and the ability of cells to internalize the molecule.[125, 126] Consequently, the incorporation of other protein sequestering biomolecules into collagen matrices is a promising alternative strategy that has also been explored. Many studies have investigated covalent or non-covalent addition of GAG chains to collagen matrices to enhance protein sequestration. For example, Wissink, et al. created "heparinized" collagen substrates by covalently tethering heparin chains to collagen films using EDC/NHS cross-linking; when loaded with FGF-2, heparinized substrates exhibited improved HUVEC proliferation compared to unmodified collagen substrates.[127] Heparinized gelatin microparticles, also fabricated using EDC/NHS chemistry, demonstrated reduced BMP-4 and TPO release compared to gelatin microparticles and stimulated greater differentiation of ESCs down the mesoderm lineage[120] (Fig 3.) and into hematopoietic progenitors[121] than soluble morphogen delivery. Alternatively, heparin has been non-covalently incorporated into collagen matrices through physical entanglement of GAG chains and collagen fibers,[128, 129] leading to stable incorporation of heparin within the scaffold, as well as attenuated VEGF release and prolonged bioactivity.[128]

Aside from the presence of specific protein binding domains, other material properties can also influence the ability of ECM-based materials to deliver morphogens to *in vitro* and *in vivo* cellular environments. For a more thorough examination of the concept of material properties that influence biological function, the reader is directed to several other recent reviews.[1, 130, 131] In the context of the protein sequestration properties of collagens, one factor that is widely explored is the effect of collagen fiber and cross-linking density on protein retention. Multiple studies have demonstrated that EDC/NHS cross-linking of collagen fibers can result in dense collagen matrices that are more resistant to collagenase-mediated degradation; this is also often accompanied by increased retention of molecular cargo within the collagen scaffold.[124, 132] We have previously demonstrated a similar effect of gelatin microparticles loaded with BMP-4 and FGF-2, in which increased methacrylate cross-linking density resulted in decreased morphogen release and reduced susceptibility to collagenase degradation.[133]

GAG-based Materials

The strong binding affinity of GAGs to a variety of proteins make GAG-based materials a promising strategy for delivering morphogens with high spatial and temporal control. Numerous studies have investigated methods to fabricate biomaterials that immobilize GAG chains onto other materials or consist of modified GAG species that can be cross-linked to form pure GAG materials. The ability to maintain growth factor bioactivity and prolong growth factor half-life, while remaining biodegradable *in vivo*, provides GAGs with clear advantages as drug delivery systems. Heparin is most commonly used in GAG-based materials because of its high charge density, which confers its strong protein binding capacity, and well-studied interactions with potent morphogens. Although chondroitin sulfate and heparan sulfate typically exhibit lower affinity for heparin binding proteins due to their decreased sulfation, biomaterials containing these GAG species have also been developed. Since CSPGs are abundant in cartilage ECM, chondroitin sulfate-containing materials have been frequently pursued for biomimetic cartilage tissue engineering applications.[134, 135] Moreover, given the natural anticoagulant ability of heparin, which stems from its strong affinity for antithrombin III, heparan sulfate presents an alternative GAG-based protein delivery strategy for situations where heparin's anticoagulant activity may pose a risk.[136]

Similar to methods for heparinizing collagen matrices as described above, heparin and other GAGs can be non-covalently or covalently incorporated into a number of other bulk materials, including polyethylene glycol (PEG), poly-lactic-co-glycolic acid (PLGA), chitosan, and fibrin. For example, heparin incorporation into PEG-based hydrogels can be accomplished by cross-linking methacrylamide or methacrylate-modified heparin with PEG dimethacrylate or diacrylate using free radical polymerization. Heparin incorporation into PEG hydrogels has been applied in bulk gels to enhance proliferation and osteogenic differentiation of MSCs either in the absence or presence of exogenous morphogens, such as FGF-2 and BMP-2.[137-139] Importantly, methacrylamide and methacrylate-modified heparin species retain their ability to extend morphogen bioactivity and enhance morphogen presentation.

Promising results have also been achieved with GAG incorporation into naturally derived materials such as chitosan and fibrin. Non-covalent incorporation of chondroitin sulfate into chitosan sponges has been used to modulate release of PDGF,[140] while fibrin gels containing covalently tethered heparin exhibit reduced BMP-2 release.[141] Finally, efforts have also been made to fabricate pure GAG materials, in order to further maximize the growth factor holding capacity of biomaterials. We have recently demonstrated methods to synthesize pure heparin microparticles and pure chondroitin sulfate nanoparticles and microparticles through the use of methacrylamide and methacrylate modification and free radical polymerization.[98, 142] Heparin microparticles exhibited reduced BMP-2 release while maintaining morphogen bioactivity,[98] while chondroitin sulfate microparticles similarly retained the majority of loaded TGF-β1.[142] Low molecular weight heparin species (< 8 kDa), such as fragmin, have also been used in conjunction with the highly cationic peptide protamine to make GAG

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microparticles through stable ionic complexing, resulting in similar attenuation of FGF-2 release and enhancement of bioactivity.[143]

