PROTEOLYTICALLY DEGRADABLE MICROPARTICLES FOR 
ENGINEERING THE EXTRACELLULAR MICROENVIRONMENT 
OF PLURIPOTENT STEM CELL AGGREGATES 

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The Academic Faculty 

by 

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PROTEOLYTICALLY DEGRADABLE MICROPARTICLES FOR
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OF PLURIPOTENT STEM CELL AGGREGATES

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To Moopie, Bosie, Saphoo and Doddie
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<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>αSMA</td>
<td>Alpha Smooth Muscle Actin</td>
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<tr>
<td>BMP4</td>
<td>Bone Morphogenetic Protein-4</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
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<tr>
<td>EB</td>
<td>Embryoid Body</td>
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<td>ESC</td>
<td>Embryonic Stem Cell</td>
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<td>EMT</td>
<td>Epithelial-to-Mesenchymal Transition</td>
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<tr>
<td>FN</td>
<td>Fibronectin</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>GMA</td>
<td>Gelatin Methacrylate</td>
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<tr>
<td>GA</td>
<td>Gelatin Glutaraldehyde</td>
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<tr>
<td>iPSC</td>
<td>Induced Pluripotent Stem Cell</td>
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<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
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<td>MA</td>
<td>Methacrylate</td>
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<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<td>MP</td>
<td>Microparticle</td>
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<td>PCA</td>
<td>Principal Components Analysis</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PMMA</td>
<td>Poly (Methyl) Methacrylate</td>
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<td>PS</td>
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<td>SEM</td>
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SUMMARY

Embryonic stem cells offer tremendous potential for regenerative medicine and tissue engineering approaches due to their pluripotent capacity to differentiate into all cell types within the body. It is anticipated that one day, understanding of pluripotent stem cells will enable functional restoration of damaged tissues as well as the ability to cure diseases that are currently only treated through symptom mitigation or through stratagems to delay organ deterioration. Clinical translation of stem cell research has been hampered despite advances in research because of the inherent difficulty in controlling differentiation potential to ensure safety and prevent carcinogenesis. Studying the developing embryo and adult stem cell niches has been invaluable for understanding stem cell function regulation, as these environments involve a complex variety of biochemical and biophysical cues, especially within the extracellular environment. Pluripotent stem cells are commonly differentiated as 3-dimensional aggregates, termed embryoid bodies, which recapitulate many of the complex morphological events that occur during embryological development and facilitate the production of extracellular matrix and cell-cell connections common to tissue structures in vivo. Thus, studying pluripotent stem cell aggregates enables analysis of morphological processes that generate the descendants to all 3 germ lineages and this knowledge can be employed to generate cell types of interest.

Manipulation of the stem cell microenvironment is increasingly being explored as a strategy to promote lineage specific differentiation due to the ability of biomaterials to engineer the complex properties of the extracellular milieu. Incorporation of biomaterial microparticles enables control of spatial presentation of the material to the cellular
environment, which is lost during encapsulation of aggregates within hydrogels. The magnitude of cell-material contact within the aggregate can be controlled through regulation of the number and size of MPs, while aggregate encapsulation restricts contact with the material to the external aggregate boundary. Incorporation of biomaterial microparticles (MPs) composed of gelatin, poly(lactic-co-glycolic acid), and agarose, within pluripotent stem cell aggregates has demonstrated that materials are able to modulate stem cell pluripotency and differentiation, even in the absence of delivered biomolecules. However, understanding how a material, especially gelatin, is able to affect changes in gene and protein expression patterns of endodermal and mesodermal phenotypic markers has still not been elucidated. Previous studies have demonstrated that introduction of proteolytically degradable materials within cellular environments have enhanced expression and often activity of the protease specific to the introduced substrate. Furthermore, modulating the material degradation rate and physical properties, such as stiffness and mesh sizes, has been demonstrated to additionally affect the proteolytic activity. It was hypothesized that introduction of gelatin MPs within pluripotent stem cell environments may enhance the activity of the gelatinase matrix metalloproteinases, MMP-2 and MMP-9. The gelatinases have been implicated in many stages of embryonic development, especially in epithelial-to-mesenchymal transition (EMT), which enables the generation of all 3 germ lineages, however, comparatively little has been studied about their role in pluripotent stem cell differentiation. The goal of this work was to develop gelatin microparticles with tunable degradation rates and analyze their ability to modulate MMP activity. The overall hypothesis of this study was that protease-dependent cellular morphogenic processes could be augmented by the
introduction of materials that enhance proteolytic activity. Namely, introduction of gelatin MPs within spheroids may enhance gelatinase activity and thus mesendodermal EMT, and that tuning MP degradation properties may lead to differences in MMP activity and subsequently affect differentiation trajectories.

Gelatin MPs were synthesized with gelatin methacrylate (GMA) chemistries, which enabled tunability of cross-linking density, from as low as 15% cross-linked, to almost 100% cross-linked. The advantage of GMA MPs from the traditional glutaraldehyde (GA), genipin, or dehydrothermal cross-linked MPs, is that MA substitution affords greater control over hydrogel cross-linking density because the methacrylate groups on GMA restrict the maximum cross-linking density achievable, independent of soluble factors that are difficult to control, such as the amount of radical initiator added, or experimental conditions, such as time and temperature of cross-linking. GMA MPs with 15%, 50% and 90% MA cross-linking were synthesized with tunable degradation rates, despite their similar size ranges and comparable gelatin content. Higher methacrylated MPs took over double the time to fully degrade in collagenase compared to the lower methacrylated MPs (96 hours compared to 48 hours, respectively). Moreover, the highest methacrylated MPs had an order of magnitude greater elastic moduli and an order of magnitude smaller mesh size than both the 15% and 50% MA MPs.

Lightly methacrylated (20% MA MP) and highly methacrylated (90% MA MP) GMA MPs were introduced within embryoid bodies to determine their effects on MMP activity and subsequent differentiation. Incorporation of both MPs enhanced total MMP as well as MMP-2 levels, with the low MA MPs enhancing MMP-2 expression compared
to untreated spheroids throughout the entire course of differentiation, and the high MA MPs increasing MMP-2 expression primarily during later stages of differentiation. Spheroids with the high MA MPs had enhanced MMP-2 activity compared to untreated and low MA MPs, although both MP spheroids had greater activity than untreated groups. Additionally, total overall MMP levels were also increased in spheroids with both MP groups through the period of differentiation during which point EMT and mesoderm induction occurs. Furthermore, simply plating spheroids on GMA coated plates did not yield greater MMP-2 levels than spheroids plated on tissue culture grade polystyrene plates, suggesting that gelatin MP incorporation promotes greater MMP-2 activity that cannot be recapitulated by simple contact of the spheroids and gelatin. Furthermore, incorporation of other methacrylated MPs or methacrylated extracellular matrix MPs did not yield increased MMP-2 activity, signifying that MMP activity is specific to the substrate presented. pSMAD inhibition also reduced MMP-2 activity, suggesting that GMA MPs may promote MMP activity through binding BMPs that enhance SMAD phosphorylation as well as a substrate sensing mechanism.

MP incorporation increased EMT processes as well as enhanced mesendodermal differentiation by day 7 of differentiation within spheroids, when compared to untreated spheroids. Furthermore, spheroids with incorporated MPs also had enhanced ECM remodeling as well as mesenchymal morphogenesis by day 7. Small molecule inhibition of MMPs on day 5 of differentiation reduced EMT and early mesoderm and endoderm markers, as well as mesenchymal phenotypes and abrogated the mesenchymal ECM expression patterns in MP groups specifically by day 7, indicating that enhancement of EMT and mesendodermal differentiation by MP incorporation is MMP-dependent.
Furthermore, MMP-2 expression was localized to the MPs within the spheroids, suggesting that proteolytic activity and resultant remodeling and differentiation effects may be localized to MP regions. Moreover, MP incorporation led to enhanced cardiomyogenesis, indicating that the differentiation trajectory of ESCs can be controlled by early presentation of proteolytically enhancing material MPs.

Introduction of proteolytically sensitive materials facilitate the study of how enzymatic activity can modulate stem cell differentiation, since a large majority of tissue development and morphogenesis require matrix remodeling and cell migration. Furthermore, these studies establish an ECM material platform with tunable properties, which can be modulated to deliver growth factors in a temporally sensitive manner. Overall, the introduction of enzymatically-degradable MPs offers an avenue to engineer the ECM microenvironment, which plays a critical role in maintaining almost all aspects of cell behavior, for regulation of stem cell differentiation.
Embryonic stem cells (ESCs) offer tremendous promise for tissue engineering and regenerative medicine applications due to their capacity to generate all bodily cell types. ESCs are typically differentiated as 3-dimensional aggregates, termed embryoid bodies (EBs), which recapitulate many biomolecular and biochemical events that occur during embryogenesis and facilitate the extracellular matrix production present in in vivo tissues that aid in stem cell regulation. However, EB differentiation is very heterogeneous, and manipulation of the EB microenvironment is increasingly being investigated to promote lineage specific differentiation, especially the potential of biomaterials to engineer the complex properties of the extracellular milieu. Incorporation of biomaterial microparticles (MPs) within EBs has demonstrated that materials are able to modulate stem cell pluripotency and differentiation, even in the absence of delivered biomolecules, however the mechanisms are not well known. Previous studies have demonstrated that introduction of proteolytically degradable materials within cellular environments have enhanced proteolytic activity specific to the introduced substrate [1–6]. Additionally, protease activity has been highly implicated during stem cell differentiation, notably during the formation of all 3 germ lineages. The objective of this work was to engineer gelatin microparticles with tunable degradation rates and analyze their ability to modulate MMP activity and cellular differentiation. The central hypothesis was that incorporation of degradable gelatin MPs within EBs would enhance the proteolytic activity of the spheroids and would lead to enhanced mesodermal induction. The objective was
accomplished and the central hypothesis was tested through completion of the following specific aims:

**Specific Aim 1. Determine the effects of MP cross-linking density on MP bulk properties, degradation, and growth factor release.** The *working hypothesis* was that modifying the cross-linking densities of gelatin MPs would modulate their degradation rates, gelatin content, and growth factor capacity and release rates. Gelatin MPs with varying cross-linking densities were synthesized from free radical cross-linking of gelatin substituted with varying levels of methacrylate. Size analysis, gelatin content per particle volume, and degradation rates in response to collagenase were evaluated for each of the MP formulations. MP loading of the growth factors, BMP4 and bFGF, and the subsequent release rates from the MPs were also investigated.

**Specific Aim 2. Evaluate the incorporation of MPs with different cross-linking densities on protease activity, extracellular remodeling, and lineage differentiation.** The *working hypothesis* was that incorporation of MPs would increase MMP-2 activity, basement membrane extracellular matrix remodeling, and mesendodermal differentiation through enhancement of epithelial-to-mesenchymal transition (EMT) processes. The gelatin MPs were incorporated within EBs in comparable volumes and the cell-mediated protease remodeling response to the introduced exogenous ECM MPs was analyzed via zymography. EMT and mesoderm specific gene expression was analyzed via qPCR, and ECM remodeling was analyzed via histology and immunohistochemistry. MMP
involvement in differentiation processes was analyzed via small molecule inhibition of MMP and mesenchymal differentiation.

This work is significant because it is the first to evaluate proteolytic responses in response to biomaterials within embryonic stem cell aggregates, as well as the resultant effects of the proteases on pluripotent stem cell extracellular remodeling and differentiation, which has not been well characterized. Understanding the proteolytic effects of materials may yield insights into the design and development of biomaterial systems to direct stem cell differentiation. Inducing proteolytic responses may be a novel avenue to trigger or enhance stem cell differentiation processes, since tissue development and morphogenesis commonly require advanced matrix remodeling and cell migration. It is anticipated that proteolytic inductive materials can be used in tandem with other cues, such as soluble factors, to efficiently direct stem cell differentiation within aggregates.
CHAPTER 2
BACKGROUNDS

Embryonic stem cell differentiation

Embryonic stem cells (ESCs) are a promising cell source for regenerative medicine therapies because of their unlimited proliferative capacity in vitro and potential to become all somatic cell types. In addition, ESCs recapitulate many aspects of cellular and molecular morphogenesis, and ESC aggregates have been used as models to analyze patterns of development because of their sustenance of morphogen gradients and differentiation into cells of all three germ layers, endoderm, mesoderm, and ectoderm [7]. Thus, strategies to differentiate ESCs are vastly explored in order to obtain desired cell types that have limited regeneration potential in situ to treat a variety of debilitating diseases and improve quality of life [8].

ESCs are derived from the inner cell mass of blastocyst stage embryos and are typically cultured under conditions necessary to maintain the stem cell phenotype [9,10]. In particular, soluble cues are often necessary to maintain ESC “stemness,” such as basic fibroblast growth factor (bFGF) for human ESCs [11] and leukemia inhibitory factor (LIF) [12] for mouse ESCs cultured on gelatin-coated substrates. Additionally, ESCs have been cultured on inactivated mouse embryonic fibroblast (MEF) layers [9] or on Matrigel™, [13] which contains many soluble driving forces towards stem cell proliferation and self-renewal over differentiation. However, studies have been moving towards defined media over the unknown and often variable xenogenic substrates in order to exert greater control over the maintenance of stem cell phenotype [14].
Culture platforms: 2-dimensional vs. 3-dimensional

Maintenance of ESCs in an undifferentiated state mainly occurs on monolayer, and further differentiation on 2-dimensional platforms is often performed in an effort to provide cell accessibility to soluble factors in a more uniform manner. ESCs are increasingly being differentiated as 3-dimesional aggregates, termed embryoid bodies (EBs) based on their ability to recapitulate in vivo cell-cell connections and extracellular matrix (ECM) interactions, as well as the intercellular signaling present in early embryogenesis. ECM plays an important key role in stem cell differentiation, especially by cellular adhesive interactions via surface receptors such as integrins, enabling cell attachment, spreading and migration, and offering mechanical support [15]. Additionally, the ECM sequesters and stores growth factors in a bioactive state, which can then be cleaved by matrix metalloproteinases (MMPs) during matrix remodeling [16]. In vitro studies comparing between 2-D and 3-D models demonstrate that cells have morphologies and characteristics more closely resembling the native tissue, including advanced cell adhesion complexes and basement membrane development, when cultured in a 3-D environment [17,18]. Moreover, specialized cell types obtained through 3-D stem cell differentiation have enhanced engraftment into the in vivo environment compared to single differentiated cells during transplantation [19,20]. For example, engraftment of cardiomyocyte-differentiated embryonic stem cell aggregates is enhanced compared to differentiated single cells, due potentially to the enhanced cell-cell connections allowing their survival and integration [19,20]. Additionally, the authors hypothesize that the aggregates may be “pre-conditioned” to survive in the infarcted environment since their centers are exposed to a slightly hypoxic environment and
already experience limited nutrient diffusion *in vitro*, however this hypoxic preconditioning is dependent on aggregate size [19,20].

Furthermore, spheroid culture requires much less tissue culture surface area per volume compared to monolayer culture, enabling translation to scalable bioprocessing platforms [21]. Previous studies have demonstrated that rotary suspension formation and culture of EBs enable more uniform EB populations and greater EB yield over static formation and culture [22]. Additionally, rotary cultured EBs have much greater throughput over hanging drop culture, an alternative method that commonly produces uniform EB populations [22]. Moreover, novel methods to produce EBs in a more throughput manner have emerged, such as EB formation using Ultra-High Throughput AggreWell™ technology [23], honeycomb microwell arrays [24], dielectrophoresis [25], microfluidics [26], with avidin-biotin cross-linking [27], and with methylcellulose medium induction [28]. However, culture in the 3-D environment introduces high levels of complexity, such as homotypic and heterotypic cellular interactions, as well as increased ECM secretion, and greater paracrine and autocrine signaling [29]. The most common method of EB differentiation is soluble factor delivery to the EB differentiation media. However, previous reports have demonstrated the development of a dense “shell-like” layer on the exterior of the EB, and this layer may serve as a barrier to soluble factor diffusion [30–34]. The presence of this exterior EB layer has prompted the development of differentiation technologies aimed to overcome soluble factor transport limitations, such as microparticle (MP) incorporation within stem cell aggregates.

**Microparticle platforms for stem cell aggregate differentiation**

Microparticles have been incorporated within stem cell aggregates to influence
differentiation from within the aggregate, overcoming soluble factor diffusion limitations. Several different types of MPs have been incorporated within both pluripotent and multipotent stem cell aggregates for directed differentiation approaches. Studies have incorporated MPs composed of PLGA, agarose, and gelatin within mouse EBs, and they have reported that the biomaterial properties themselves influence differentiation, although suggesting that MP delivery of soluble factors may enhance directed differentiation strategies [35]. Furthermore, sizes of MP vehicles can influence incorporation within aggregates as well as the course of differentiation [36]. PLGA MPs have been loaded with VEGF, PDGF, and bFGF for human EB differentiation into vascular lineages [37]. Additionally, another study has reported osteogenic differentiation in aggregates with PLGA MPs loaded with simvastatin and BMP-2, as well as endothelial differentiation in aggregates containing VEGF loaded MPs [38]. Interestingly, incorporation of retinoic acid loaded PLGA MPs within mouse ESC aggregates led to development of EBs with cystic interiors with an outer epiblast-like layer, reminiscent of a pre-implantation blastocyst stage embryo [31]. The delivery of thrombopoietin and BMP4 within gelatin MPs also enhanced hematopoietic differentiation in pluripotent SC aggregates over soluble delivery of the two factors [39]. Moreover, MPs have been synthesized from highly negatively charged glycosaminoglycans, which can tightly sequester oppositely charged growth factors and prevent them from degradation. Incorporation of chondroitin sulfate (CS) MPs within EBs has previously been demonstrated and offers a platform to locally deliver cationic growth factors for directed stem cell differentiation [40]. Additionally, incorporation of magnetic MPs within stem cell aggregates enables spatial patterning control of multi-cellular assembly for bottom-
up tissue engineering [41–43]. Thus, tailoring properties of MPs, such as size, charge, and magnetic ability facilitates both spatial control as well as growth factor controlled delivery for directed differentiation strategies within embryonic stem cell aggregates.

In addition to pluripotent aggregate differentiation, MP differentiation approaches have also been efficacious in obtaining more specialized cell types within multipotent stem cell aggregates. Several groups have also incorporated MPs within mesenchymal stem cell aggregates for directed differentiation approaches down the adipogenic, chondrogenic, and osteogenic lineage. Incorporation of hyaluronic acid microspheres within MSC aggregates has been employed for adipose tissue regeneration for soft tissue reconstruction purposes [44]. Furthermore, incorporation of gelatin MPs within rat MSC spheroids resulted in higher aggregate viability and cell proliferation due to greater aerobic metabolism and oxygen permeability. Additionally, they reported that the presence of gelatin MPs promoted osteogenic differentiation yet inhibited chondrogenic differentiation of MSC aggregates [45]. The incorporation of unloaded PEG MPs also modulated chondrogenic differentiation in human MSC aggregates, thus suggesting the impact of MPs alone during differentiation [46]. However, in other studies, incorporating TGF-β-loaded gelatin MPs within bone marrow stromal cell aggregates increased chondrogenesis [47,48], and modifying the MP cross-linking density also affected degree of chondrogenesis [49]. In addition, introducing gelatin MPs within hMSC spheroids significantly impacts their mechanical properties [50], which could be modulated as a novel platform for directing differentiation through alterations in cytoskeletal organization and subsequent intracellular signaling. Thus, tuning cross-linking density, mechanical properties and growth factor delivery of incorporated MPs offers a promising
differentiation strategy for obtaining specialized cell types within both multipotent and pluripotent stem cell aggregates.

Matrix metalloproteinases (MMPs)

The matrix metalloproteinase (MMP) family consists of over 20 extracellular matrix proteinases that regulate many embryonic developmental processes and are also involved in many aspects of stem cell differentiation [51]. MMPs degrade ECM molecules for cellular migration, alter the cellular environment for modulation of cellular behavior, and alter biomolecule activity by their or their inhibitor cleavage from bound glycosaminoglycans [51–54]. These proteases can be divided into several subgroups based on their substrates or similarity in structure: collagenases that cleave fibrillar collagen, gelatinases that cleave denatured collagen, stromelysins that cleave noncollagen ECM, and transmembrane – type MMPs (MT-MMPs) [52]. Only collagenases MMP-1, MMP-8, MMP-13, and MMP-14 can degrade the type I, II, and III fibrillar collagens in their triple helical form efficiently. Once, the triple helical forms are cleaved, they are rendered thermally unstable, and then denature, or unwind to form gelatin. The gelatinases, MMP-2 and MMP-9 can then further degrade the gelatin [51].

Most MMPs consist of two domains, a protease domain and an ancillary helper domain, connected by a proline-rich flexible hinge peptide [51,52,55]. The protease domain consists of a signal peptide, followed by a pro-domain and then catalytic domain (His-Glu-X-Gly-His-X-X-Gly-X-X-His-Ser) [56]. The catalytic site has a “specificity pocket” called “S1’”, which accommodates the side chain of the substrate residue. The varying sizes of the S1’ pocket in MMPs confer substrate-enzyme binding specificity.
The ancillary domain has sequences similar to those in hemopexin, the heme-binding protein, and also vitronectin [57]. The hemopexin/vitronectin-like domains are arranged as blades in a propeller-like structure, and the asymmetry in the different “blade” components also plays a role in substrate specificity, MMP activation and dimerization [58]. Thus, different MMP domains confer substrate specificity through substrate affinity interactions with specific domains or through size exclusion and “fit” of substrate moieties.

Although most MMPs are secreted and exist in a soluble state, many MMPs can be anchored to the cell surface by a membrane-type- matrix metalloproteinase (MT-MMP) or other membrane-bound integrins, such as α,β, or complexes such as the hyaluronan receptor CD44, cell-surface heparin-sulfate proteoglycans like syndecan, or the extracellular matrix metalloproteinase inducer (EMMPRIN/CD147) [59–61]. MT-MMPs consist of a transmembrane domain and a short cytosolic segment of 20 amino acids that is highly conserved between species [51]. Most MT-MMPs are type I transmembrane proteins with the amino terminus oriented outside of the cell except for MMP-23, a type II transmembrane protein, and they can exist with or without a glycosylphosphoinositol (GPI) anchor for binding to the plasma membrane [62,63]. Thus, most non-membrane type MMPs can exist both in a soluble state and anchored to the cell membrane during their lifetime, and MMP assessment typically involves tissue homogenization steps to remove any MMP still bound to the plasma membrane.

Both gelatinases, MMP-2 and MMP-9, contain three tandem fibronectin type II repeats to facilitate gelatin binding [64], with MMP-9 further encompassing a type V collagen-like domain, whose function is currently unknown [51]. MMP-2 is the most
widespread of all MMPs [65], and its activation, which is one of the most well characterized, requires both MT1-MMP (MMP-14) and TIMP-2 [66]. Active MT1-MMP first binds and inhibits TIMP-2, whose hemopexin C-terminal domain then binds ProMMP-2. MT1-MMP can then cleave and partially activate MMP-2, which then dissociates from the membrane and becomes fully activated through intermolecular processing [58]. Activated MMP-2 is then is located to the cell surface through binding of integrin αvβ3 by its carboxyl terminus or through binding to the MMP-14/TIMP-2 complex [61]. MMP-9 is typically inhibited by TIMP-1, and the α2 chains of collagen IV bind MMP-9 with high affinity in both its active and inactive form [51]. The enzyme and substrate are able to bind with such high affinity despite the inactive state of the enzyme, which allows for sequestered protease to be immediately available in the case of necessary remodeling events, such as wound healing after injury.

**Endogenous MMP regulation**

Since MMPs have such a high potential for tissue breakdown, they are tightly regulated in the body at three different levels, the transcript level, activation level and inhibition level. Additionally, regulation of their location inside or outside of the cell and endocytosis internalization contributes to this strict level of control [51]. MMPs are Ca$^{2+}$ and Zn$^{2+}$ dependent endopeptidases, so the proteases are secreted and maintained in a latent form by the interaction of the cysteine residue in the propeptide with the zinc moiety in the protease active site, which excludes water from the active site [51]. At the transcription level, transcription of many MMPs, including MMP-9, are regulated by their upstream promoter sequence, which include the transcription factors, NFκB and
activator protein 1 (AP-1). NFκB and AP1 are activated by the nuclear translocation of c-Jun and c-Fos to AP-1, and p65 and p50 to NFκB in response to numerous growth factors, cytokines, or mitogens [67,68].

At the activation level, MMPs are secreted as an inactive zymogen with a pro-domain that must be cleaved for enzyme activation [69]. The MMPs can be activated by other proteases or thiol-modifying agents (4-aminophenylmercuric acetate, N-ethylmaleimide), oxidized glutathione, SDS, reactive oxygens, or even low pH and heat by disrupting the cysteine-zinc pairing (cysteine-switch mechanism) conformation, followed by cleavage of the propeptide by other proteases or intermolecular signaling cascades [70]. Furthermore, oxidative stress and cellular glutathione can glutathiolate the cysteine residue and disrupt its binding to the catalytic zinc, forming an intermediate active form before the entire propeptide is hydrolyzed or removed by further proteolysis [71]. Many pro-inflammatory cytokines can enhance MMP production, including TNFα, IL-17, oncostatin M, and IL-1, which bind cell surface receptors and enhance MMP mRNA upregulation through signal transduction pathways [69]. However, furin can intracellularly activate many MMPs, such as membrane-type MPs and MMP-11, by recognizing a specified furin motif in the prodomain, KX(R/K)R [58]. Intracellularly activated membrane-type MMPs can further initiate activation cascades, which in turn activate other MMPs [72].

Lastly, at the inhibition level, tissue inhibitors of metalloproteinases, or TIMPs, bind to activated MMPs to suppress their action [58]. The TIMP molecules are shaped like wedges and mimic the substrate’s entrance into an MMP active-site cleft. Four TIMPs are ubiquitous in vivo, with all known cell types expressing at least one member.
Each of the TIMPs can mostly non-selectively bind all MMPs, and TIMP-2 is the most ubiquitously expressed at high levels [73]. However, TIMP-1 can inhibit most MMPs except the majority of the membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-24), suggesting that TIMP-1 may exclude membrane-type specific elements, such as the carboxy-terminal transmembrane and the cytoplasmic domains. For the most part, the 4 mammalian TIMPs are 21 kDa in size, have 6 disulfide bonds, and a N-terminal domain and C-subdomain [58,74]. Since the TIMPs predominantly bind MMPs in a non-selective manner, their differences, observed mainly in the the N-terminal domains, lead to clear effects in their phenotypic roles on cell growth and survival, which are not always clearly tied to their ability to abrogate MMP activity [58,74]. Additionally, RECK, a membrane-anchored glycoprotein, can inhibit MMP-2, MMP-9 and MMP-14 [69,73]. Furthermore, a2-macroglobulin can inactivate active MMPs through a “mouse-trap”-like mechanism: a2-macroglobulin has protease-sensitive sites, and when they are cleaved, the inhibitor closes around the protease and prevents it from interacting with other substrates [74]. Thus, the balance of MMPs and inhibitors plays a significant role in ECM production and degradation, maintaining homeostasis and promoting tissue morphogenesis during development.

**Small molecule inhibition of MMPs**

Like TIMPs, synthetic inhibitors were initially designed to bind tightly at the active site to block proteolytic activity. The inhibitors have very similar peptide sequences to those sequences around the substrate’s cleavage site, however a chelating group, composed of thiol, carboxylic acid, hydroxamate, or phosphorous, replaces the
substrate’s scissile bond, which is where the protease specifically cleaves. The chelating group is then bound to the zinc in the protease’s active site, providing effective inactivation [69]. With the advent of crystallography analysis that enables visualization of protein structure, specifically the composition of the active site clefts, inhibitors could be produced with increased specificity for the active sites of individual MMPs [65,69]. Some of the substrate specificity variation in MMPs is due to differences in the active site cleft among the six specificity subsites and their surrounding sequences. In particular, the S1’ pocket, which is the first subsite on the carboxy-terminal side of the substrate scissile bond, is larger in MMP-8 and deeper in MMP-3 than it is in MMP-1. Differences have been reported out to the S’4 position and can be utilized to design more specific synthetic inhibitors [65,69].

Figure 2.1. Design of synthetic MMP inhibitors. Synthetic inhibitors (C) can be designed to fit within the 6 different protease subsites (A) similar to the manner in which the substrate can bind (B), and tightly chelate the active site zinc to prevent activity. The differences in the sizes and composition of protease subsites can be exploited to design inhibitors that target either or both (Ci) the S’ side (Ci) or S region (Cii). Figure adapted from Catterall et al. Arthritis Research and Therapy, 2003.
Based on exploiting the differences in the 6 specificity subsites in the MMP active site cleft, a variety of small molecules have been synthesized to selectively inhibit groups of MMPs. Two nonpeptidyl hydroxamate inhibitors are Trocade (Roche Ro-32-3555), which potently inhibits MMP-1, MMP-8, and MMP-13 [75,76], as well as MMP-2 and MMP-9 at a lesser level, and prinomastat (Agouron AG-3340), which inhibits gelatinases more potently than collagenases [65]. Trocade can effectively inhibit MMP activity in mouse embryonic stem cells in a non-cytotoxic manner [77], and it could abrogate MMP-2 activity at 50 μM. Moreover, hydroxamate inhibitor MMI270B (Novartis CGS 270231) can strongly inhibit MMP-1, 2, 3, 9, 12 and 14 [78], while Tanomastat, a non-peptidyl biphenyl inhibitor (Bayer BAY 12-9566) targets MMP-2, 3, 8, 9 and 13 [79]. Thus by understanding the MMP active site conformation and composition through crystallography, inhibitors can be designed for more preferential inhibition towards certain MMPs, as determined by low Ki values. However, the small molecule inhibitors still may potentially inhibit untargeted MMPs, although to a lesser extent. Further elucidation of MMP structure can lead to potential other targets for small molecule inhibition, such as targeting C-terminal hemopexin domains, which also play a role in interaction with the substrate during proteolysis [65].

MMPs in embryonic development

The gelatinases, type I MT-MMPs, and all TIMPs are universally and abundantly expressed throughout all tissues in the embryo, as determined through expression analysis of the entire MMP and TIMP gene families during mouse tissue development. Except for MMP-20, which is later expressed in tooth pulp tissue and odontoblasts, all of the MMPs
were expressed in at least one embryonic tissue [73,80]. This extensive characterization suggests that MMP and their endogenous inhibitors play important roles during early development. [73,80].

The gelatinases, MMP-2 and MMP-9, are highly involved in developmental processes, such as cell migration and tissue morphogenesis. Their large role in embryonic differentiation ranges from blastocyst uterine implantation to epithelial-to-mesenchymal transition and gastrulation, to the development of organ systems. The essential roles of the two MMPs were typically determined via knockout mouse studies. As the blastocyst implants into the uterine wall, the trophoblasts highly express MMP-9 for invasion of the maternal decidua to establish connection with the maternal circulation [81]. Additionally, MMP-9 plays a large role in neural extensions over long distances to establish the developmental of the mature nervous system [82] and in endochondral ossification in early long bone development [83]. Regarding tissue morphogenesis, pancreatic islet developmental organization is dependent on TGF-β activation of MMP-2, which degrades collagen type I, III, and IV during stages E17 and E19 to allow endocrine cells to migrate into the developing islet, which organizes into a structure with complex architecture [84]. Abrogation of MMP-2 activity still enables endocrine cell differentiation, yet islet morphogenesis into organized clusters is completely abolished [84]. In terms of regulating branching morphogenesis, MT-1-MMP activation of MMP-2 and TIMP-2 modulates epithelial-to-mesenchymal transitions within the metanephros to sustain the development of the ureteric kidney bud [85]. Furthermore, MMP-2 expressed in the neural tube is highly involved in the detachment and motility of cells from the neural tube epithelium during epithelial-mesenchymal transformation (EMT) that
generates the neural crest [86]. MMP-2 is involved in mesodermal and endodermal lineage development, as day 15 mouse embryo had highest active form in rib and mandible, and active form in lung, kidney, intestine, heart [66]. However, MMP-9 is not as highly expressed, although its RNA is present at a low level in all tissues that have been examined [73]. MMP-3, MMP-7, and MMP-13 have also previously been implicated in EMT activity [87–89].

**MMPs in embryonic stem cell differentiation**

Embryonic stem cells recapitulate many aspects of embryonic developmental pathways, however only a few studies have investigated MMP activity in ESCs. A previous study has demonstrated that MMP-3 is involved in cardiomyocyte differentiation of mouse ESCs [90]. Furthermore, MMP-3 plays a role in regeneration of ESCs derived odontoblast-like cells treated with Il-1β [91]. Also ESCs induced toward neural differentiation within PEG hydrogels with MMP-degradable cross-linkers had greater motor neuron outgrowths compared to ESCs cultured in regular PEG hydrogels, indicating that MMP production is a critical component of the differentiation process [92]. mESCs can also be cultured in an undifferentiated state in a LIF-independent manner via addition of MMP1, which enables mESC self-renewal by cleavage of the ciliary neurotrophic factor (CNTF), normally trapped in the endogenous ECM, allowing activation of the JAK-Stat3 pathway and promotion of self-renewal markers [77]. These studies illustrate the importance of proteolytic cleavage of the ECM to regulate signal transduction and ultimately stem cell fate.
The proteolytic release of growth factors and cytokines from the ECM reservoir has the potential to activate downstream signaling cascades to alter cellular phenotype. For example, FGF and BMP4 are involved in many differentiation pathways, and they often bind to glycosaminoglycan (GAG) sidechains of proteoglycans within the extracellular matrix. Proteolytic release of sequestered bioactive growth factors enables cellular binding and subsequent changes in signaling and gene expression, ultimately regulating differentiation [93–95]. Additionally, proteolytic cleavage also alters cell-ECM interactions or releases bioactive ECM fragments, which can both directly affect integrin signaling. Integrin signaling plays a large role in lineage commitment during early embryogenesis, and ECM signaling is predominantly transmitted via integrins to effect changes in cellular phenotype during differentiation [15]. Furthermore, exposure of ECM “cryptic sites” via proteolytic cleavage can alter integrin signaling and lead to cell motility and processes such as cell migration [96]. Thus, MMPs play a large role in embryonic differentiation, from blastocyst uterine implantation to EMT and gastrulation, to the development of organ systems. Similar to these developmental processes, MMPs facilitate stem cell differentiation mainly through modulation of cell-ECM or cell-growth factor interactions leading to changes in gene expression and cellular phenotypic changes.