The large number of GAG-modified materials that now exists to improve morphogen retention and enhance bioactivity highlights the broad utility of GAGs in biomaterial-based strategies for protein sequestration. The majority of GAG-based materials that have been fabricated thus far have been applied to growth factor delivery for *in vivo* tissue regeneration, and thus fewer studies directly address the effects of protein sequestering materials on stem cell fate. Several studies undertaken by Anseth and colleagues elucidated the stimulatory effects of morphogen-laden heparin-PEG hydrogels on MSC proliferation and differentiation, showcasing dramatic increases in alkaline phosphate (ALP) activity and osteogenic gene expression in the presence of heparin-functionalized hydrogels compared to PEG hydrogels alone.[137, 138] PLGA microspheres coated with heparin-complexed FGF-2, BMP-7, IGF, and TGF-β3 have also been used as substrates for MSC culture, resulting in differential effects on MSC lineage commitment as evaluated by gene and protein expression, cell morphology, and biochemical assays.[144] Specifically, BMP-7 induced osteogenic differentiation, dual delivery of FGF-2 and IGF stimulated adipogenic differentiation, and TGF-β3 delivery resulted in chondrogenic differentiation, highlighting the versatility that heparin affords delivery systems by enhancing presentation of various morphogens contributing to tri-lineage MSC differentiation.

As mentioned previously, the sulfation patterns of GAG chains play a key role in the presentation of morphogens, by modulating the electrostatic charges responsible for protein binding. Consequently, the ability to selectively desulfate GAG chains offers a means of further manipulating morphogen binding and sequestration properties. Several studies have investigated chemical methods of desulfating GAG chains and its effects on morphogen binding. Fully sulfated heparin consists of 2-O-sulfate, 6-O-sulfate, and N-sulfate groups, and common desulfation methods result in removal of one, two, or all of these sulfate groups.[94, 145, 146] Ratanavaraporn, et al. demonstrated that heparin chains desulfated at the 2-O-sulfate position retained significantly more BMP-2 than native heparin and other desulfated species, and that MSCs cultured in gelatin hydrogels containing BMP-2 and non-covalently entangled 2-O-desulfated heparin derivatives enhanced early osteogenic differentiation.[145] The sulfation patterns of heparin-containing microparticles can similarly modulate heparin-BMP-2 binding and subsequent BMP-2 bioactivity.[146] The importance of GAG sulfation patterns in ESC fate determination has also led to investigation into the effects of exogenous GAG addition in ESC cultures. For example, sulfating the 6-O position of chitosan disaccharides can modulate its overall sulfation pattern and charge. When used as a substrate for ESC differentiation, sulfated chitosan can increase levels of neural differentiation without the addition of exogenous morphogens.[147]

Synthetic Materials

The unique properties of ECM components make their addition into various biomaterials an attractive strategy for creating biomimetic cell culture substrates and delivery vehicles. However, synthetic materials can also emulate the properties of the native ECM by mimicking their cell adhesive sites, structural properties, and protein binding abilities. In terms of morphogen binding, many strategies have been employed to impart ECM-inspired properties into synthetic polymer materials. The following section highlights several ECM-inspired synthetic materials; for a more thorough summary of synthetic approaches to biomimetic sequestration, the reader is directed to the following recent review.[148]

With the promising results achieved through the use of GAG-based biomaterials, a number of methods have evolved to fabricate synthetic GAG mimetics that possess similar structure and protein binding functions to native GAG chains. The ability to artificially synthesize polysaccharides with GAG properties may provide new possibilities for biomaterial design, by presenting reproducible, scalable methods of obtaining large amounts of well-characterized GAG material that can be engineered to promote morphogen binding while minimizing anticoagulant abilities.[149] Thus far, several studies have investigated the effects of morphogen binding to GAG mimetics, revealing similar binding affinity compared to native GAG species. For example, the heparin mimetic polysodium-4-styrenesulfonate (PSS) bound similar amounts of FGF-2 as heparin,[150] while heparin mimetic peptide amphiphiles demonstrated robust VEGF, HGF, and FGF-2 binding.[151, 152] GAG mimetics have also successfully been used to direct stem cell fate. The heparin mimetic OTR4120, which consists of sulfate-modified dextran, can promote MSC proliferation, migration, and osteogenic differentiation *in vitro*, and can also be non-covalently attached to bone substitute scaffolds for *in vivo* delivery.[153] Furthermore, polyacrylamide hydrogels covalently cross-linked with PSS demonstrated the ability to maintain human ESC proliferation and pluripotency similarly to culture on Matrigel, in part due to the adsorption of FGF-2 into the matrix.[154]

Synthetic versions of fibronectin and tenascin have recently d promise for their ability to strongly sequester bioactive morphogens. Although fewer biomaterials incorporate glycoproteins compared to GAGs into their matrices, the recent development of purified fibronectin and tenascin molecules by Hubbell and colleagues presents the opportunity to incorporate high binding glycoproteins into bulk materials.[78, 79] As mentioned previously, fibronectin and tenascin consist of cell adhesion and growth factor domains in close proximity to each other and exhibit an increased affinity for numerous morphogens, including VEGF, TGF-βs, PDGF, BMPs, FGFs, and IGFBPs. Fibronectin fragments can increase migration and proliferation of HUVECs, smooth muscle cells, and MSCs in the presence of VEGF and/or PDGF; fibronectin can also enhance differentiation, including VEGF-mediated HUVEC tube formation, PDGF-mediated smooth muscle cell sprouting, and BMP-2-mediated MSC osteogenesis.[155] Finally, fibronectin fragments can be incorporated into fibrin hydrogels through physical entanglement during hydrogel cross-linking for *in vivo* growth factor delivery.[155]

Protein sequestration can also be accomplished with synthetic materials that are not specifically based on the structure of ECM molecules. The thermosensitive polymer, poly-N-isopropylacrylamide (pNIPAm), has demonstrated utility in absorbing proteins from *in vitro* cell cultures and protein-rich body fluids.[156, 157] Although pNIPAm materials have not typically been used for morphogen delivery to direct cell fate thus far, their unique protein sequestration properties may lend them to this application. pNIPAm hydrogels are highly porous, charged materials, that exhibit temperature-dependent swelling and collapse that enable them to rapidly imbibe fluid and soluble factors; their tunable mesh sizes allow them to act as molecular sieves to capture biomolecules of specific sizes while excluding others. pNIPAm materials can also be further functionalized with charged moieties in order to attract specific proteins via electrostatic interactions. Consequently, pNIPAm microparticles have been successfully used as molecular sieves to separate and concentrate dilute proteins of interest such as insulin, SDF-1, and myoglobin from complex protein mixtures, while excluding more abundant proteins such as albumin and immunoglobins on the basis of size.[162] Furthermore, pNIPAm has been used to isolate proteins biomarkers for diseases from body fluids. The most notable of these is PDGF, which can be captured and concentrated 10-fold from a large volume

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Table 1. Summary of Biomaterial Strategies for Growth Factor Delivery and Sequestration

Material	Growth Factor(s)	Purpose	Reference(s)
Collagen Scaffolds	BMP-2, BMP-7	*In vivo* delivery for bone regeneration	118
	VEGF	HUVEC proliferation	122
	TGF-β2	*In vivo* delivery for cell proliferation	123
	FGF-2, TGF-β1	MSC proliferation and chondrogenic differentiation	119, 124
Gelatin Microparticles	BMP-4, Noggin	Mesoderm differentiation of ESC aggregates	120
	BMP-4, TPO	Hematopoietic differentiation of ESC aggregates	121
	BMP-4, FGF-2		133
Heparinized Collagen/Gelatin Matrices	BMP-2	MSC osteogenic differentiation	145
	BMP-2	*In vivo* delivery for bone regeneration	129
	FGF-2	HUVEC proliferation	127
	BMP-4, TPO	Mesoderm and hematopoietic differentiation of ESC aggregates	120, 121
	VEGF	Endothelial cell proliferation	128
Chondroitin Sulfate Particles	TGF-β1		142
Chondroitin Sulfate Chitosan Sponges	PDGF-BB	Osteoblast migration and proliferation	140
Heparin-Containing Microparticles	BMP-2	C2C12 osteogenic differentiation	98, 146
	BMP-2	MSC osteogenic differentiation	144
	FGF-2, IGF	MSC adipogenic differentiation	144
	TGF-β3	MSC chondrogenic differentiation	144
Heparin-Containing Hydrogels	BMP-2	*In vivo* delivery for bone regeneration	141
	BMP-2	MSC osteogenic differentiation	158
	FGF-2	MSC proliferation	137
	TGF-β1	*In vitro* cardiac progenitor cell proliferation, differentiation, and soluble factor secretion	159, 160
	TGF-β1	*In vivo* cardiac progenitor cell survival	159
	No exogenous GF	MSC proliferation and osteogenic differentiation	137-139, 158
Heparin Mimetics	FGF-2	C2C12 proliferation and myogenic differentiation	150
	VEGF, FGF-2	HUVEC tube formation	151, 152
	HGF		151
	NGF	Neurite extension from neural crest cells	151
	No exogenous GF	MSC proliferation, migration, and differentiation	153
	No exogenous GF	ESC pluripotency maintenance	154
Fibronectin Fragments	VEGF, PDGF, FGFs	HUVEC, MSC, and smooth muscle cell migration and proliferation	78, 155
	VEGF	HUVEC tube formation	155
	PDGF-BB	Smooth muscle cell sprouting	78, 155
	BMP-2	MSC osteogenic differentiation	155
	BMP-2, PDGF-BB	*In vivo* delivery for bone regeneration	155
	VEGF, PDGF-BB	*In vivo* delivery for skin wound healing	155
Sulfated Chitosan	No exogenous GF	ESC neural differentiation	147
Heparin-Binding Peptides	No exogenous GF	HUVEC proliferation	161
	No exogenous GF	MSC proliferation and osteogenic differentiation	161

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of human serum into a small volume of pNIPAm microparticles and subsequently shielded from proteolytic degradation prior to analysis.[157]