**Epithelial-to-mesenchymal transition (EMT)**

Epithelial to mesenchymal transition (EMT) is a fundamental developmental step that occurs during the majority of organogenesis processes to facilitate complex tissue development, and EMT is characterized by the loss of cell-cell adhesion and polarity, and the acquisition of migratory properties [97]. EMT processes enable a polarized and
stabilized epithelial cell, with apical-basal polarity, to obtain a migratory mesenchymal cell phenotype, possessing migratory capabilities, invasiveness, apoptosis resistance, and enhanced ECM production [98]. Epithelial cells are characterized as adherent cells that form intercellular adhesion complexes, such as tight junctions, adherens junctions, desmosomes, and gap junctions [99]. The cell-ECM interactions allow the basal side to be anchored to the basement membrane, and the cell-cell interactions enable the apical and basal surfaces to have different functions. On the other hand, mesenchymal cells are non-polarized and lack intercellular junctions, allowing them to move as individual cells through the ECM. Mesenchymal cells can also synthesize more ECM and participate in its reorganization by secretion of MMPs [98,99].

The EMT process is reversible, in which a cell with mesenchymal phenotype can revert to a more stable epithelial form, termed mesenchymal-epithelial-transition (MET), in order to derive transient epithelia that form structures such as the notochord, somites, and somatopleure [100]. Besides the notochord, all of the mesoderm derived embryonic structures can undergo successive EMT and MET events to obtain the final architecture of tissues and organs. For example, paraxial mesoderm, or the area of mesoderm that runs alongside and forms simultaneously with the neural tube, is obtained via EMT through the primitive streak, and then undergoes MET to form the somites, which are organized as epithelial cell clusters. The somites then undergo EMT again to form the sclerotome, which eventually differentiate into the vertebrae and the skull. Thus, successive EMT and MET events occur in cycles throughout development to define tissue architecture [97,100,101]. The vast majority of all bodily structural components derived from the
early mesoderm goes through several EMT and MET events to obtain fully mature tissue and organs.

There are three types of EMT, categorized by the biological settings in which they occurred [98]. The first type, “type I,” is associated with embryonic development, involving uterine implantation, gastrulation and organ development. Mesenchymal cells derived from type I EMT have the potential to become epithelia through subsequent MET processes. Additionally, type I EMT does not cause inflammation, fibrosis, or induce invasive phenotypes that lead to metastatic spreading. Type II EMT is initiated as part of the repair process that occurs to reconstruct tissues after injury and inflammation. Type II EMT is thus associated with inflammation in the context of wound healing and tissue regeneration, and typically ceases once inflammation is ameliorated. However, in the case of chronic inflammation, the continuation of Type II EMT can lead to fibrosis and potential organ destruction. Lastly, type III EMT is specific to cells that have undergone genetic mutations and become carcinogenic, invading other tissues and metastasizing, typically present in the final stages of terminal cancer [97,98,101].

Despite the differences in the three types of EMT, they all seem to express common genetic and biomolecular elements. Among these biomolecular processes involved to signal the start of EMT as well as usher it towards completion, transcription factor activation, cell surface protein expression, cytoskeleton protein expression and reorganization, expression and activation of proteases to degrade ECM, and even modifications in microRNA expression are involved [99].
EMT during embryonic development

Type I EMT occurs during vertebrate embryonic development and is characterized by primary, secondary and tertiary EMT. Primary EMT involves embryo implantation, gastrulation, in which epiblast epithelium forms mesoderm (axial, paraxial/somites, intermediate, lateral plate) and endoderm, and neural crest formation, in which the dorsal neural tube epithelium forms neural crest mesenchyme. Secondary EMT encompasses somite decondensation into the sclerotome and myotome, further development of the somatopleure and splanchnopleure, as well as pancreas and liver development. Tertiary EMT is characterized by formation of the cardiac cushions from cardiac endothelium [97–99,101]. The first instance of primary EMT occurs during implantation of the embryo in the endometrium, which commences placenta formation. The embryo trophectoderm cells undergo an EMT to invade and anchor into the endometrium to facilitate proper nutrient exchange from the mother’s womb [97,101].

Another key event characteristic of primary EMT, gastrulation, enables the formation of all 3 germ layers and begins to establish the organism’s structure and architecture. Gastrulation is initiated through Wnt signaling-dependent formation of primitive streak, which is a linear structure forming in the posterior part of the embryo that transverses the embryo along its antero-posterior axis [102]. Wnt signaling also renders cells competent to respond to signals from the TGF-β superfamily, specifically Nodal and Vg1, which along with FGF, facilitate EMT through activation of the Snail family of transcription factors [97,98,101]. Snail transcription factors facilitate downregulation of E-cadherin so that cells can lose their cell-cell contacts and become free to migrate [103]. Rapid downregulation of E-cadherin is also mediated by
eomesodermin, which increases the ability of Snail to repress E-cadherin, and P38 MAPK and p38IP, which promote active and rapid degradation of E-cadherin by mediating its trafficking through the Golgi Apparatus [97,104].

In addition to repression of E-cadherin, the Snail transcription factors also repress epithelial phenotype, namely genes encoding tight junction compartments, claudins and occludins, and apico-basal polarity, Crumbs3 and Ephrin1 [97,101]. Snail is able to abolish localization of polarity complexes to the tight junctions, which reduces the cell-cell connections and enables cell motility [89,103]. In addition, EMT processes are characterized by the ability of cells to pass through the basal membrane and delaminate from the epithelial cell layer. Thus, Snail transcription factors also activate MMP-2, 3 and 9 for basal membrane breakdown as well as repression of the construction and receptors of ECM components such as laminin-5 [89,103]. Moreover, E-cadherin is connected to the actin cytoskeleton by β-catenin at the adherens junctions. In response to Wnt signaling and Snail, β-catenin is translocated from the cell membrane to the nucleus where it complexes with lymphoid enhancer factor-1 (LEF-1), and the resulting β-catenin-LEF-1 transcription complexes enhance expression of the target genes vimentin, fibronectin, and αSMA that are characteristic of the mesenchymal phenotype [101,105].

Thus, primary EMT is mediated by a complex signaling network, which facilitates repression of epithelial phenotype, breakdown of the basement membrane, and mediates ingression and development of mesenchymal phenotype.

**EMT processes in embryonic stem cell differentiation**

Human and murine embryonic stem cells have increasingly been utilized as a model system for elucidating mechanisms involved in development, especially since
ESCs can recapitulate many of the EMT events occurring in the mammalian embryo, particularly during gastrulation, [87,106–108]. EMT processes in monolayer human embryonic stem (ES) cells is characterized by downregulation of E-cadherin concurrent with upregulation of N-cadherin, up-regulation of Snail and Slug (E-cadherin repressor molecules), enhanced vimentin expression, and increased gelatinase (MMP-2 and MMP-9) activity and cellular motility [107,109]. Additionally, in a manner similar to embryogenesis, Wnt signaling is required for upregulation of EMT, mesendoderm and mesoderm gene expression in mESCs [110]. Studies in ESCs have elucidated the role of the 5T4 oncofetal antigen, which when expressed on the cell membrane, promotes mesenchymal-like cell motility through mediating DECMA-1-dependent E-cadherin internalization [109]. In human ESCs, E-cadherin stabilizes cystoskeleton architecture, which prevents cell membrane localization of the 5T4 antigen. Thus, later Snail-mediated E-cadherin downregulation promotes 5T4 antigen membrane expression, stimulating further E-cadherin internalization [107,109]. The roles of several other molecules involved in EMT processes occurring during ESC differentiation have been identified. Ell3, a testis-specific RNA polymerase 2 elongation factor, modulates differentiation by enhancing gene expression associated with EMT, such as Zeb1, and suppressing apoptosis through repressing p53 [111]. Furthermore, although Snail transcription factors can repress epithelial phenotype in differentiating ESCs, one potential mechanism is through its regulation of members of the ESC-associated microRNA-200 family. The 5 members of the microRNA-200 family can activate the transcription factors, Zeb1 and Zeb2, which lead to E-cadherin repression [112]. Thus, ESCs recapitulate many EMT processes present during embryonic gastrulation, and continuing to study EMT in ESCs
can elucidate the roles of ESC-specific EMT molecules, which may aid in developing directed differentiation strategies.

Roles of MMPs in regulation of EMT processes

MMP cleavage of the ECM has increasingly been characterized, and byproducts of the cleavage have been determined to impart specific biological function. In particular, MMPs can cleave and release “cryptic sites” as well as “neo-epitopes” from the ECM that possess bioactivities and functions separate from their parent molecules [113]. For example, MMP-2 and its closely associated activator, MT1-MMP, can release the $\gamma_2$ chain of laminin-5, which has been demonstrated to induce migration of epithelial cells [114]. Furthermore, MMP-2 and MMP-9 can expose cryptic sites in Collagen IV and laminin that regulate cell migratory behavior [55,115]. A laminin-111 fragment cleaved by MMP-2 modulates EMT events in human and murine ESCs at the gene and protein expression through an $\alpha_3\beta_1$-integrin/extracellular matrix metalloproteinase inducer complex (EMMPRIN) [108]. Additionally, fibronectin fragments exposed by MMP cleavage also induce cellular migration, since a truncated isoform of fibronectin containing Fib1/Hep1, the gelatin binding regions, and the first portion of module III1, termed the migration-stimulating factor (MSF), is able to induce cellular migration, which cannot be attained with the full length fibronectin molecule. Motogenic activity by MSF is dependent on the IGD motif located within the gelatin-binding domain. [116]. Further evidence that fibronectin fragments can direct EMT processes have been demonstrated in a study, which found that epithelial cells cultured with mainly the RGD fragment (FnIII10) displayed a more mesenchymal phenotype, characterized by
formation of stress fibers, loss of cell-cell connections and reduction in circularity and elongated fibroblast-like morphology [117,118]. Meanwhile, epithelial cells cultured with the RGD fragment as well as the synergy (PHSRN) site (FnIII9’10) maintained cell-cell contacts and their epithelial markers and morphology [117,118].

The ECM also serves as a reservoir to store latent growth factors and cytokines in bioactive conformations, and proteolysis can release and activate these factors, enabling them to bind to the cell membrane and facilitate downstream signaling processes [119–121]. For example, TGF-β, which drives EMT through stimulation of Snail family of transcription factors, is typically maintained in a latent complex with latency-associated peptide (LAP), which is bound to the ECM through covalent attachment between LAP and a latent TGF-β binding protein (LTBP). MMP-2, 3, and 9 can cleave LTBP, effectively releasing the latent TGF-β complex, and additionally the MMPs can also activate TGF-β by cleavage of LAP. MMPs can likewise release and activate molecules such as BMP4, through cleavage of its inhibitory complex with chordin, which enables BMP to bind to its ALK receptors and promote downstream SMAD phosphorylation and mesoderm induction [103,113]. Additionally, MMPs can modify the interactions cells have with the ECM, either through building stronger interactions of the cytoskeleton to the adherens junctions to maintain an epithelial phenotype, or to reduce the interactions to facilitate migration.

Thus MMPs play a large role in EMT processes, through both exposure of moieties that possess biological functions, as well as release and activation of potent biomolecules that can facilitate downstream transcription. The role of MMPs in EMT processes can be further elucidated by studying embryonic stem cells, which have been
increasingly utilized as models for studying developmental processes because they recapitulate many relevant signaling morphogenic events occurring during embryogenesis, including gastrulation.
CHAPTER 3

GELATIN METHACRYLATE MICROPARTICLE SYNTHESIS AND CHARACTERIZATION

Introduction

Gelatin has been used as a delivery vehicle for the controlled release of biomolecules due to its ability to form polyion complexes with charged therapeutic compounds such as proteins, nucleotides, and polysaccharides [122,123]. Gelatin is obtained from denaturation of collagen via alkaline or acid treatment to yield gelatin with either a net negative (isoelectric point (IEP) = 5) or net positive (IEP = 9) charge, respectively, at pH 7.4. Modulating the net charge of gelatin allows for sequestering of biomolecules of the opposite charge while maintaining their bioactivity. While molecules may be released from the gelatin via diffusion, gelatin’s proteolytic degradability offers an additional mechanism to facilitate release of growth factors [123,124].

Gelatin microparticles (MPs) have been extensively studied for their ability to deliver growth factors for diverse applications such as therapeutic angiogenesis [124,125], cartilage tissue engineering [126–128], and post-myocardial infarction therapy [129], as well as stem cell differentiation within scaffolds [47], embedded within a self assembling cell sheet [130], or within aggregates [35,45,49,50]. Gelatin MPs are typically formed via a water-in-oil emulsion and subsequent cross-linking of gelatin microspheres with reagents such as glutaraldehyde (GA) [128,131], genipin [49,130], or carbodiimides [132].

Modified from:
Nguyen, AH.* McKinney, J. Miller, T. Bongiorno, T. McDevitt, TC. Accepted in Acta Biomaterialia.
The most common method for cross-linking gelatin MPs is via GA cross-linking, which occurs primarily through the reaction of GA aldehyde groups with the ε-amine groups of lysine or hydroxylysine residues, resulting in a Schiff base intermediate that cross-links gelatin through an aldol condensation reaction [133]. However, the Schiff base intermediates are unstable, and can react further to form products such as secondary amines and 6-membered dihydropyridines, which can form other types of cross-links within the MP, such as aliphatic crosslinks and quaternary pyridinium-type cross-links, among other classes of molecules [133–135]. Thus, GA cross-linking can lead to a variety of possible cross-linking entities, conceivably creating more heterogeneous hydrogels, although few studies have characterized the frequency of the various cross-links and whether they produce adverse events. Despite the use of a wide range of GA concentrations (from 0.05 to 2.5 wt%), cross-linking below 60% is rarely attained, with GA above 0.5 wt% typically yielding 100% cross-linking within 24 hours [136]. Furthermore, GA treatment yields similarly cross-linked MPs despite employing reaction times from 1 to 24 hours and different temperatures (4-37°C) [130,132,133,137]. Experimentally controlling the low GA concentration required to obtain lower cross-linking densities may be difficult and thus may not have been explored if the studies did not require MPs cross-linked below 60%. Since a fifty-fold difference in GA concentration, as well as vast variations in temperature and reaction time, yield only a small range of cross-linking, very little correlation can be drawn between input GA and cross-linking density. These inherent caveats in GA cross-linking procedures make it difficult to accurately predict cross-linking a priori or fabricate MPs with varying levels
of cross-linking density, which limits applications for varying release rates of loaded biomolecules. Furthermore, GA cross-linking significantly reduces the degradability of collagen-based materials, and debris from cross-linked hydrogel abrasion or breakdown in addition to remaining GA molecules have demonstrated cytotoxicity and inflammation in vivo and can lead to calcification, possibly due to unpaired aldehyde functional groups, where calcification is initiated, after GA treatment [136,138–140].

In order to circumvent the limited control over cross-linking density and cytotoxic complications of GA cross-linking, methacrylation of gelatin has been increasingly studied based on its reduced cytotoxicity and ability to support a broader range of cross-linking densities [141]. Amine groups on gelatin can be substituted with glycidyl methacrylate (GyMA), methacryloyl chloride (MC) or methacrylic anhydride (MA) [141–144]. However, GyMA contains a hydrolytically degradable ester group, producing less stable hydrogels, and MC less efficiently facilitates methacrylate substitution compared to MA [141]. Methacrylate groups can be reproducibly introduced into gelatin with MA to achieve a wide range of methacrylation values. MA substitution affords greater control over hydrogel cross-linking density than other systems such as glutaraldehyde, genipin, or dehydrothermal cross-linking because the methacrylate groups on GMA restrict the maximum cross-linking density achievable, independent of soluble factors that are difficult to control, such as the amount of radical initiator added, or experimental conditions, such as time and temperature of cross-linking.

Although GMA bulk hydrogels have been characterized extensively [141,143–145], this study is the first to characterize GMA MP physical properties and growth factor binding and release. In this study GMA MPs were fabricated from gelatin with a
wide range of methacrylate substitution. Resulting MPs were analyzed for size range, degradability, and their ability to bind and release growth factor compared to the conventional GA cross-linked MPs. These studies demonstrate that modulation of MP cross-linking density via gelatin methacrylation facilitates greater control over critical MP properties compared to conventional glutaraldehyde cross-linked MPs, and this enhanced control enables tailoring of particles to deliver growth factors for tissue engineering purposes.
Methods

Gelatin methacrylate synthesis

Gelatin B (pI = 5.0) (Bovine skin, Sigma Aldrich, St. Louis, MO) was dissolved in water (10% w/v) at 60°C. Gelatin methacrylate (GMA) was produced by reacting the amine groups on gelatin type B with methacrylic anhydride (MA), similar to previously described methods [141–146]. Briefly, MA (Sigma Aldrich) was added dropwise to the gelatin solution at a 1:3, 2:3, or 1:1 ratio (mol MA: mol unsubstituted amines on gelatin). The pH was continuously monitored and adjusted to ~pH 7.4 to promote efficient substitution of amines to methacrylate groups. The GMA solution (15 mL) was added to 60 mL PBS and dialyzed (SpectraPor, MW cutoff 12-14 kDA, Spectrum Labs, Santo Dominguez, CA), for 5 days against 2 L deionized water, with water changed twice daily, followed by lyophilization and storage at -20°C until further use.

Gelatin methacrylate characterization

A fluorescamine assay was performed to determine the degree of substitution of free amine groups via methacrylation. Lyophilized GMA at molar concentrations of 1:3, 2:3, and 1:1 MA to unsubstituted amine groups on gelatin, as well as completely unsubstituted gelatin type B, were solubilized in 500 μL of deionized water and reacted with 1 mL of fluorescamine solution (7 mg fluorescamine (Sigma Aldrich) in 25 mL dimethylsulfoxide (DMSO)). Samples were read on a Synergy H4 plate reader (Biotek, Winooski, VT) at excitation 390 nm, emission 465 nm. The number of free amine groups

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in each GMA formulation was calculated using a glycine standard curve and compared to unsubstituted gelatin type B to determine the final degree of substitution.