Since a variety of ECM molecules play essential roles in creating morphogen gradients that drive tissue development, ECM-based and ECM-mimetic materials can also be used to create synthetic gradients to direct stem cell fate and tissue regeneration. A number of studies have attempted to establish stable morphogen gradients using various natural and synthetic biomaterials.[163] For example, Dodla, et al. demonstrated that an anisotropic distribution of laminin and NGF in an agarose hydrogel was more effective than isotropic distribution in promoting axonal regeneration across nerve gaps in rats.[164] Furthermore, opposing gradients of encapsulated BMP-2, TGF-β1, and IGF-1 in PLGA and silk fibroin scaffolds have been used to direct MSC differentiation down osteogenic and chondrogenic lineages with spatial control to create osteochondral tissue constructs.[165, 166] Finally, stable gradients of covalently linked sonic hedgehog and cillary neutrotophic factor in agarose hydrogels have demonstrated the ability to promote neural precursor cell migration and induce activation of respective signaling pathways in retinal precursor cells.[167]

Materials for Complex Endogenous Protein Sequestration

The success of numerous recombinant protein binding systems for *in vitro* stem cell fate regulation and *in vivo* growth factor delivery has recently led to the development of new strategies to sequester and present soluble factors that are endogenously produced during *in vitro* cell culture. The sequestration of endogenous growth factors has long been considered a possible mechanism by which GAG-based biomaterials enhance morphogen effects on stem cell populations beyond the potency of the morphogens themselves; however, it is only recently that studies have attempted to more systematically test this hypothesis. GAG-based protein sequestration aims to mimic and subsequently harness the naturally occurring ECM-morphogen interactions that take place in the stem cell niche and contribute to spatial and temporal control of cell signaling. The ability to capture and concentrate numerous cell-secreted and serum-borne morphogens using biomaterials may provide novel methods of more easily analyzing cell-secreted products and potentiating cell growth and differentiation through dynamic feedback between cells and biomolecules. Although this is an emerging concept in the field of stem cell engineering, several studies have begun to elucidate the effects of presenting complex mixtures of proteins on stem cell fate. This following discussion will highlight some of the key studies that have contributed to recent knowledge in this field of research.

Because many morphogens are considered heparin binding growth factors, GAG-based biomaterials are prime candidates for sequestering cell-secreted proteins. Through the use of heparin-PEG hydrogels, Anseth and colleagues demonstrated the ability of GAG-based biomaterials to enhance the effects of exogenously added FGF-2 and BMP-2 on MSC proliferation and differentiation.[137, 138] Further investigation within this system next aimed to modulate the presentation and availability of both serum-borne and cell-secreted morphogens. By depleting heparin binding proteins from serum-containing media and selectively adding back in fibronectin and BMP-2, the necessity of serum-borne heparin binding proteins in inducing MSC osteogenesis as well as the role of exogenous heparin chains in concentrating fibronectin and BMP-2 was elucidated. A subsequent study investigated the role of heparin-PEG hydrogels in concentrating cell-secreted morphogens, by incorporating fluvastatin, a small molecule known to increase cellular BMP-2 secretion, into the gels.[158] It was found that, while all fluvastatin-containing hydrogels increased MSC osteogenesis, the additional presence of heparin in fluvastatin-containing hydrogels significantly increased osteogenic gene expression, likely due to the heparin-mediated concentration of cell-secreted BMP-2.

Similar results have also been obtained using other strategies for GAG-mediated morphogen sequestration. Hudalla, et al. recently described the covalent tethering of a novel heparin binding peptide, derived from the heparin binding domain of FGF-2, at varying densities to oligoethylene glycol self-assembling monolayers using EDC/NHS chemistry; this peptide can sequester serum-borne heparin to subsequently in turn modulate the presentation and availability of morphogens in culture.[161, 168] Sequestration of soluble heparin enhanced presentation of serum-borne FGF-2, resulting in increased HUVEC and MSC proliferation, and was hypothesized to similarly enhance presentation of endogenous, MSC-secreted FGFs (Fig. 4). Investigation of this heparin binding peptide in osteogenic media has also revealed a potential role of heparin sequestration in potentiating osteogenic differentiation mediated by MSC-secreted BMPs, corroborating results obtained by Anseth and colleagues.

Some of the latest work by Healy and colleagues further illustrates the benefits of GAG-based morphogen sequestration.[159, 160] In a recent study, hyaluronic acid hydrogels were fabricated with covalently cross-linked heparin using acrylated hyaluronic acid and cleavable peptide cross-linkers via Michael type addition. Cardiac progenitor cells (CPCs) seeded onto hydrogels containing heparin-bound TGF-β1 exhibited robust proliferation, differentiation, and formation of tubular structures. This was accompanied by dramatic increases in several matrix-bound morphogens, which contributed to endothelial cell mobilization (IGFBPs, VEGF, HGF) and vascular invasion (IL-10, endostatin), suggesting that heparin presentation both concentrated and amplified cell-secreted signals to enhance CPC maturation. Moreover, heparin-mediated presentation of soluble signals also led to positive effects *in vivo*, where heparin-containing hydrogels enhanced CPC survival and differentiation following hindlimb implantation.

Heparin has also been used to concentrate cell-secreted morphogens for delivery to primary myoblast and neural progenitor cell cultures, as well as damaged tissues.[52] While whole ESC conditioned media induced proliferation and survival of both cell types, it was found that these mitogenic effects were mainly caused by the heparin binding fraction of the media (Fig. 5). Heparin binding proteins were concentrated from conditioned media using heparin-agarose beads and eluted prior to *in vitro* delivery to primary myoblasts and *in vivo* delivery to sites of muscle injury; in both scenarios, heparin binding proteins enhanced proliferation, while conditioned media depleted of heparin binding proteins failed to induce proliferation of primary myoblasts in culture.

While the aforementioned work has begun to directly address the abilities of GAG-based biomaterials to contribute to endogenous and serum-borne protein sequestration, a number of other studies have alluded to these phenomena following observations that highlight surprising control of cellular fate in the presence of GAGs. For example, enhanced MSC proliferation, migration, and differentiation has been demonstrated in the presence of GAG-based scaffolds and the absence of exogenous morphogens,[139, 153] while GAG mimetics can maintain ESC self-renewal and pluripotency without the need for additional growth factors or feeder cells.[154, 169] We have also observed enhanced cellular effects due to endogenous morphogen sequestration, such as increased hematopoietic progenitor differentiation in the presence of BMP-4-loaded gelatin

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microparticles and endogenous VEGF secretion.[121] Overall, emerging research in this area highlights the fact that GAG and ECM-based biomaterials may provide an alternative method of controlling stem cell fate through material sequestration properties alone and without the need for exogenous morphogen addition.

Conclusions and Future Outlook

The dynamic environment of the stem cell niche involves many biological processes and signaling events that are potentiated by spatial and temporal control of morphogen presentation. Thus, the ability to exert control over stem cell fate using protein sequestering biomaterials represents a promising biomimetic strategy to improve stem cell maintenance and directed differentiation *in vitro* and prolong paracrine effects following stem cell transplantation *in vivo*. Despite the prevalence of ECM and GAG-based biomaterials, the majority of research in the field thus far has focused on improving the delivery of exogenously added morphogens, with numerous accomplishments in fabricating materials that provide sustained release of recombinant growth factors to robustly direct stem cell fate; however, fewer studies have investigated the capabilities of biomaterials to preserve endogenously secreted factors and enhance their activity. Consequently, additional research is necessary to clarify the role(s) that endogenous morphogen sequestration plays in regulating stem cell fate, in order to harness it to create more effective biomaterial systems for stem cell control.

ECM-based biomaterials have been employed to sequester and present endogenous morphogens to enhance paracrine effects during stem cell culture and to concentrate morphogens for delivery to other cell populations both *in vitro* and *in vivo*. Studies by Jha, et al. and Yousef, et al. demonstrate the potential for future stem cell-based therapies to employ paracrine-based approaches to tissue regeneration, in which the effects of stem cell-secreted factors can be strengthened with the use of protein sequestering biomaterials that prolong growth factor bioactivity.[52, 159] Ultimately, stem cell-based therapies in the future may not rely solely on the ability to deliver viable cells, if the stem cell-secreted biomolecules that induce paracrine effects on endogenous cells and tissues can be identified and concentrated for *in vivo* delivery.

ECM-based biomaterials may also induce similar sequestration of cell-secreted proteins *in vivo*, resulting in enhancement of local signaling molecules; this concept has been recently explored in a study by Liu, et al., in which chitosan hydrogels delivered following myocardial infarction increased both local SDF-1 concentrations and c-kit$^+$ cell recruitment.[170] Considering the propensity of ECM-based biomaterials to sequester a myriad of cell-secreted proteins, *in vivo* protein sequestration by delivered biomaterials also deserves further attention. Finally, since endogenous protein sequestration results in the collection of a complex mixture of cell-secreted factors, the identification of the specific factors and relative amounts being captured presents a challenge that must also be addressed. Proteomic analysis using antibody-based and mass spectrometry approaches can help identify stem cell-secreted morphogens that are potent modulators of other cell populations and may elucidate potential synergistic effects between endogenously secreted and exogenously added morphogens. Overall, material-based protein sequestration presents a vital facet of biomaterial interactions with stem cell populations; the knowledge derived from this research will eventually lead to improved biomaterials strategies for *in vitro* stem cell maintenance and *in vivo* stem cell transplantation in the future.

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Figure Captions

Figure 1. Disrupted VEGF patterning in developing mouse embryo in absence of heparin-binding VEGF isoforms. Asterisks denote hindbrain midline. Embryos were imaged at day 10.5 of development. A) VEGF gene expression near hindbrain midline. B) Spatial localization of VEGF (red) near hindbrain midline, surrounded by $PECAM^{+}$ blood vessels (green). Comparison of VEGF distribution and intensity in wild-type (C,E) and VEGF 165/189 deficient (D,F) mouse embryos. VEGF was highly localized and intensely expressed around the hindbrain midline in wild-type embryos compared to the VEGF 165/189 deficient embryos, which expressed lower levels of dispersed VEGF, indicating a disruption of spatial patterning. Scale Bar = 50 µm. Reproduced with permission.[99] Copyright 2002, Cold Spring Harbor Laboratory Press.

Figure 2. Methods of morphogen sequestration and presentation demonstrated in the native stem cell niche (left) and employed in engineered biomaterials for protein delivery (right). The stem cell niche displays soluble, ECM-bound, and GAG-bound morphogens that are typically secreted by other supporting cell types within in the niche. Biomimetic materials make use of these natural cell-material interactions, including both covalent morphogen presentation strategies (morphogen tethering to materials) and non-covalent presentation strategies (ECM based and GAG-based materials).