$^1$H NMR was also performed to determine the degree of substitution of gelatin. Each lyophilized gelatin derivative (5-10 mg) was dissolved in deuterium oxide (D$_2$O, Cambridge Isotope Laboratories, Inc., Andover, MA, USA). $^1$H NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer and each resulting spectrum was phase corrected, baseline subtracted and integrated with ACD NMR processor 12.0 software. The degree of substitution was determined according to the method of Hoch et al [141]. In brief, the signal of protons resulting from aromatic amino acids in the polymer at $\delta=7.0$ ppm to $\delta=7.5$ ppm were used as a reference in each spectrum. The signal of methylene protons ($\delta=2.7 – 2.9$ ppm) neighboring the lysine amino acid was used for quantification of the integrated signal areas. The integrated area of methacrylated gelatin relative to unmodified gelatin was used to determine the degree of substitution. The degree of methacrylate substitution for $^1$H NMR was determined by the following equation: $[1-(\text{lysine integration signal of methacrylated substituted gelatin/lysine integration signal of unsubstituted gelatin})]$. 

**Gelatin methacrylate microparticle fabrication**

Lyophilized GMA was dissolved in water (10% w/v) at 37°C. Sixty mL of corn oil (Mazola) was heated to 37°C prior to the addition of 1 mL of polysorbate 20 (Promega, Fitchberg, WI) and homogenized at 1500 rpm (Polytron PT-3100 homogenizer) for 3 minutes. The 10% GMA solution was mixed with 3 μL of 0.3 M ammonium persulfate (APS) (Bio-Rad, Hercules, CA) and added dropwise to the corn oil
phase. The oil and water emulsions were homogenized for 5 minutes at 1800 rpm for 15% and 50% GMA, and 1500 rpm for 90% GMA (to obtain MPs of similar size as 15% and 50% GMA MPs), and placed on a hotplate set to 45°C with agitation via stir bar. N₂ gas was bubbled through the emulsion for 20 minutes to purge oxygen. Under constant stirring, the hotplate was increased to 100°C to initiate the thermal cross-linking reaction and allowed to proceed for 40 minutes. The particle/corn oil mixture was centrifuged at 2500 rpm at 4°C to harvest the microparticles, and excess corn oil was removed with four successive washes with deionized water. In order to visualize the hydrogel microparticles in some cases, fluorescent labeling was performed by incubation with Alexa Fluor ® succimidyl ester 594 (Invitrogen, Carlsbad, CA) in 0.1 M sodium bicarbonate buffer followed by four deionized water washes. All particles were stored at 4°C in dH₂O until further use.

**Gelatin glutaraldehyde microparticle fabrication and cross-linking determination**

Glutaraldehyde cross-linked gelatin particles were synthesized as previously described [137]. A 10% gelatin solution in water (2 mL) was added dropwise to 60 mL of corn oil and homogenized at 2500 rpm at room temperature. The corn oil/gelatin mixture was cooled to 4°C and incubated for 10 minutes before 35 mL of acetone was added and the whole mixture was homogenized at 2500 rpm at room temperature. The mixture was re-cooled to 4°C and centrifuged at 2500 rpm to pellet the particles. Excess corn oil and acetone were removed via 3 water washes. The particles were separated into 1.5 mL tubes and 1 mL gluteraldehyde (10 mM) was added to each tube to crosslink the particles overnight. Glycine (25 mM) was added 15 hours later to quench any remaining reactive
aldehyde groups. The particles were then washed with water 3 times, lyophilized, and stored at -20°C until further use. In order to determine GA cross-linking, an equal weight of lyophilized GA cross-linked MPs and unsubstituted gelatin type B were solubilized in deionized water and reacted with fluorescamine reagent in a manner analogous to determining GMA degree of substitution as described above. Free amine groups in GA MPs were compared to amine groups in 0% cross-linked unsubstituted gelatin type B to determine the degree of cross-linking.

**Scanning electron microscopy**

MPs were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (Electron Microscopy Sciences (EMS), Hatfield, PA). The MPs were then thoroughly rinsed and incubated in 1% osmium tetroxide (EMS) followed by graded ethanol dehydrations and critical point dried using a Polaron E3000 critical point dryer (Quorum Technologies Inc., Guelph, ON, Canada). Samples were sputter coated for 2 minutes at 2.2 kV using a Polaron SC7640 sputter coater and imaged using a Hitachi S-800 scanning electron microscope (Hitachi High Technologies, Pleasanton, CA).

**Microparticle size analysis**

MPs were suspended in ISOTON II diluent (Beckman Coulter, Hialeah, FL), a phosphate-buffered saline solution, at an approximate concentration of 0.1 mg/ml. Volume size distribution was determined via Coulter Counter (Beckman Coulter Z2, Brea, CA) with a 70 μm aperture. Size bin, or category, distributions were defined by the
percentage of MPs that fell within the ranges of less than 5 \( \mu \text{m} \), between 5 and 15 \( \mu \text{m} \), and greater than 15 \( \mu \text{m} \).

**Gelatin microparticle content**

The amount of gelatin per MP was quantified using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). For each of the different MP formulations, 1 million particles in 50 \( \mu \text{L} \) of PBS were reacted with 400 \( \mu \text{L} \) BCA working reagent for 30 min at 37\(^{\circ}\)C. Absorbance readings were taken at 562 nm on a Synergy H4 Biotek plate reader and total gelatin content was calculated by comparing the absorbance readings against a standard curve generated using a range of gelatin type B concentrations (0-2000 \( \mu \text{g/mL} \)).

**MP degradation analysis**

Collagenase 1A (Sigma Aldrich) was adsorbed to magnetic polystyrene beads (4-5 \( \mu \text{m} \) in diameter, Spherotech, Lake Forest, IL), as previously described [147]. Briefly, a 25 \( \mu \text{L} \) aliquot of Spherotech bead solution was incubated in 1 mL of 2 mg/mL of collagenase for 2 hours at room temperature in a low retention 1.5 mL tube (Axygen, Pittsburgh, PA). Collagenase-coated beads were separated from solution via a magnet, the supernatant removed, and unbound collagenase was removed via 3 PBS washes. A BCA assay was used to determine the amount of beads necessary to obtain 1 \( \mu \text{g} \) collagenase. Beads with 1 \( \mu \text{g} \) of collagenase were added to 0.5 mg of each MP formulation in 1 mL PBS with 0.036 mM CaCl. The collagenase beads and MPs were rotated continuously at 37\(^{\circ}\)C, and the collagenase coated-beads were replaced with freshly coated beads under sterile conditions every 48 hours. At specified time points,
collagenase-coated magnetic beads were removed and the MP suspension was transferred to a new tube with 0.5 mL of 0.03% fluorescamine in DMSO. Increasing values, indicating the generation of new amino terminus groups by peptide bond cleavage by collagenase, were determined on a Biotek plate reader with an excitation of 390 nm and emission of 465 nm, and compared to non-degraded MPs and completely degraded MPs (50 µg collagenase for 48 hours).

**MP swelling studies**

An equal volume of each MP formulation (100 uL) was deposited in a 1.5 mL microcentrifuge tube, and all excess water was aspirated and blotted from the MPs before the swelling weight was recorded. MPs were lyophilized for 48 hours before recording the dry weight of the MPs. Swelling ratio (q) = (swollen MP weight)/(dry MP weight). Equilibrium swelling ratio (Qₘ) = 1 + (ρₐgelatin/ρₜwater)(q+1), with q = density. Water content was determined as (Wₛ-Wₕ)/Ws x 100, with Wₛ= swollen weight of MPs and Wₕ = dry weight of MPs.

**MP elastic moduli measurement**

The elastic moduli of MPs were measured via atomic force microscopy (AFM). MPs were conjugated to glass coverslips prior to AFM measurements. Coverslips were functionalized with amine groups via incubation with 1% (3-aminopropyl)trimethoxysilane (APTMS) in absolute ethanol for 2 hours at room temperature under gentle agitation and then washed with dH₂O to remove excess APTMS. MPs were incubated with 2 mM 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride.
(EDC) and 5 mM N-hydroxysulfosuccinimide (sNHS) in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) activation buffer for 15 minutes, and subsequently quenched with 1.4 uL of 2-mercaptoethanol. Each MP sample was centrifuged to remove activation buffer, resuspended in dPBS, incubated on a coverslip for 2 hours at room temperature (23°C), and washed with dPBS and placed in a 50 mm plastic dish (BD, Franklin Lakes, NJ) in dPBS before mechanical characterization was performed with an atomic force microscope (Asylum Research, Santa Barbara, CA) stationed on a vibration isolation table (Herzan, Laguna Hills, CA). A brightfield microscope (Eclipse Ti, Nikon, Melville, NY) was used to locate and position a tipless silicon nitride cantilever (MLCT-O10, Bruker, Camarillo, CA, Cantilever E, k=50-200 pN/nm) with a 5.5 μm polystyrene bead (Bangs Labs, Fishers, IN) over the center of each MP. The cantilever spring constant (k=110.56 pN/nm) was determined by thermal calibration, and a probe velocity of 2 μm/s was used. Indentations of approximately 25 nm for typical particles were obtained using a 5 nN force trigger. The Young’s modulus of each particle was determined using IGOR software (Wavemetrics, Portland, OR), which applies the Hertzian contact model to the extension force-displacement curves from 60-95% of the maximum indentation, over which range the Young’s modulus was largely independent of indentation variability during the early contact of the cantilever bead with the MP due to softness of the sample. The average Young’s modulus of two measurements was calculated for each particle, assuming cellular Poisson’s ratio, ν=0.5 and using indentation offset as a free variable.
Mesh size calculation

Mesh size of the MPs was determined from MP elastic moduli measurements via AFM and swelling ratios as previously described [146]. Briefly, molecular weight between crosslinks (M_c) was determined as equivalent to 3ρT/E, where E = the elastic modulus as determined via AFM, ρ = gelatin density, R = gas constant (8.3145 J/Kmol), and T = absolute temperature (K). The mesh size (ξ) was calculated via the formulation:

$$\xi = 2\alpha(M_c/M_r)^{1/2}(2.21\text{Å})(Q_m)^{1/3}$$

with M_r = 100 g/mol, Q_m as the equilibrium swelling ratio, and α, the expansion factor, as 2 for gelatin.

Microparticle growth factor loading

MPs (0.5 mg) were added to 1 mL PBS with 1% BSA and incubated overnight with 10, 50, 100, 150, 200, or 300 ng/mL sterile solutions of recombinant human BMP-4 or recombinant human FGF-2 (R&D) under rotational agitation at 4°C. After 15 hours, the tubes were centrifuged to separate out the MPs with loaded growth factor, and the supernatant was removed and assayed for BMP-4 or FGF-2 content via ELISA (R&D Duoset). Growth factor loading was determined by subtracting the amount of growth factor in the supernatant from the input amount. Loading efficiency at each concentration was determined via total amount loaded divided by total growth factor initially added to the MPs.

Growth factor release from microparticles

GMA MPs of varying degrees of methacrylation, as well as GA MPs (0.5 mg) were incubated for 15 hours at 4°C with equal concentrations of growth factor (10 ng/mg
MP for BMP4 and 50 ng/mg MP for bFGF). After the incubation period, the MPs were centrifuged, and the supernatant was replaced with fresh buffer consisting of 1% BSA solution in PBS with or without collagenase 1A under sterile conditions. At each time point thereafter, 300 uL of the supernatant was collected for analysis and replaced with an equal volume of fresh buffer. Enzymatically treated samples were either treated with 100 ng/mL or 1 µg/mL collagenase 1A. Collagenase buffer was changed every 3 to 4 days as previously reported [127,128]. The amount of growth factor collected in the supernatant was determined via BMP4 and bFGF ELISA (R&D duoset). The percent of cumulative growth factor release was determined via normalization of total growth factor released at each time period with the total growth factor initially loaded onto the MPs.

**Statistical analysis**

All values are reported as mean ± standard error, with a minimum of triplicate experimental samples. Before statistical analysis, a Box-Cox power transform was used to process all non-normal data to a Gaussian distribution. Statistical significance was determined using one-way ANOVA with Tukey’s post hoc analysis with 95% confidence intervals after performing Levene’s equality of variances test. P-values < 0.05 were determined to be statistically significant.
Results

Gelatin methacrylate modification

GMA degree of substitution was determined via both $^1$H NMR and a fluorescamine assay. Based on $^1$H NMR analysis, GMA synthesized with low, medium, and high degrees of methacrylation (molar ratios of 1:3, 2:3, and 1:1 of MA to unsubstituted groups on gelatin) corresponded to approximately 7%, 39%, and 100% methacrylate substitution, respectively (Figure 3.1B,D). Similarly, fluorescamine analysis indicated that the three GMA formulations were approximately 15%, 50% and 90% substituted (Figure 3.1C,D). The two substitution analysis methods were highly correlated, and the GMA formulations were referred to thereafter by the fluorescamine-determined substitution values. In comparison to the GMA MPs, GA MPs were 93.4 ± 4.5% cross-linked as determined by assaying the remaining unsubstituted amines after GA treatment via the fluorescamine assay.
Figure 3.1. Gelatin methacrylate characterization. A) Schematic of methacrylate substitution of the primary amines of gelatin. B) H$^1$ NMR spectra was recorded for unsubstituted gelatin and GMA with 1:3, 2:3, and 1:1 mol methacrylic anhydride: mol unsubstituted amines on gelatin. The MA modification of lysine residues with increasing methacrylic anhydride addition can be confirmed by the continuous decrease in the lysine signal at $\delta = 2.9$ ppm (y), and increase in the methacrylate vinyl group signal at $\delta =5.4$ ppm and 5.7 ppm (x) and methyl group signal at $\delta=1.8$ ppm. C,D) Degree of methacrylate substitution was also determined for the GMA formulations via a Fluorescamine assay normalized to unmodified gelatin determined as 0% substitution.

Gelatin methacrylate particle characterization

Microparticle size analysis

All MPs had a smooth, round morphology as illustrated via phase microscopy of the MPs (Figure 3.2 A-D). SEM analysis confirmed this morphology, however, the 15% substituted MPs appeared to fuse together during preparation for SEM and thus their
individual morphology could not assessed by SEM (Figure 3.2 E-H). MP sizes were analyzed via Coulter Counter and the average diameters of hydrated 15%, 50% and 90% GMA MPs were 4.9 ± 3.6 μm, 5.5 ± 5.2 μm, and 5.0 ± 6.9 μm, respectively, while GA MPs were determined to be 5.1 ± 7.7 μm (Figure 3.3 A-D). No significant differences were found in the size distribution of MPs across methacrylated groups. Overall, the methacrylated MPs could be reliably produced with higher proportions of monodisperse particles (< 5 μm) compared to GA MPs, which were the most polydisperse (Figure 3.3 E).

Figure 3.2. Gelatin microparticle morphology. A-D) Phase and E-H) scanning electron microscopy images of GMA and GA cross-linked MPs indicate round morphology and smooth surfaces. A-D) Scale bar: 100 μm. E-H) Scale bar: 10 μm.
Figure 3.3. Gelatin microparticle size analysis. MP size bin analysis was performed via Coulter Counter for A) 15% MA MPs, B) 50% MA MPs, C) 90% MA MPs, and D) GA MPs. Volume percentage distribution of MPs spread out further across all size bins with increased methacrylation, with the greatest distribution across size bins occurring in the GA MPs. E) No differences were found between MP sizes despite different extents of MA substitution. GMA MPs can be fabricated with a greater proportion of smaller sized MPs and lower quantities of large sized MPs than can be achieved through GA formulations. *: denotes statistical significance, n≥3, p<0.05.
Microparticle gelatin content analysis

The GA MPs contained significantly more gelatin per particle (2.5 μg/μm$^3$) than any of the GMA formulations (<1 μg/μm$^3$). However, increasing the methacrylation decreased the gelatin content of the fabricated MPs, as the 15% and 50% MA MPs had two-fold greater gelatin content than the 90% MA formulation (Figure 3.4).

![Figure 3.4. Microparticle gelatin composition.](image)

Figure 3.4. Microparticle gelatin composition. Gelatin content per volume of MPs was determined for each of the 4 gelatin MP formulations. The GA MPs had the highest gelatin content and for the GMA formulations, increasing the degree of methacrylation appeared to decrease the gelatin content.