Figure 3. BMP-4 delivery via gelatin or heparinized gelatin microparticles to embryoid bodies induces robust mesoderm differentiation. A) Schematic depicting culture conditions of embryoid bodies treated with soluble BMP-4, soluble BMP-4 and microparticles, or BMP-4-loaded microparticles. B) Images demonstrating mesoderm differentiation via Braychury-T expression (green) in embryoid bodies treated with soluble BMP-4 (sBMP-4), sBMP-4 and microparticles (red), and BMP-4 bound to microparticles. BMP-4 delivery using microparticles resulted in localized Braychury-T expression with 12-fold less growth factor. Reproduced with permission.[120] Copyright 2013, Elsevier.

Figure 4. Self-assembling monolayers containing heparin-binding peptides sequester serum-borne heparin and growth factors to induce MSC proliferation. A) Schematic representing sequestration of serum-borne heparin and growth factors onto self-assembling monolayers, followed by subsequent MSC growth and differentiation. B) MSC growth over 72 hours in response to heparin-binding peptide and RGDSP immobilization (red) versus scrambled peptide immobilization (blue); MSCs were incubated in 0.01%, 0.1%, 1.0%, and 10% FBS. MSC proliferation significantly increased in the presence of the heparin-binding peptide at higher serum concentrations. Reproduced with permission.[130] Copyright 2011, Royal Society of Chemistry.

Figure 5. Heparin-binding fraction of ESC-conditioned medium induces myoblast proliferation and inhibits myoblast differentiation. A) Images of primary myoblasts cultured for 24 hours in 50% differentiation (fusion) medium and 50% ESC-conditioned medium, heparin depleted ESC-conditioned medium, eluted heparin binding proteins, basal medium (OptiMEM), or conditioned medium from differentiated ESCs, as specified in images. Nuclei (blue), myosin heavy chain (green), and BrdU (red) are as denoted. B) Quantification of BrdU expression (indicative of proliferation) and myosin heavy chain (MyHC) expression (indicative of myoblast fusion) in myoblast cells cultured in the aforementioned media combinations. ESC-conditioned medium enhanced myoblast proliferation and inhibited myoblast differentiation compared to basal medium and medium from differentiated ESCs. This effect appeared to be attributed to eluted heparin binding proteins and was not observed in heparin depleted ESC-conditioned medium. Reproduced with permission.[52] Copyright 2013, Impact Journals.

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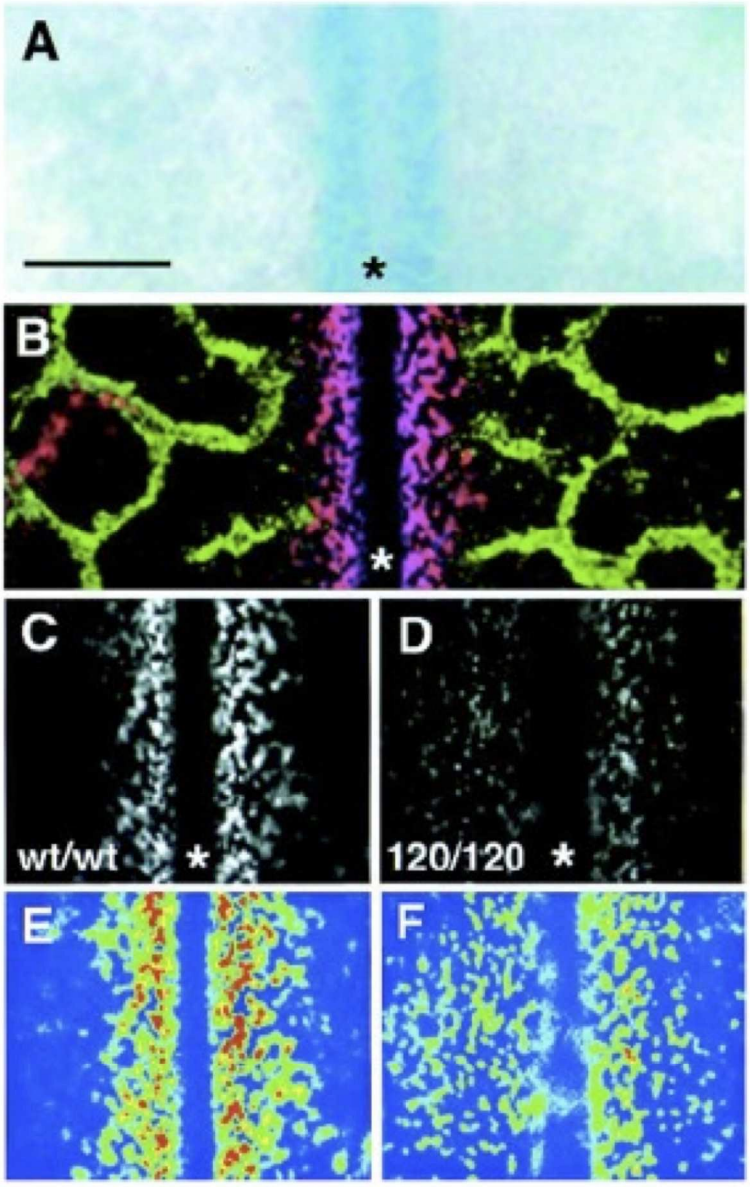

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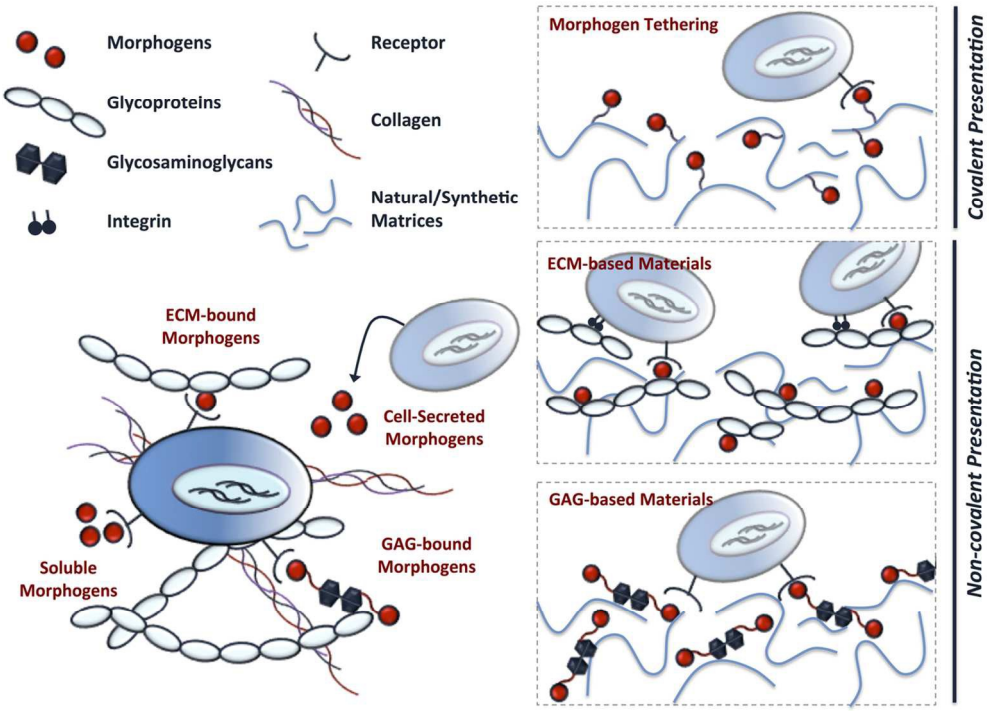

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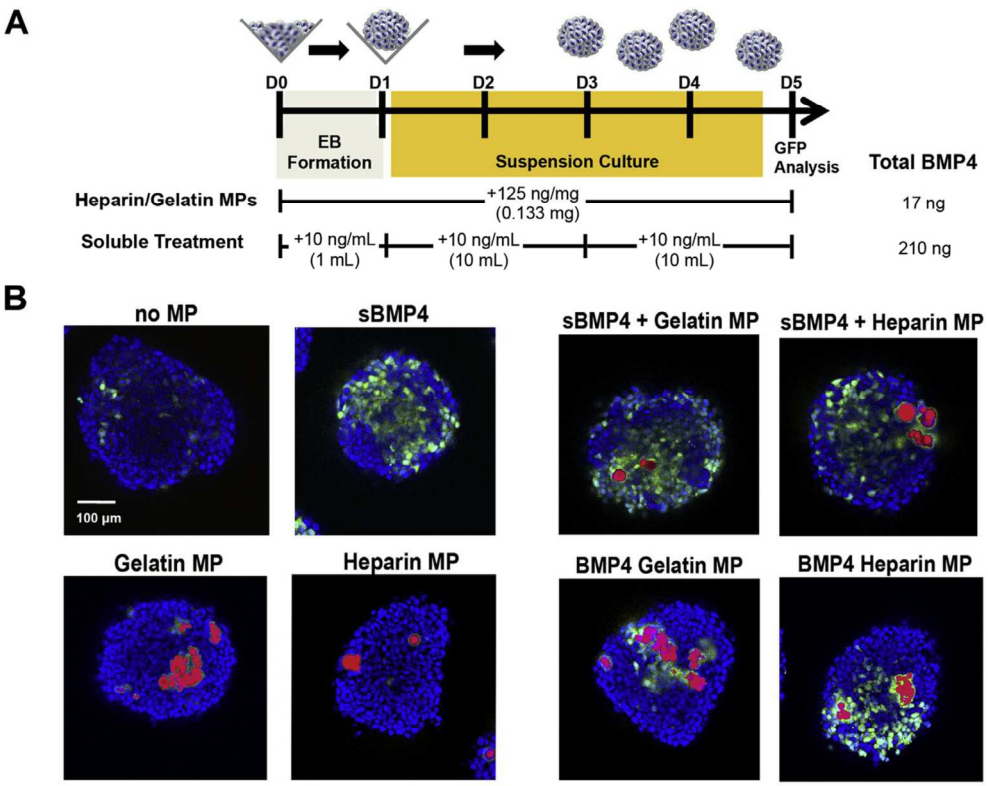