Microparticle degradation analysis

Degradation studies of the MPs upon exposure to 1 μg/mL of collagenase 1A exhibited an inverse correlation between degradation and methacrylation levels (Figure 3.5). By 6 hours, almost half of the GA formulation had degraded compared to about one fourth of the higher methacrylated MPs (50% and 90%). Furthermore, the GA MPs were completely degraded by 48 hours, whereas the higher methacrylated MPs took over 96 hours to reach 100% degradation.
Figure 3.5. Gelatin microparticle degradation. A) Degradation profiles were also determined via incubation of the MPs with collagenase. Increasing the methacrylation level resulted in an increase in MP degradation time, and GA MPs were comparable to the lowest methacrylated MP formulation in degradation profile. Degradation kinetics below 12 hours are expanded in B) for clearer visualization. The 15% MA and GA formulations were the most degraded by 12 hours, degrading completely by 48 hours. The higher methacrylated MPs were completely degraded by 96 hours. n≥3, *: denotes statistical significance with p<0.05 *: 90% MA vs. 15% MA and GA. ^: 15% MA vs. 50% MA. #: 50% MA vs. 15% MA and GA. +: 90% MA vs. 50% MA.
Microparticle elastic moduli and swelling analysis

Elastic moduli of the MPs were determined via AFM on surface-immobilized MPs. The lower methacrylated MPs (15% MA and 50% MA) were significantly less stiff (39.5 kPa and 54.0 kPa, respectively) and were almost an order of magnitude smaller modulus than the 90% MA MPs, which had an equivalent stiffness to GA MPs with moduli of 222.0 and 243.6 kPa, respectively (Figure 3.6 A). Moreover, the ability of all methacrylated MPs to swell in a hydrated environment was significantly greater than that of the GA MPs with the lowest methacrylated MPs (15% MA) exhibiting over 1.6 fold increased swelling volume compared to the other two methacrylated MPs (Figure 3.6 B). Thus, gelatin particle stiffness and swelling potential could be modulated via methacrylate modification.

Microparticle mesh size analysis

Mesh size was calculated for all of the MPs based on their elastic moduli and swelling ratio values. The lower methacrylated MPs had a significantly larger molecular weight between crosslinks ($M_c$) (Table 3.1), and therefore greater mesh size than the GA MPs, with mesh sizes of 157.1 ± 7.1 nm and 124.2 ± 9.6 nm for 15% MA and 50% MA, respectively, compared to 54.5 ± 5.4 nm for GA MPs. In addition, 90% MPs had a mesh size of 57.9 ± 4.1 nm, which was comparable to those of GA MPs (Figure 3.6 C). A decrease in mesh size was observed with increased methacrylation, thereby enabling control of particle mesh size through modulation of the degree of methacrylate substitution.
Figure 3.6. Microparticle mechanical properties and mesh sizes. A) Elastic moduli were determined for MPs, which were conjugated to a glass coverslip and analyzed via AFM. The GA MPs and highest methacrylated MPs (90% MA) were significantly stiffer than the two lower methacrylated MPs (15% and 50%) by almost a magnitude. B) Swelling studies of MPs determined that all GMA MPs had a greater ability to swell in a hydrated environment than the GA MPs, with the 15% MA MPs swelling to a greater extent than all other MPs. C) Mesh sizes of the MPs were determined based on their elastic moduli and swelling ratios. The 15% and 50% MA MPs had larger mesh sizes than the GA MPs, as well as the highest methacrylated MPs (90% MA). E: elastic modulus, $Q_m$: equilibrium swelling ratio, $\xi$: mesh size, $n = 15$, *: denotes statistical significance with $p<0.05$.

Table 3.1: Table of microparticle mechanical properties

<table>
<thead>
<tr>
<th>MP formulation</th>
<th>E (kPa)</th>
<th>$q$</th>
<th>$Q_m$</th>
<th>Water Content (%)</th>
<th>$M_c$ (g/mmol)</th>
<th>$\xi$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% MA</td>
<td>39.5 ± 3.8*</td>
<td>27.8 ± 2.3*</td>
<td>37.7 ± 1.8*</td>
<td>96.4 ± 0.2*</td>
<td>289.1 ± 25.7*</td>
<td>157.1 ± 7.1*</td>
</tr>
<tr>
<td>50% MA</td>
<td>54.0 ± 9.5*</td>
<td>17.2 ± 0.7*</td>
<td>23.2 ± 0.5*</td>
<td>94.2 ± 0.1*</td>
<td>263.8 ± 43.8*</td>
<td>124.2 ± 9.6*</td>
</tr>
<tr>
<td>90% MA</td>
<td>222.0 ± 33.7</td>
<td>15.3 ± 1.4</td>
<td>20.6 ± 1.1</td>
<td>93.4 ± 0.3</td>
<td>61.3 ± 8.7</td>
<td>57.9 ± 4.1</td>
</tr>
<tr>
<td>GA</td>
<td>243.6 ± 40.7</td>
<td>12.9 ± 0.3</td>
<td>17.3 ± 0.3</td>
<td>92.2 ± 0.1</td>
<td>64.9 ± 15.5</td>
<td>54.5 ± 5.4</td>
</tr>
</tbody>
</table>

E: elastic modulus, $q$: swelling ratio, $Q_m$: equilibrium swelling ratio, $M_c$: molecular weight between cross-links, $\xi$: mesh size, $n = 15$, *: denotes statistical significance to all other groups, #: denotes statistical significance to 90% MA MPs and GA MPs, with $p<0.05$. 
**Gelatin microparticle growth factor loading**

The capacity of the different gelatin MPs to bind BMP4 (pI = 9.0) and bFGF (pI = 9.6) was examined via an overnight loading assay. Even at the highest loading concentration of 300 ng/mg, GA MPs bound relatively low quantities of BMP4 (8.1 ng), whereas the lower (15% and 50%) GMA MPs, had peak loading efficiencies at 99.6% and 63.1%, with average efficiencies across all loading concentrations of 69.2% and 43.9%, respectively (Figure 3.7 A,B). At the lower loading concentrations, 50 and 100 ng/mg, the GA MPs bound significantly less BMP4 than all of the GMA formulations. Similarly, at the two highest loading concentrations, GA bound less BMP4 than the two lower methacrylated MPs, 15% (p < 0.01) and 50% MA (p < 0.038). Decreasing the degree of methacrylation resulted in a significant increase in BMP4 binding capacity, with the 15% MPs binding more of the growth factor than the 90% MPs at the highest loading concentrations (150-300 ng/mL). The GA MPs also bound significantly less bFGF than all GMA MPs at the lowest (10 and 50 ng/mg) and highest (200 and 300 ng/mg) loading concentrations (Figure 3.7 C,D). Similar to BMP4 results, reduced methacrylation levels enabled an increase in bFGF binding capacity, with greater binding of the two lowest methacrylated MPs compared to the 90% MPs at all of the loading concentrations.
Figure 3.7. Microparticle growth factor loading capacity. Gelatin MPs were incubated with six concentrations of A) BMP4 and B) bFGF to determine the growth factor binding capacity. A) GA MPs bound less BMP4 than all GMA formulations at the lower loading concentrations (50 and 100 ng/mg) and bound less than the two lowest GMA formulations at the highest loading concentrations (200 and 300 ng/mg). B) While GA MPs bound low quantities of BMP4, even at the highest loading concentration, the lowest GMA MPs had a peak binding efficiency at 99.6%. C) The GA MPs also bound less BMP4 than all GMA formulations at the lowest and highest loading concentrations (50,100 ng/mg and 200,300 ng/mg). D) bFGF loading efficiency peaked at 100 ng/mL for all MP formulations. n≥3, symbols denote statistical significance with p<0.05. *: GA vs. all GMA MPs, #: GA vs. 15% MA and 50% MA, $: GA vs. 15% MA, &: 15% MA vs 90% MA, %: 50% MA vs 90% MA.
Gelatin microparticle growth factor release

Each MP formulation was loaded with 10 ng of BMP4 and 50 ng of bFGF per mg of MP. No significant differences were observed in passive release of BMP4 between any of the MP formulations (Figure 3.8 A). However, low collagenase treatment (100 ng/mL) enabled a greater release of BMP4 from the GA MPs compared to the 50% MPs at 50 hours (Figure 3.8 B), whereas high collagenase treatment (1 μg/mL) enabled greater BMP4 release at the earliest time point (3 hours) of the GA MPs compared to both the 50% and 90% MA MPs (Figure 3.8 C). Furthermore, GA MPs did not release any more BMP4 in response to protease treatment compared to passive release, except for the initial release at 3 hours (Figure 3.9 A). However, the release of BMP4 from the higher methacrylated MPs, 50% MA and 90% MA, was more sustained, as it increased with collagenase treatment at all time points assayed, even up to 170 hours (Figure 3.9). Treatment with 1 μg/mL collagenase facilitated the release of 49.5% and 54.7% BMP4 by the 50% and 90% MA MPs, respectively, compared to 79.2% released by the GA MPs by 3 hours. In this case, while the GA MPs released nearly all (91.1%) of bound BMP4 by 11 hours, the 50% and 90% MA MPs had only released 75.4% and 72.1% BMP4, respectively, by 170 hours (Figure 3.10). Thus, the higher methacrylated MPs afford a more sustained release of BMP4 compared to the GA MPs, and varying the degree of methacrylation enables modulation of BMP4 release kinetics.

Passive release of bFGF from 50% MPs was less than release from all other MP formulations after 25 hours and remained less than GA MPs throughout the assayed time period (Figure 3.8). Compared to passive release, collagenase treatment resulted in an increase in bFGF released by the 50% MA MPs until 25 hours and the 90% MA MPs
until 170 hours, further illustrating the tunability of the methacrylated MPs (Figure 3.10). While low collagenase treatment enabled greater release of bFGF from the 15% MPs compared to the 50% MA formulation after 11 hours, and the 90% MA MPs released more bFGF from 11 hours to 170 hours (Figure 3.8 E), no differences were found in growth factor release between MP formulations under high collagenase treatment (1 μg/mL) (Figure 3.8 F). Importantly, there were no differences between bFGF passive release and release after proteolytic treatment from GA MPs. Moreover, the 50% MP formulation offered a more sustained release of bFGF compared to all other MPs, releasing only 72.4% by 170 hours with higher collagenase treatment (1 μg/mL) compared to 94.0%, 97.6%, and 92.3% from the 15% MA, 90% MA, and GA MPs respectively (Figure 3.8 F). Therefore, higher levels of MP methacrylation enables greater sustained release of growth factors compared to the GA formulation and demonstrates that modification of gelatin cross-linking density enables control over growth factor release upon protease treatment.
Figure 3.8. BMP4 and bFGF release from microparticles.

The MPs were incubated with 10 ng/mg MPs of BMP4 (A-C) and 50 ng/mg MPs bFGF (D-F), and release kinetics was obtained over the course of 170 hours. A) Passive release of BMP4 was similar between all MP formulations. B) Greater release of BMP4 in 100 ng/mL collagenase was observed in the GA MPs compared to the 50% MA MPs at 60 hours. C) Larger BMP4 burst release at 3 hours was observed in the GA formulation compared to the higher methacrylated MPs with 1 μg/mL collagenase treatment. D) Less bFGF was released passively in the 50% MPs compared to the GA MPs after 25 hours. E) Greater bFGF was released from the GA MPs compared to the 50% MA MPs by 170 hours following treatment with 100 ng/mL collagenase. F) High collagenase treatment resulted in no observable differences in bFGF release between MP formulations. n≥3, symbols denote statistical significance with p<0.05. *: GA vs. 50% MA, %: GA vs 15% MA, $: GA vs 90% MA, #: 50% MA vs 15% MA, &: 50% MA vs 90% MA. Diamond (red): 15% MA, square (blue): 50% MA, triangle (black): 90% MA, circle (green): GA.
Figure 3.9. Time-point specific collagenase-mediated BMP4 release from MPs. Besides the initial release at 3 hours, GA MPs under protease treatment did not release any more BMP4 compared to passive release. On the other hand, the release of BMP4 from the higher methacrylated MPs, 50% MA and 90% MA, increased with collagenase treatment at all time points assayed, and this effect was sustained up to 170 hours. *: denotes statistical significance, n≥3, p<0.05. Light grey bar: no collagenase, dark grey bar: low collagenase treatment (100 ng/mL), white bar: high collagenase treatment (1 μg/mL).
Figure 3.10. Time-point specific collagenase-mediated bFGF release from MPs. No differences were observed between bFGF passive release and proteolytic-mediated release of GA MPs. However, an increase in bFGF released by the 50% MA MPs and 90% MA MPs were increased with collagenase treatment up to 25 hours and 170 hours, respectively. *: denotes statistical significance, n≥3, p<0.05. Light grey bar: no collagenase, dark grey bar: low collagenase treatment (100 ng/mL), white bar: high collagenase treatment (1 μg/mL).
Figure 3.11. Chart of GMA MP properties. MP bulk properties and growth factor interactions can be modulated by tuning MP methacrylation.
Discussion

GMA MPs can be synthesized from a wide range (15-90%) of methacrylate substituted gelatin species with methacrylation enabling highly reproducible fabrication of relatively monodisperse MPs while allowing control over a range of critical parameters. Compared to conventional glutaraldehyde cross-linking, varying the degree of methacrylation directly influences the amount of gelatin/particle, thereby allowing greater control over degradation kinetics. Additionally, the degree of methacrylation positively correlates to MP elastic moduli and inversely correlates with swelling potential and mesh size, with GA MPs having comparable mechanical properties to the methacrylated MPs with similar cross-linking densities (>90%). Furthermore, decreasing the degree of methacrylation increases growth factor binding and promotes more complete growth factor release, with GA MPs behaving similarly to the lowest methacrylated MPs in terms of bFGF and BMP4 release.

GMA MPs offer greater control over cross-linking density compared to glutaraldehyde MPs. Methacrylation of gelatin can be controlled a priori by introducing the vinyl moiety responsible for the cross-linking, thus offering a predetermined number of reactive sites for cross-linking. Conversely, GA MPs have all amine sites available for cross-linking, and soluble addition of glutaraldehyde for modification of cross-linking density results in varying intermediate reaction compounds, making the overall reaction difficult to control [133]. Additionally, although bulk GMA hydrogels at lower substitution percentages (20%) lack sufficient physical integrity to be handled [148], stable GMA MPs can be formed with cross-linking densities as low as 15% MA substitution. In contrast, despite manipulation of GA molecule concentration as well as
reaction time and temperature, the lowest cross-linked GA MPs still have greater than 60% cross-linking [130,132,133,136,137]. Also of note, no significant differences in size distribution between GMA MPs were observed under similar fabrication conditions. A previous study has reported that increasing the degree of methacrylation resulted in a decrease in gelatin viscosity [141]. Therefore, despite differences in substitution, methacrylated MPs can be synthesized with similar size distributions by raising the emulsification speed for lower substituted, or more viscous GMA. In contrast to glutaraldehyde cross-linking methods, which typically result in more polydisperse MPs, GMA MPs can be reliably fabricated with reduced polydispersity. Highly uniform GMA microdroplets have recently been synthesized using a T-junction microfluidic device, although the minimum size of reported droplets was greater than 30 μm, despite tuning GMA concentration and flow rate [149]. Since bulk properties and growth factor interactions of the microdroplets have not yet been characterized, this study represents the first report on these critical properties of GMA microspheres [149].

GA MPs degraded more than the 50% and 90% methacrylated MPs over the first 48 hours of collagenase treatment. Previous studies have reported GA molecule diffusion limitations through chitosan gel beads during GA cross-linking, resulting in a more heavily cross-linked MP exterior [150]. Thus, non-homogeneous GA cross-linking of gelatin microspheres could likely occur, since gelatin sphere formation via a water-in-oil emulsion is required before the addition of any cross-linking reagents. Although not examined in these particular studies, despite lower cytotoxicity and a greater range of cross-linking attainable [130], genipin cross-linked MPs may also be non-homogenously cross-linked, especially since genipin has a higher molecular weight than GA. Once
proteases degrade the exterior surface of GA MPs, the remainder of the MPs can easily degrade, potentially explaining the rapid degradation rate of the GA MPs after the first 6 hours of collagenase incubation. Conversely, increasing the methacrylation level above 15% results in a more sustained degradation of GMA MPs over 96 hours and prevents the rapid degradation observed with the GA MPs. Moreover, since thermal initiators are mixed in with the gelatin during the water-in-oil emulsion of GMA MP synthesis, free radicals can be distributed more uniformly within the bulk of the material, possibly promoting more homogenous cross-linking throughout the MPs [151,152]. Although dehydrothermal cross-linking may also be more uniform than GA cross-linking, dehydrothermally modified MPs also degrade rapidly in all reported studies, persisting no more than 24 hours in a variety of environments, possibly due to the presence of only physical cross-links that may more readily dissociate compared with chemical cross-linking [153,154]. Despite differences in degradation assay buffer conditions, GMA MPs with above 50% methacrylation can persist for up to 96 hours in the presence of collagenase. Thus, increasing the methacrylation of GMA MPs can enable slower and more sustained MP degradation than compared to other gelatin MP cross-linking methods.

The GMA MP results reported herein corroborate with previous studies of GMA bulk hydrogels, which demonstrate that in enhanced methacrylation increases bulk stiffness but decreases the molecular weight between crosslinks, therefore decreasing hydrogel pore/mesh size [141–143,146,155]. Less cross-linked MPs bound greater concentrations of oppositely charged growth factor from solution, suggesting that reducing the methacrylation of MPs increases the distance between cross-links,
facilitating greater swelling and greater mesh size, so that growth factors can more freely enter the hydrogel. The increase in mesh size could explain why lower methacrylated GMA MPs bind higher amounts of growth factor than the GA cross-linked MPs, despite the significantly lower gelatin content per volume of GMA MPs. As previously mentioned, since GA MPs are synthesized by adding GA to pre-formed gelatin spheres, the GA MPs may have a dense shell of cross-linking at the surface [150]. The presence of a highly cross-linked outer shell region may restrict growth factor binding primarily to the MP surface and sterically hinder molecules from entering the interior of the microsphere during growth factor loading [156,157]. More homogenous gelatin network formation, as seen during GMA synthesis where the initiator is added to the gelatin solution before spheres are formed, would facilitate greater access of the growth factors to the charged sites within the particle interior, and thus enhance biomolecule loading. Modulation of physical factors, such as mesh sizes and swelling potential, via methacrylation appears to play a larger role in growth factor binding than the reduction in charge inherent to methacrylation. Increasing methacrylation reduces the net positive charge on the MP, and although the two growth factors assessed in the study are both positively charged at physiological pH, the increasingly methacrylated MPs did not have enhanced binding as would be expected with reduced electrostatic repulsion. Since enhanced binding was not observed with increased methacrylation, it is postulated that physical factors modulated by the degree of methacrylation may play a more significant role in growth factor binding than simply a change in the net charge.

Decreased growth factor release from GMA MPs was also observed with increasing gelatin methacrylation. In agreement with earlier reports, these studies indicate
that increasing methacrylation reduces hydrogel swelling [148], physically limiting the solvent and growth factor interaction that enables release [158]. Reduced swelling results in smaller hydrogel mesh sizes [155], within which the biomolecules encounter more steric hindrance contributing to the more sustained release from the higher methacrylated GMA MPs. Increasing the collagenase treatment concentration accelerated the release rate from the MPs, with the majority of release occurring 24 hours before the release in MPs without collagenase treatment. Furthermore, in the higher (90%) methacrylated MPs, increased growth factor release occurred only after exposure to higher collagenase concentrations, thus illustrating that delivery can be mediated by proteolytic cleavage. Modulating the degree of gelatin methacrylation also resulted in varying degradation and release profiles, both passive and active. Overall, the sustained release profiles of two positively charged growth factors from the negatively charged GMA MPs presented herein, suggest that modulation of GMA cross-linking density can provide a platform for controlled release of different growth factors. Thus, GMA MP technology can be engineered for in vivo and in vitro applications where smaller sized biomolecule delivery vehicles are desired, such as wound bed packing for release of anti-inflammatory agents [159,160] or where controlling timeframe of release is desirable, such as within stem cell aggregates to direct differentiation with morphogenic factors [31,39]. Hence, GMA MPs can be easily customized for the controlled delivery of charged biomolecules for a variety of tissue engineering and regenerative medicine applications.

GMA MPs were synthesized and characterized in this study, and they offer greater control over cross-linking density than traditional glutaraldehyde cross-linked gelatin MPs. Modification of GMA MP cross-linking density modulates gelatin content,
proteolytic degradation kinetics, mesh sizes, and the ability to bind and release growth factors. GMA MPs therefore provide a robust platform for controlled release of electrostatically coupled growth factors, particularly within a proteolytic environment, for emerging tissue engineering technologies.
CHAPTER 4
GELATIN METHACRYLATE MICROPARTICLES ENHANCE MATRIX METALLOPROTEINASE-DEPENDENT MESENCHYMAL MORPHOGENESIS OF PLURIPOTENT STEM CELL AGGREGATES

Introduction

Pluripotent stem cells hold tremendous promise for regenerative medicine and tissue engineering due to their potential to become any cell type. Study of pluripotent stem cell aggregates provides valuable insight into the multifaceted milieu required for differentiation, since the aggregates can form the complex cell-cell interactions and promote extracellular matrix (ECM) production with parallels to embryogenesis and in vivo tissue development [7]. Aggregate differentiation is typically directed through soluble factor addition to the surrounding culture medium, however tight junctions and dense cell packing within the aggregates may serve as barriers to free diffusion, limiting the access of the molecules to the interior of the aggregate [30,31]. Incorporation of biomolecule-loaded microparticles (MPs) within aggregates promotes more homogenous presentation of delivered biomolecules compared with soluble treatment, and incorporation of biomaterial MPs within stem cell aggregates can impact differentiation propensity based on cell-material interactions, which can regulate cell fate even in the absence of delivered molecules [31,35]. In particular, extracellular matrix (ECM) based materials are increasingly utilized to direct stem cell differentiation, based on their innate ability of ECM to dynamically provide cues for differentiation and maintenance of
differentiated tissues [16,65,161–163] through remodeling by matrix metalloproteinases (MMPs), which are a class of proteases that can cleave all aspects of the ECM [58].

Direct ECM contact with cells has the potential to modulate MMP activity through substrate-protease specific feedback response, as demonstrated in studies where fibroblast cell culture on 2-D surfaces coated with molecules such as decorin, vitronectin, and fibronectin, can trigger enhanced MMP activity [1–3]. Additionally, subtle changes in ECM composition can strongly impact MMP activity, as culture on the RGD-region on fibronectin enhances fibroblast MMP-9 activity, which was highly reduced with culture on additional fibronectin fragments, suggesting that specific regions of molecules have permissive or inhibitory effects on MMP activity [2]. Furthermore, mesenchymal stem cells (MSCs) cultured in an RGD-alginate gel with MMP-cleavable peptides had greater activity of MMP-2 than MSCs cultured only in an RGD-alginate gel [4]. Additionally, enhancing MMP-cleavable peptide cross-linker in PEG hydrogels enhances MMP-2 activity of cultured MSCs in a proportional manner [5]. Moreover, MSCs cultured within type I collagen gels had enhanced activity of 5 different collagenase MMPs compared to cells cultured on a plastic dish, and specifically MT1-MMP plays a significant role in MSC osteoblastic differentiation [164]. Thus, cell interactions with ECM materials can enhance MMP activity, which may modulate differentiation in addition to facilitating tissue remodeling.

MMPs are able to modulate many differentiation processes through ECM cleavage, which releases sequestered bioactive growth factors and exposes isoforms of ECM-based components that activate receptor-mediated downstream signaling cascades to promote transcriptional activity [113]. In particular, remodeling of the ECM by matrix
metalloproteinases (MMPs) is strongly involved in epithelial-to-mesenchymal transition (EMT) that occurs in the mammalian embryo after primitive streak formation, which gives rise to all 3 germ layers during gastrulation [52,165]. Specifically, MMPs facilitate epiblast cell ingression through the primitive streak to form mesoderm or definitive endoderm through MMP-mediated breakdown of the basement membrane [97,101], which not only enable epithelial cell delamination and migration but also release potent biomolecules and “cryptic sites” in ECM that can induce transcriptional repression of epithelial markers, such as E-cadherin, and activation of mesodermal markers, including N-cadherin, fibronectin, and MMP 2 and 9 [55,108,115,119–121,166]. MMPs are also highly involved in branching morphogenesis, as well as other key developmental processes such as generation of the neural crest and somitogenesis [101].

Pluripotent stem cell aggregates recapitulate much of the cellular morphogenesis occurring during embryological development, and although the roles of MMPs have been extensively characterized within mammalian and avian embryogenesis [86,101,102,119], little has been reported on how MMP-mediated ECM remodeling can regulate differentiation within pluripotent stem cells [106]. Additionally, although cell-contact with substrate-specific materials can trigger enhanced MMP activity, this increase in activity has also been demonstrated to be dependent on 2-D vs. 3-D presentation [6], and studies have yet to investigate whether MMP activity can be similarly enhanced by material presentation within a 3-D spheroid environment or even within pluripotent stem cells. It is hypothesized that incorporation of enzymatically degradable MPs composed of gelatin within pluripotent stem cell aggregates will increase MMP activity and may facilitate EMT-like processes, ultimately enriching mesendodermal differentiation. The
critical roles of MMPs and extracellular remodeling in early embryogenesis strongly
suggest that developing tools to engineer remodeling of the ECM microenvironment will
offer enhanced control over directed differentiation strategies.
Methods

Mouse ESC culture

Undifferentiated mouse embryonic stem cells (mESCs) (D3) (passages 23-33) were cultured on 0.1% gelatin coated tissue culture dishes in ESC media composed of DMEM (Mediatech Inc., Herndon, VA) supplemented with 15% FBS (Hyclone, Logan, UT), 1x non-essential amino acids (Mediatech), 2mM L-glutamine (Mediatech), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin (Mediatech), 0.1 mM 2-mercaptoethanol (Fisher Scientific, Fair-lawn NJ), and $10^3$ U/ml leukemia inhibitory factor (LIF) (Millipore, Billerica, MA). The cells were routinely passaged with 0.05% trypsin every 2-3 days before reaching 70% confluency.

Spheroid formation and culture

mESCs were treated with 0.05% trypsin to obtain a single cell suspension, and forced to aggregate within AggreWell™ 400 inserts (Stem Cell Technologies, Vancouver, CA). Briefly, 1.2 million cells in 0.5 mL media were inoculated into the microwell inserts and centrifuged at 200 g for 5 min, dividing the cells uniformly between 1200 microwells. For MP incorporation, 1.2 million cells were homogeneously mixed with 0.4, 1.2, or 3.6 million MPs, followed by addition to the microwell inserts. After 18 hours of culture, a wide-bore pipette was used to gently remove cell aggregates from the microwells and transfer them to a rotary orbital shaker (45 rpm) for suspension culture to maintain the population homogeneity. Spheroids were collected by sedimentation every two days, and 90% of the media was exchanged with fresh mESC media (without LIF). In the case of Ro32-3555 MMP inhibitor (Tocris Biosciences,
Bristol, UK) supplementation, the inhibitor was initially added at 50 μM on day 5 and additionally every other day during media exchange. Supplementation of pSMAD inhibitor, LDN-193189 (Stemgent, Cambridge, MA) was initiated on day 3 and continued every other day during media exchange.

**MP incorporation analysis**

GMA MPs were fabricated with 5 μm diameters via a water-in-oil emulsion as previously described in Chapter 3 and fluorescently labeled by incubation with Alexa-Fluor 594 succidimidyl ester (Invitrogen, Carlsbad, CA) in a 0.1 M sodium bicarbonate buffer followed by 4 deionized water washes. Heparin methacrylate MPs were also synthesized as previously described [167], and poly (methyl) methacrylate (Polysciences, Warrington, PA), and polystyrene MPs (Spherotech, Lake Forest, IL) with similar sizes as the GMA MPs (5-10 μm) were purchased. Polystyrene MPs were coated with GMA through incubation with 1% sterile filtered GMA solution for 2 hours at room temperature, followed by 3 washes in PBS with 1% BSA.

Quantification of fluorescently labeled GMA MPs was determined after incorporation within spheroids at ratios of 1:3, 1:1, or 3:1 MPs: cells. Spheroids were collected from microwells after 18 hours, and the spheroids were counted and lysed in a lysis solution containing 5% SDS and 0.1 NaOH for 2 hours at room temperature. The spheroid lysate was analyzed at emission 570 nm and excitation 620 nm on a Biotek fluorescent plate reader. The number of microparticles per spheroid was determined using a standard curve obtained with a known number of microparticles suspended in the lysis solution. Incorporation of non-labeled GMA, heparin methacrylate, poly (methyl)
methacrylate (Polysciences, Warrington, PA), and polystyrene MPs (Spherotech, Lake Forest, IL) were similarly measured by lysing a known amount of spheroids in a 5% SDS solution and manually counting the incorporated MPs on a hemocytometer.

**MMP activity analysis**

Quantitative analysis of overall protease activity was performed with the Sensolyte® 520 Generic MMP Assay Kit (AnaSpec, San Jose, CA) as previously described [168,169]. The kits employ a quenched FAM/QXL FRET-conjugated universal MMP sensitive peptide sequence. Upon MMP cleavage, detectable fluorescence directly correlates to MMP activity. Spheroids were lysed via homogenization on ice in 0.1% Triton-X in the provided assay buffer, followed by centrifugation and collection of the supernatant lysate. Lysates were activated with 1 mM amino-phenyl mercuric acetate (APMA) for 2 hours and assayed alongside their unactivated counterparts to quantify pro-form and active-forms. Fifty μL of spheroid lysate was incubated with 50 μL of FRET-conjugated substrate in a black 96 well plate for 1 hour at room temperature, stop solution was added, and then the plate was read on a Synergy H4 plate reader (Biotek, Winooski, VT) at excitation/emission: 490nm/520 nm. A standard curve was created using 5-FAM-Pro-Leu-OH to convert fluorescence values into amount of substrate cleaved. Values were normalized to total protein content determined via a BCA assay and plotted compared to the no microparticle control group.

Analysis of MMP-2 protease activity was performed with a zymography assay. mESCs from day 0 monolayer culture and spheroids with and without MPs from days 1, 4, 7, 10, and 14 were homogenized on ice for 5 seconds with lysis buffer (20 mM Tris-
HCl pH 7.5, 5 mM EGTA, 150 mM NaCl, 20 mM Glycerol-phosphate, 10 mM NaF, 1 mM Sodium Orthovanadate, 1% Triton X-100, 0.1% Tween 20). Protein concentration in the supernatant was quantified, and 15 μg of protein was mixed with 5X non-reducing loading buffer (0.05% bromophenol blue, 10% SDS, 1.5 M Tris, 50% glycerol) prior to loading onto a 10% SDS-polyacrylamide gel containing 5 mg/mL gelatin. The gel was run at 4°C at 115 V, and then the gels were removed and the enzymes were renatured in 2.5% Triton X and incubated overnight in an assay buffer containing 50 mM Tris-HCl, 10 mM CaCl₂, 50 mM NaCl, and 0.05% Triton X-100. Gels were then rinsed with deionized water and stained with Coomassie Blue for 1 hour at room temperature. The gels were imaged using Imagequant 4010 (GE Healthcare, Amersham, UK). Band intensities were analyzed by densitometry on the ImageJ software (NIH, Bethesda, MD).

**Gene expression analysis**

Total RNA was extracted from the ESC monolayer and spheroid samples at days 7, 10, and 14 of differentiation with the RNeasy Mini kit (Qiagen Inc, Valencia, CA). Complimentary cDNA was obtained via the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and analyzed using SYBR green technology and real time PCR (MyIQ cycler, Bio-Rad). Sequences and annealing temperatures for *Mixl1, Brachyury-T, E-Cadherin, N-Cadherin, Snai1, Slug, Mesp1, MMP-2, Flk1, Pax6, FoxA2, Nanog,* and *18S* are provided in Table 4.1. Gene expression levels were normalized to the housekeeping gene 18S and calculated with respect to expression levels of mESCs using the Pfaffl method [170].
### Table 4.1. Primer sequences and conditions for qPCR

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Histology and immunostaining

Spheroids were fixed in 10% formalin, embedded in Histogel (Thermo Scientific, Rockford, IL), and processed and embedded in paraffin. Paraffin processed sample blocks were sectioned to 5 μm thickness (Microm HM 355S) and adhered to Superfrost Plus slides (VWR, West Chester, PA). The sections were deparaffinized, stained with hematoxylin and eosin (H&E), and imaged with a Nikon 80i upright microscope and an SPOT Flex camera (15.2 64 MP Shifting Pixel, Diagnostic Instruments, Sterling Heights, MI).

For whole-mount immunofluorescent staining, spheroids were permeabilized for 30 minutes in 1.5% Triton X-100 in blocking buffer consisting of 2% serum of the same animal origin as the Alexa-Fluor-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, goat or donkey, 1:200) against N-cadherin (Dako, Glostrup, Denmark, mouse monoclonal, 1:30), E-cadherin (Sigma, St. Louis, MO, rat polyclonal, 1:200), fibronectin (Millipore, Temecula, CA, rabbit polyclonal, 1:80), and α-smooth muscle actin (Dako, mouse monoclonal, 1:100). The spheroids were incubated with primary antibody in blocking buffer overnight at 4°C, rinsed with 0.1% Tween in PBS, and incubated with secondary antibody in blocking buffer for 4 hours at 4°C. Spheroids were counterstained with Hoechst (1:100), washed 3 times in 0.1% Tween with PBS and imaged using a Zeiss LSM 700 confocal microscope (Carl Zeiss Inc., Heidelberg, Germany).

Principal Components Analysis

Images were digitized using Cell Profiler and processed by Douglas White, from the Kemp lab at Georgia Tech. Briefly hematoxylin and eosin histological images were
loaded into cell profiler [171] and the cytoplasmic versus nuclear staining was separated using the spectral separation algorithm. The resulting cells were located via nuclei, and extended to match cytoplasmic boundaries using CellProfiler’s expand function. Various metrics related to shape were measured using the measure_object_size_shape module. The number of neighbors was then measured using get_nearest_neighbors module. The data was exported and processed using custom python files to create networks containing cells with information on their shape.

The resulting networks were run through a machine learning algorithm to extract features which could provide classification information about the cell type. The resulting classifiers were trained using both information on cell physical shape and morphology as well as metrics derived from the network analysis such as number of neighbors, connection length, etc. Various classifiers from the sklearn package were tested, although ultimately a SVM based approach was utilized. The SVM algorithm implemented from sklearn is an interface to the popular libsvm written in C language.

Principal component analysis (PCA) was performed using the sklearn python package. This algorithm performs PCA based on the algorithm outlined in Tipping et al. [172], causing a probabilistic approach coupled with singular value decomposition. In this specific case, PCA was not used as predictive model but was used in its capacity as a dimensional reduction algorithm. Data management and loading heatmaps were generated using custom python code. The scores plots were generated using the scatter function from Matplotlib in python.
**Contractile assessment**

At 7 days of suspension culture, spheroids from each condition were plated individually onto 48-well tissue culture treated dishes coated with 0.1% gelatin. Individual spheroids were selected from a petri dish by pipetting 100 μL of media containing the spheroid and placing it within a well. An additional 400 μL was added per well, with a total of 500 μL per well with at least one EB. The presence of contractile spheroids was determined on days 8, 10, 12 and 14. Merged EBs were not included in contractile analysis, with a minimum of 30 EBs per experimental sample (n), with n ≥ 3. The percentage of spheroids with greater than 3 contractile foci was also calculated. Furthermore, a score was assigned to spheroids based on their beating levels, with ✓- as a few small beating regions, ✓ as multiple smaller beating regions that beat independently, and ✓+ as a large beating region that beats connectively.