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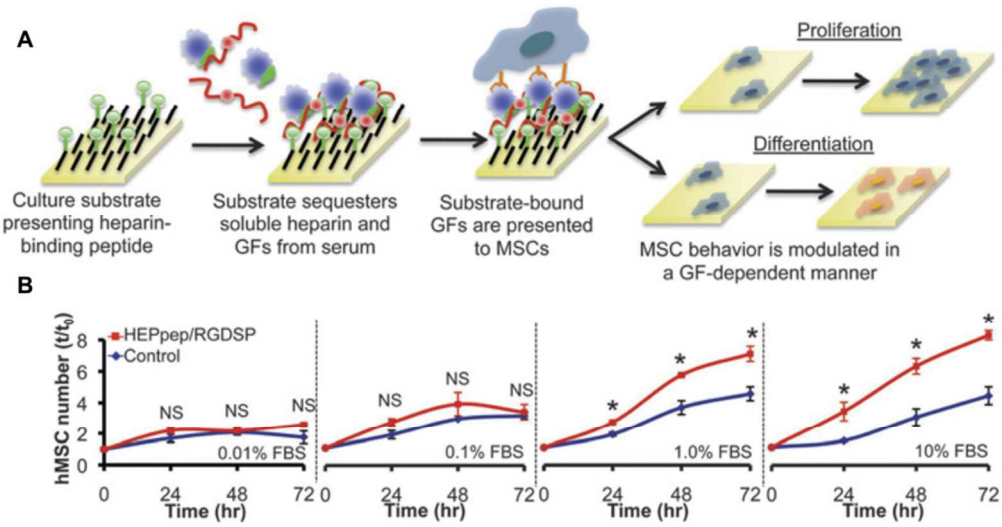

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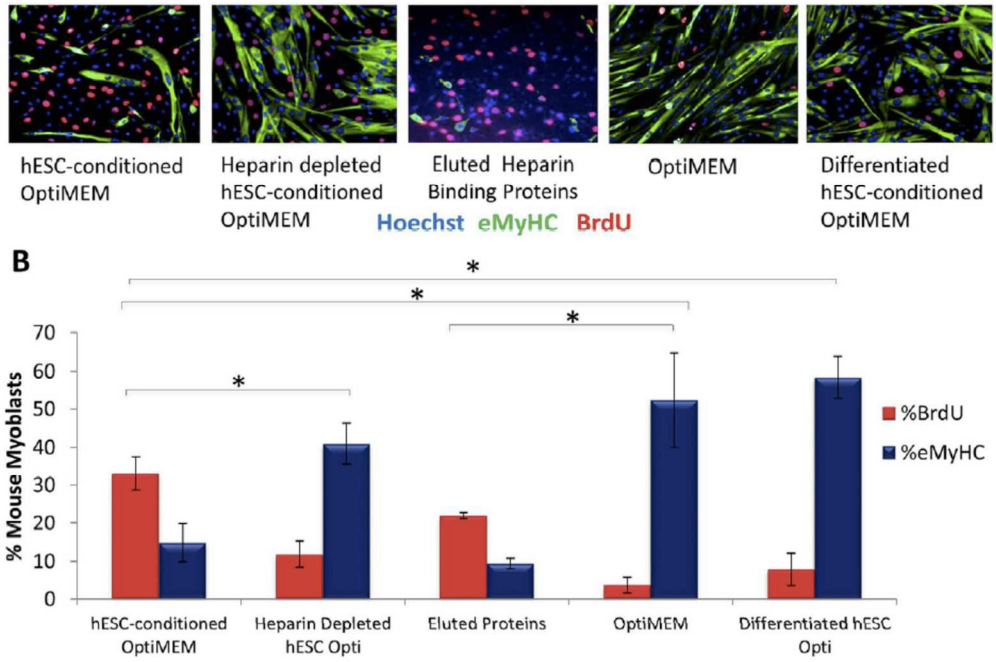

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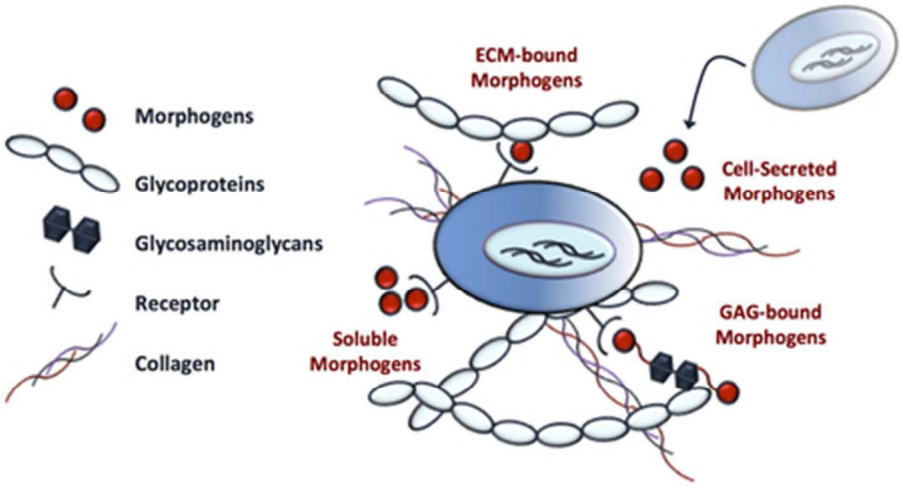

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