**Flow cytometry**

Spheroids plated for the contractile assessment assay were washed 3 times with PBS and dissociated to a single cell suspension with trituration in 0.05% trypsin-EDTA for 5 min. The single-cell suspension was rinsed in PBS, fixed in formalin for 10 minutes at room temperature, and thoroughly rinsed again in PBS by pelleting at 1000 rpm for 5 min between rinses. The cell pellet was then washed in working buffer (2% goat serum and 0.01% Tween-20) and permeabilized with 0.05% Triton X-100 in working buffer for 20 minutes at room temperature. The cell pellet was then resuspended in 300 μL of monoclonal mouse anti-α-sarcomeric actin antibody (5c5, Sigma, St. Louis, MO,) or isotype control IgM (1:500) in working buffer for 2 hours at room temperature. After 3
rinses in working buffer, the cell pellet was resuspended in 300 μL of goat-anti-mouse Alexa-Fluor conjugated secondary antibody in working buffer and incubated at room temperature for 1 hour, followed by successive rinses with working buffer. Flow cytometry was performed with an Accuri C6 cytometer (Accuri Cytometers, Ann Arbor, MI), with a minimum of 10,000 events per sample collected within the FSC/SSC gate for live cell populations (n = 3 independent experimental samples per condition). The 20% MA MP and 90% MA MP IgM control cells alone were used to establish appropriate gates and compensation. Within the FSC/SSC gate, polygonal gating was used to limit less than 4% of the IgM treated undifferentiated population via FlowJo software (Tree Star, Inc., Ashland, OR). The whole cell population was gated to include less than 1% 20% MA MPs and 4% 90% MA MPs.

**Statistical analysis**

All values are reported as mean ± standard error, with at least triplicate independent biological samples. Before statistical analysis, all non-normal data was processed to a Gaussian distribution with a Box-Cox power transform on MATLAB software (Natick, MA). Statistical significance was determined via one-way ANOVA with Tukey’s post hoc analysis with 95% confidence intervals after performing Levene’s equality of variances test on SYSTAT 12 software (Chicago, IL). P-values < 0.05 were determined to be statistically significant.
Results

Matrix metalloproteinase activity in response to MP incorporation

Both the 20% MA MP and 90% MA MP formulations (Figure 4.1A) incorporated within spheroids similarly, with 1:3, 1:1, and 3:1 MPs: cells incorporating in 1000 cell spheroids at 200, 600 and 1200 MPs per spheroid, corresponding to 1:5, 3:5, and 1.2:1 MPs: cells, respectively (Figure 4.1B, Figure 4.2). However, upon commencement of rotary culture, spheroids with greater than 1:3 MP: cells merged into a few distinct clusters, thus the 1:3 MP: cell incorporation ratio was utilized for the remainder of the study to avoid spheroid agglomeration. Both MP formulations incorporated at a 1:3 MP: cell ratio persisted within the spheroid up to 14 days of rotary culture, as illustrated by the continued presence of Alexa-Fluor-labeled MPs in the aggregates (Figure 4.1C).

Overall MMP levels in spheroids were assessed with FRET-conjugated MMP cleavable peptides (Figure 4.3A). Total generic MMP levels peaked at D10, while active levels appeared to remain constant through the course of differentiation. On day 4, the 20% MA MPs induced greater total and active MMP levels compared to no MP spheroids. On day 7, both MP formulations appeared to have increased active MMP levels compared to no MP spheroids, with greatest total MMP levels in spheroids with 90% MA MPs.

The activity of MMP-2 was further assessed via zymography and densitometry. The pro form of MMP-2 was detected throughout the entire time course analyzed, and the active form was observed by day 7 (Figure 4.3B,C). Incorporation of the lower MA MPs (20%) led to an increase in pro form over both no MP and higher MA MP (90%) spheroids after 24 hours. By day 7, spheroids with both MP formulations had greater pro
and active form compared to no MP spheroids, with the 90% MA MPs inducing more than double the active form compared to no MP spheroids. Furthermore, all MMP-2 pro forms and active forms peaked by day 10 of differentiation, with both MA MP groups having increased MMP-2 levels compared to no MP aggregates. By day 10, spheroids with lower MA MPs had the greatest pro form, with a 3.1 fold increase over no MP spheroids (Figure 4.3C). Moreover, spheroids with higher MA MPs had the highest active form, with a 1.5 fold increase over no MP spheroids. MMP-2 levels strongly decreased by day 14 and were similar in all groups at this late stage of differentiation.

Moreover, although MMP-2 levels were enhanced in both MP spheroids compared to spheroids without MPs on days 7 and 10, spheroids with 20% MA MPs had continuously enhanced pro form over 90% MA MPs, notably in the first 4 days of differentiation and at a later stage (day 10). However, MMP-2 active form was enhanced 3 fold in spheroids with 90% MA MPs compared to 20% MA MPs on day 7 and remained highest in 90% MA MP spheroids on day 10.

To determine whether introduction of GMA to spheroids on monolayer culture is sufficient to elicit a similar MMP-2 response, day 4 spheroids were plated on either tissue culture polystyrene (TCPS) or TCPS plates coated with cross-linked 20% or 90% GMA. Interestingly, all spheroids expressed comparable pro and active forms of MMP-2 despite culture on 2-D TCPS or GMA coated plates (Figure 4.3D). In order to determine whether the MMP-2 response is unique to GMA MP incorporation, MPs of similar size but different chemical composition to the GMA MPs, were incorporated within spheroids at comparable amounts when seeded at 1 MP: 3 cells (Figure 4.4). Unlike the increase of pro form and active form induced by incorporation of the 20% GMA MPs and 90%
GMA MPs, respectively, none of the other MPs composed of polystyrene (PS), PS coated with both 20% and 90% GMA, poly (methyl) methacrylate (PMMA), or heparin methacrylate (Hep) were able to enhance MMP-2 levels on day 1 or 7 of differentiation (Figure 4.3E).
Figure 4.1. GMA MP incorporation and persistence in ESC aggregates. A) 20% MA and 90% MA MPs were incorporated into ESC aggregates via seeding alongside ESCs in microwells to form aggregates and subsequently transferred to rotary culture. B) Various MP to cell ratios were used at seeding and resulted in similar amounts of MPs being incorporated into aggregates in a proportional manner. C) Alexa Fluor 594 labeled-MPs (red) were seeded with ESCs at 1:3 (MP: cell) ratio in Aggrewell™ for 18 hours to form aggregates, followed by suspension culture. The MPs persisted within the aggregates for up to 14 days.
Figure 4.2. MP incorporation within spheroids. MPs incorporated within ESC aggregates increased proportionally when seeded within microwells at ratios of 1:3, 1:1 and 3:1 MP: cell. Aggregates were formed after 18 hours of incubation within microwells. After formation, spheroids were transferred to rotary culture and imaged via confocal microscopy.
Figure 4.3. MP incorporation enhances matrix metalloproteinase levels in ESC aggregates. A) Generic MMP levels within lysates of spheroids with and without MPs was analyzed using a FRET-conjugated cleavable peptide-based assay. MP incorporation led to significant increase in total MMP and active form MMP at both D4 and D7 compared to no MP aggregates. B) The expression of both pro (arrow) and active (arrowhead) forms of MMP-2 in spheroid lysates were analyzed by gel zymography. C) Densitometry analysis depict that the expression of both forms of MMP-2 peaked at D10 during 14 days of culture, and the expression in MP-incorporated aggregates was significantly higher than in no MP aggregates. D) No difference in MMP-2 expression was observed in lysates of D7 spheroids plated on either TCPS or GMA coated plates. E) Neither MPs coated with GMA nor MPs composed of PMMA or heparin methacrylate were able to induce enhanced MMP-2 levels as observed with GMA MP incorporation. * denotes statistical significance, with n ≥ 3 and p ≤ 0.05.
Figure 4.4. MPs of various materials incorporate similarly within spheroids. A) Similarly sized MPs (~5-10 μm) composed of polystyrene (PS), PS coated with GMA, poly (methyl) methacrylate (PMMA), and heparin (Hep) were incorporated within spheroids at comparable amounts at a 1 MP: 3 cell ratio.
EMT-related protein expression patterns and gene expression in response to MP incorporation

The expression patterns of EMT related proteins were evaluated via immunostaining. No MP spheroids had strong membrane expression of E-cadherin while MP treated spheroids had punctate expression (Figure 4.5A). Additionally, N-cadherin was observed more strongly within the interior of the MP-spheroids compared to within no MP samples on day 10 (Figure 4.5B). Furthermore, fibronectin expression patterns were heavily observed throughout the MP spheroids, with 90% MA MP spheroids having sizable fibrillar regions (Figure 4.5C). Moreover in MP spheroids, MMP-2 expression was strongly localized to regions with circular morphology, strongly indicative of MPs (Figure 4.5D). Fibronectin also co-localized heavily to MMP-2 expression found throughout the spheroids except at the MP-like locations.

Incorporation of MPs led to a decrease in expression of primitive streak genes, Mix11 and Brachyury-T, on day 4 compared to no MP spheroids; however, this MP-based difference was no longer observed by day 7 (Figure 4.6A). Although incorporation of MPs did not affect E-cadherin, the levels of the E-cadherin repressor gene, Slug, was increased by MP incorporation, most highly in the 20% MA MP spheroids, on day 7. Additionally, incorporation of the 90% MA MPs enhanced expression of other EMT related genes, N-cadherin and Gata4, while both MPs enhanced levels of MMP-2 and Mesp1 by day 7 (Figure 4.6B). Pluripotency was equally decreased across all spheroid groups over the first 7 days of differentiation, as assessed by reduction of Nanog gene expression. Also by day 7, mesendodermal differentiation was enhanced with GMA MP incorporation, based on increased gene expression of mesoderm marker, Flk1, and
endoderm marker, FoxA2. Flk1 levels were further increased by day 10 in MP spheroids, whereas no differences between groups were observed towards ectodermal differentiation (Pax6) (Figure 4.6C).
Figure 4.5. MP incorporation modulates expression patterns of cadherins, fibronectin, and MMP-2 in day 10 ESC aggregates. A) More labeling of E-cadherin in a punctate distribution was observed in MP-incorporated aggregates than no MP aggregates. B) N-cadherin was observed more strongly around the cellular membranes in the MP treated spheroids compared to those in no MP spheroids. C) Fibronectin expression was observed throughout greater regions of MP-incorporated aggregates compared to no MP spheroids. D) MP incorporation also increased local MMP-2 staining, especially at regions of spherical morphology evocative of MPs (white arrow). MMP-2 expression independent of the strongly stained spherical regions is largely co-localized with fibronectin, as indicated by a yellow color and white arrowhead.
Figure 4.6. MP incorporation modulates EMT-related and lineage specific gene expression. A) MP incorporation reduces expression of primitive streak genes, Mixl1 and Brachyury-T, on day 4 of differentiation. B) Although the expression of E-cadherin was unmodified in the presence of MPs, Slug, N-cadherin, MMP-2, Mesp1, and Gata4 levels were higher than MP groups on day 7. C) Endodermal marker FoxA2 had increased expression in MP groups on day 7, while levels of mesodermal marker Fkh1 was increased in MP groups on days 7 and 10. No difference in ectodermal marker, Pax6, expression or pluripotency marker, Nanog, was observed among groups. Relative fold change over ESC gene expression level was calculated for all samples. * denotes statistical significance, with n ≥ 4 and p ≤ 0.05.
MP-induced EMT-related gene expression in response to MMP inhibition

Based on the zymogram appearance of MMP-2 active form beginning on day 7, treatment of spheroids with MMP inhibitor, Ro32-3555, was initiated on day 5. The MMP inhibitor treatment was able to reduce MMP-2 active form in all spheroid groups beginning at day 7 and persisting up to day 14 of differentiation (Figure 4.7). Inhibition appears to reduce spheroid breakdown and remodeling of GMA, since GMA plated spheroids treated with MMP inhibitor had a large buildup of cells at the visible spheroid boundaries by day 10 compared to uninhibited spheroids that spread further on GMA (Figure 4.8).

MMP inhibition resulted in a three-fold increase in expression of the primitive streak gene, Mixl1, in the 90% MA MP spheroids compared to corresponding MP spheroids as well as non-MP spheroids, while Brachyury-T expression was unaffected (Figure 4.9A). Expression of the E-cadherin repressor gene, slug, was decreased three-fold in both MP treated groups after inhibition, while N-cadherin, MMP-2, and Gata4 expression was reduced two-fold in highest methacrylated MP spheroids (Figure 4.9B). Additionally, inhibited spheroids with 20% MA MPs had a two-fold reduction in Mesp1 expression. Despite reduced expression in inhibited MP groups compared to their corresponding uninhibited counterparts, EMT gene expression of all inhibited MP groups was comparable to uninhibited non-MP groups. Moreover, Flk1 expression was reduced in inhibited spheroids with both MP formulations compared to their uninhibited counterparts, while FoxA2 reduction was observed in inhibited spheroids with high methacrylated MPs as well as no MP spheroids (Figure 4.9C). Thus, mesendodermal differentiation in inhibited MP groups was also comparable to uninhibited non-MP
groups. Pluripotency of MP groups was not affected by MMP inhibition by day 7, although inhibition of 20% MA MP spheroids led to enhanced Nanog expression compared to uninhibited non-MP spheroids. Additionally, ectodermal differentiation was decreased in inhibited 90% MA MP spheroids, which had reduced Pax6 expression compared to uninhibited non-MP spheroids.

**Figure 4.7. MMP inhibition abrogates MMP-2 active form.** Treatment of spheroids with MMP inhibitor, Ro32-2555, beginning at day 5 results in inhibition of MMP-2 active form (arrowhead) up to day 14 of differentiation. The pro form is indicated by a black arrow.
Figure 4.8. MMP inhibition reduces spheroid spreading on 2D surfaces. Spheroids were plated on either tissue culture polystyrene (TCPS) or TCPS plates coated with cross-linked 20% GMA or 90% GMA on day 4 of differentiation. One day after plating, spheroids plated on TCPS spread out markedly less than spheroids plated on GMA, and marked differences in spreading surface area could be observed until day 10. By day 10, spheroids treated with MMP inhibitor exhibited a dense accumulation of cells at the spheroid boundaries, suggesting reduced spreading.
Figure 4.9. Inhibition of MMP abrogates MP-induced changes in EMT-related and lineage specific gene expression in day 7 aggregates. A) MMP inhibitor treatment increased expression of Mixl1 in MP aggregates, while Brachyury-T levels were unaffected. B) In MP groups, Slug, N-cadherin, Mesp1, Gata4 and MMP-2 levels were decreased with inhibitor treatment, whereas E-cadherin was not affected. C) Flk1, FoxA2, and Pax6 were also decreased in MP groups with MMP inhibitor treatment on day 7. Relative fold change over ESC gene expression level was calculated for all samples. * denotes statistical significance between indicated groups, and # denotes statistical significance to no MP group, with n ≥ 4 and p ≤ 0.05.
MP-induced basement membrane expression patterns in response to MMP inhibition

The basement membrane ECM protein, laminin, was similarly observed between all groups, existing mainly on the periphery and within the internal cavitations observed in 90% MA MP spheroids (Figure 4.10A). However, western blot analysis indicated that the breakdown of the 210 kDa laminin-α5 chain was enhanced in MP groups, as observed by enhanced degradation product band intensities beginning at 100 kDa (Figure 4.10B). The breakdown was markedly reduced in inhibited groups, as observed by reduced band intensities similarly beginning at 100 kDa. Collagen IV, another component of the basement membrane typically bound to laminin, was expressed as clusters in the interior of MP treated spheroids, and these thick expression patterns were abrogated with inhibitor treatment (Figure 4.11).
Figure 4.10. Laminin expression in response to MP incorporation. A) The basement membrane ECM protein, laminin, was similarly observed between all groups, however B) western blot analysis indicated that laminin breakdown from the full unprocessed form (~260 kDa) to processed fragments (<100 kDa) was increased in both MP treated spheroids. MMP inhibition reduced the breakdown of laminin in the MP treated groups.
Figure 4.11. Collagen IV expression in response to MP incorporation and MMP inhibition. Collagen IV, a large component of the basement membrane with laminin interactions, was strongly expressed as large clusters in less-cell dense regions of MP treated spheroids. MMP inhibition reduced the regional expression patterns visualized with MP incorporation.
MMP-dependent mesenchymal morphogenesis in response to MP incorporation

Histological differences were observed between spheroid populations as early as day 7 of differentiation (Figure 4.12). By day 7, spheroids with higher MA MPs were less cell-dense, and spheroids with MA MPs had more cavitations and internal structures by day 10. By day 14, spheroids with MA MPs, particularly the highest MA MPs, had markedly less cell dense morphologies, which stained positively for mesenchymal marker, α-smooth muscle actin (αSMA), as well as an increased number and size of void spaces and enclosed inner structures (Figure 4.12, Figure 4.13A, Figure 4.14).

Inhibitor treatment resulted in a reduction of spheroid cavitations beginning on day 7 and occurring through day 10 (Figure 4.12). By day 14, inhibitor treatment resulted in not only marked reduction of spheroid cavitations and αSMA positive, low cell density regions, but also in overall size of the spheroids (Figure 4.12, Figure 4.13A, Figure 4.14). The variance in mesenchymal phenotype was illustrated via a PCA model based on cell type and cell cluster size and shape, which were separated along the first and second principal components, respectively (Figure 4.13B). PCA 1 and PCA 2 were able to retain 71% of the original variance present in all samples. Incorporation of both microparticles resulted in increased mesenchymal-like cell differentiation, with the 20% MA MPs having the largest areas and frequency of mesenchymal-like cell clusters. Inhibitor supplementation lead to an enhanced epithelial-like cell population in the MP samples, with the 20% MA MPs in particular having the largest size and number of epithelial-like cell clusters. Inhibition of no MP spheroids did not appear to have a strong effect on differentiation morphology.
Figure 4.12. Spheroid morphology modulated by MPs and MMP inhibition. A) Introduction of an MMP inhibitor, Ro32-2555, on day 5 of differentiation resulted in a reduction of spheroid cavitations beginning on day 7. B) By day 10, inhibitor treatment resulted in marked decrease in spheroid void spaces. C) Inhibitor treatment resulted in not only reduced cavitations in the 20% and 90% MA MP groups, but also markedly diminished sizes of spheroids by day 14.
Figure 4.13. MP incorporation enhances MMP-dependent mesenchymal morphogenesis in day 14 aggregates. A) Markedly greater enclosed inner structures and low cell dense areas, which exhibit positive staining for mesenchymal marker αSMA, were observed in spheroids with MA MPs compared to non-MP spheroids. Inhibitor treatment led to decreased frequency of cavitations and lower cell dense regions with reduced presence of αSMA in MP-spheroids. B) The variance in mesenchymal phenotype was illustrated via a PCA model based on cell type and cell cluster size and shape. Incorporation of both MPs resulted in increased mesenchymal cell differentiation, and inhibitor supplementation lead to an enhanced epithelial cell population in the MP samples.
Figure 4.14. α-smooth muscle actin expression localizes to lower density cellular regions. A) In MP treated groups on tissue sections, αSMA was localized as large clusters in lower cell density regions, clearly illustrated by nuclei-only staining. B) Inhibitor treatment reduced the large expression patterns of αSMA.
MMP-2 activity and mesenchymal morphology of spheroids in response to pSMAD 1/5/8 inhibition

Since increased MMP activity enhanced mesenchymal and mesodermal differentiation, we sought to determine whether inhibition of mesoderm induction, through treatment with SMAD 1/5/8 phosphorylation inhibitor, LDH-193189, would also inhibit MMP activity. Active forms of all treated spheroids were strongly reduced with pSMAD 1/5/8 inhibition on days 7 and 10 (Figure 4.15A). Pro forms of the no MP and 90% GMA spheroids were slightly reduced on day 7 and day 10 of differentiation, although the pSMAD 1/5/8 inhibitor did not appear to affect the strongest pro form, expressed by the 20% MA MP spheroids. The pSMAD 1/5/8 inhibitor also strongly reduced cavitations and mesenchymal phenotypes characterized by low cell density within all spheroids by day 10 (Figure 4.15B).
Figure 4.15. pSMAD 1/5/8 inhibitor reduces MMP-2 activity and mesenchymal morphology of spheroids. A) LDH-193189, a pSMAD 1/5/8 inhibitor, strongly reduced the active form of MMP-2 in all spheroids but only partially reduced pro forms in the no MP and 90% GMA spheroids on day 7 and day 10 of differentiation. B) pSMAD 1/5/8 inhibition markedly reduced the mesenchymal morphology and cavitations observed in all spheroids.
Figure 4.16. Schematic representation depicting possible mode of action of GMA MP–enhanced MMP2 activity and mesenchymal differentiation. MPs present within aggregates can increase MMP activity through a protease substrate-based feedback mechanism or locally sequestering bone morphogenic proteins, which subsequently activate the SMAD signaling pathway. The activation of MMP-2 promotes enhanced EMT processes and further mesendodermal differentiation.
Cardiomyocyte differentiation in response to MP incorporation

The ability of MP incorporation to induce additional lineage commitment was evaluated through analysis of MP spheroids to promote further mesodermal differentiation towards the cardiomyocyte lineage. In order to assess cardiomyogenesis, spheroids were plated on day 7 and observed until day 14 for contractile beating activity. Contractile assessment resulted in spheroids without MPs having the first instances of beating activity on day 8, however no differences between groups was observed on days 10, 12, and 14, with almost 90% of all spheroid groups beating by day 12 (Figure 4.17A). Although over 90% of spheroids in all groups were beating by day 14, MP spheroids, especially spheroids with 90% MA MPs, were observed to have the highest frequency of contractile foci and large sheet-like beating regions (Figure 4.17B,C). Based on the visual observation of greater contractile regions and foci in MP spheroids, plated spheroids from the contractile assay were collected on day 14, and \(\alpha\)-sarcomeric actin expression was quantitatively analyzed with flow cytometry and additionally evaluated via immunostaining. MP spheroids had greater \(\alpha\)-sarcomeric actin expression as determined via flow cytometry, with 90% MA MP spheroids having 22.5% 5c5+ cells, compared to 19.9% and 12.7% for 20% MA MP and no MP spheroids, respectively (Figure 4.18A,B). Day 14 spheroids were also analyzed for \(\alpha\)-sarcomeric actin expression via whole mount immunohistochemistry, and MP-spheroids had greater \(\alpha\)-sarcomeric actin expression over no MP spheroids. MP spheroid \(\alpha\)-sarcomeric actin expression decreased with MMP inhibitor treatment, demonstrating the dependency of MP-induced cardiomyogenesis on MMP activity (Figure 4.18C).
Figure 4.17. Contractile analysis of spheroids in response to MP incorporation. A) Spheroids without MPs had the first instances of beating activity on day 8, with no further differences between groups observed through day 14. B) The percentage of spheroids with greater than 3 contractile foci were calculated, with the highest MP MPs having the most instances of multiple contractile regions. C) A score was assigned to spheroids based on their beating levels. The no MP spheroids had the greatest percentage of ✓- spheroids, while the highest methacrylated MPs have the greatest percentage of ✓+ spheroids.
Figure 4.18. Cardiomyogenesis of spheroids in response to MP incorporation. A,B) Plated spheroids from the contractile assay were collected and MP spheroids had greater α-sarcomeric actin expression compared to no MP spheroids. The spheroids with 90% MA MPs had 22.5% 5c5+ cells, compared to 19.9% and 12.7% for 20% MA MP and untreated spheroids, respectively. C) Day 14 MP spheroids had greater α-sarcomeric actin expression over spheroids without MPs, and interior expression patterns decreased in MP spheroids with MMP inhibitor treatment.
Discussion

This study demonstrated that incorporation of gelatin methacrylate MPs within 3D ESC aggregates can promote an MMP-dependent increase in mesenchymal differentiation. The enhancement of MMP-2 activity via MP incorporation was dependent on the manner of presentation and chemical composition of the MPs. Furthermore, MP-induced EMT processes and mesenchymal morphogenesis was abrogated with MMP inhibition, suggesting these processes are MMP-dependent. Additionally, pSMAD 1/5/8 inhibition also reduced spheroid MMP-2 activity and mesenchymal phenotypes, suggesting possible new modes of action of MP-mediated mesenchymal differentiation.

MPs incorporation enhanced MMP activity, thus the conditions eliciting this proteolytic response were further investigated. MMP activity was not increased through 2-D contact of spheroids plated on GMA, suggesting the method of material presentation plays a crucial role in inducing a proteolytic response. Manner of presentation may be key in proteolytic cell responses as previously demonstrated in a study where endothelial cells had significantly higher MMP-2 activity when encapsulated within 3D collagen gels compared to when seeded on a 2D gel surface, since the collagen fibril structure may not preserved when thinly coated on the dish, preventing MMP-2 binding [6]. Additionally, MP incorporation may offer greater contact of the material with cells compared to 2-D spheroid plating, especially in the early portion of differentiation, which may be more crucial for impacting proteolytic activity and its subsequent effects. On the other hand, spheroids plated on GMA increase their contact with the material as they spread out, thus perhaps the amount of contact necessary to induce a response is not reached until a later
stage of differentiation, at which stage ESCs may no longer be as responsive to certain cues [173]. Moreover, the effect of MP incorporation on MMP activation is sensitive to the GMA content, since no other MP materials investigated were able to enhance MMP-2 activity in a manner akin to the GMA MPs. In a similar fashion, endothelial cells encapsulated only in collagen I and not Matrigel, a mix of extracellular matrix components, was able to enhance MMP-2 activity [6]. Furthermore, the methacrylate chemistry present in GMA does not appear to contribute towards MMP activity enhancement, since unlike studies in which leukocytes exposed to PMMA had enhanced MMP-2 activity [174,175], PMMA MPs were unable to increase MMP-2 levels within ESC aggregates. Furthermore, another methacrylated pure ECM-based MP formulation composed of heparin was unable to induce matrix degrading enzyme activity [167]. Surprisingly, coating polystyrene MPs with GMA was not sufficient to increase MMP activity, possibly because a 5 μm diameter polystyrene MP can only adsorb 0.35% of its weight of a gelatin-sized 50-70 kDa protein on its surface [176]. Thus, enhancement of MMP-2 activity appears to be specific to MP incorporation of its substrate, GMA, with a minimum quantity of GMA required. Moreover, the active form of MMP-2 was higher on days 7 and 10 in 90% MA MP spheroids compared to spheroids with the lower MA MPs, which had the greatest pro form throughout differentiation. MP stiffness may thus play a role in enhancing MMP activity, since epithelial cells encapsulated in 3D PEG hydrogels with 5-fold higher stiffness had 2-fold greater MMP activity compared to cells within the softer hydrogel counterparts.

MP incorporation promotes the down-regulation of primitive streak and epithelial markers and the MMP-dependent enhancement of mesenchymal phenotype by day 7.
MMP up-regulation occurs during embryonic EMT to facilitate cell migration through primitive streak during gastrulation [101]. In this system, introduction of MPs reduces MixII gene expression, which is increased with MMP inhibition, thus greater EMT occurring in MP-spheroids may explain the lower quantity of cells undergoing upstream primitive streak processes. One critical marker of EMT is internalization of E-cadherin from the plasma membranes. Although E-cadherin gene expression remained unchanged despite MP incorporation, MP groups had greater punctate expression of E-cadherin, which suggests membrane internalization [177]. Moreover, a previous study also reported that E-cadherin gene expression is uniformly expressed from day 0 to 12 of differentiation [109]. Thus, despite the ubiquitous presence of E-cadherin during early stage differentiation, MP incorporation may modulate the localization of E-cadherin expression. Consistent with the change in E-cadherin, an increase in gene expression of slug and MMP-2, which have been reported to facilitate E-cadherin internalization [87–89], was also observed.

MP-dependent enhancement of MMP activity is able to enhance EMT processes and promote mesenchymal differentiation by day 7. In corroboration with the studies reported herein, which observed MMP-mediated differences in MP-induced basement membrane molecule expression patterns, previous reports have demonstrated that MMP cleavage of the ECM exposes “cryptic sites,” which possess bioactivities separate from their parent molecule and can promote EMT processes. For example, MMP-2 and its closely associated activator, MT1-MMP, can release the \( \gamma_2 \) chain of laminin-5, which has been demonstrated to induce migration of epithelial cells [114]. In particular, a recent study in mouse and human ESCs has determined that MMP-2 can cleave laminin-111, a
major component of the basement membrane, and the resulting laminin fragment plays a role in EMT-related processes through the interaction with the $\alpha_3\beta_1$ integrin and type 1 transmembrane glycoprotein EMMPRIN (CD147) complex [108]. Furthermore, MMP-2 and MMP-9 can expose cryptic sites in laminin, and also laminin-bound collagen IV, that regulate cell migratory behavior [55,115]. Additionally, fibronectin fragments exposed by MMP cleavage also can induce cellular migration through activating biomolecules such as migration-stimulating factor (MSF) [116]. Fibronectin is a well documented EMT marker [98,106,178], and knockout studies have demonstrated the importance of fibronectin on mesoderm development [179,180]. Moreover, fibronectin has a very high affinity for gelatin [181], so incorporated gelatin MPs throughout the spheroid may explain the more pronounced fibronectin fibrillar organization observed within the EB interior, and suggest that fibronectin may be involved in increased MP-dependent mesodermal differentiation. Fibronectin also co-localized strongly with MMP-2, possibly because of fibronectin’s high affinity for gelatin, which is the primary substrate of MMP-2. MMP-2 expression was localized to the spheroid regions highly suggestive of MPs, and since MMP-2 has an isoelectric point of 5.29-6.07 [182], it is likely that MMP-2 is binding to the net negatively charged MP based on substrate affinity rather than electrostatic interaction.

MMP inhibitor treatment mainly affected EMT expression and morphology of MP-spheroids compared to spheroids without MPs. MMP inhibitor treatment on GMA 2-D plated spheroids resulted in a large buildup of cells at the visible spheroid boundaries by day 10, suggesting MMP inhibition prevented GMA breakdown and remodeling that facilitates spreading. Thus, the MMP inhibitor treatment reduces cellular interaction with
ECM, perhaps explaining why inhibition highly reduced mesenchymal differentiation in the presence of MPs and did not have as notable an impact on spheroids without MPs. Furthermore, inhibition markedly reduced mesenchymal morphology in the 20% MA MPs compared to that of the 90% MA MPs. Spheroids with 90% MA MPs had greater MMP activity compared to the lower MA MP spheroids on day 7 and day 10, thus MMP inhibition may not have been able to abrogate mesenchymal morphogenesis to the extent observed in the 20% MA MP spheroids. Additionally, MP incorporation can enhance later stage MMP-mediated mesodermal differentiation towards the cardiomyocyte lineage, suggesting that the differentiation trajectory of ESCs can be controlled by early presentation of MPs.

pSMAD 1/5/8 inhibition, which inhibits mesenchymal differentiation, also markedly reduced MMP-2 activity. Based on previous studies in Chapter 3 detailing the ability of gelatin MPs to bind bone morphogenic proteins (BMPs) [131], which can then enhance SMAD 1/5/8 phosphorylation, sequestering and binding of BMPs by the GMA MPs may also have contributed to enhanced MMP-2 activity. Moreover, MMPs promote EMT behaviors by releasing molecules such as BMPs and TGF-β from the ECM and activating them through inhibitory complex cleavage, which enables binding to ALK receptors and subsequent SMAD phosphorylation [103,113]. Furthermore, BMP4 has been demonstrated to enhance EMT and mesoderm commitment in pluripotent stem cells through activation of slug and MSX2 [183]. However, it appears that MPs promote MMP-2 activity primarily through a protease-substrate based feedback mechanism, since pSMAD inhibition could only partially reduce the MP-mediated MMP-2 response. Thus, these studies demonstrate that GMA-dependent enhancement of MMP-2, either directly
through a protease-substrate based feedback mechanism or through downstream SMAD phosphorylation from possible BMP binding, can enhance EMT processes and promote mesendodermal differentiation (Figure 4.16).

Although the role of MMPs in embryological development has been extensively studied, little has been reported on MMP involvement in embryonic stem cell differentiation. Incorporation of biomaterial microparticles has previously been demonstrated to direct embryonic stem cell (ESC) phenotype in a dose dependent manner [35], however mechanisms by which biomaterials can regulate differentiation are still largely unknown. This study also illustrates the potential to engineer the stem cell ECM microenvironment and regulate differentiation by incorporation of protease-modulating MPs. Inducing proteolytic responses may be a novel method to trigger or enhance stem cell differentiation processes, since tissue morphogenesis commonly requires matrix remodeling to facilitate cell migration. Based on the well-studied and important role of MMPs in early embryo lineage specification and in tissue morphogenesis [52,165], it is anticipated that further understanding the effect of MMPs on differentiation will inform generation of desired cell types *in vitro*. 
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

In this work, incorporation of gelatin methacrylate microparticles, formulated with tunable degradation properties, within embryonic stem cell aggregates enhanced MMP activity and promoted basement membrane remodeling and mesendodermal differentiation in an MMP-dependent manner. These studies established an ECM material platform with tunable properties that inform design of a vehicle for delivery of growth factors in a temporal manner.

Although glutaraldehyde or genipin cross-linked gelatin MPs have been utilized extensively in tissue engineering applications for over a decade [123,124,137], limited control exists over MP cross-linking density, since similarly cross-linked MPs, above 50% cross-linking, are obtained despite varying experimental conditions, such as the concentration of cross-linker and time and temperature of cross-linking. We were able to synthesize MPs composed of gelatin methacrylate (GMA) that allow for a wide range of cross-linking densities (15%-90%), since GMA can be synthesized prior to the cross-linking step with varying degrees of methacrylate groups, which restrict the maximum cross-linking density achievable despite changes in experimental conditions. GMA MPs could be synthesized with similar size distributions and gelatin content despite differences in cross-linking density. Furthermore, we illustrated that by decreasing cross-linking density, and effectively reducing the hydrogel mesh size, the lowest cross-linked MPs could load 5x as much growth factor as the highest cross-linked MPs and the traditional glutaraldehyde cross-linked MPs. Hence, GMA MPs have the ability to sequester and deliver greater amount of growth factor than the commonly used gelatin
MPs, offering a more robust delivery vehicle. Moreover, degradation rate of the MP, as well as the release of bound growth factors in the presence of proteases, could be significantly delayed by increasing the cross-linking density. Thus, compared to soluble chemical cross-linking methods, methacrylation enhances the tunability of gelatin MPs, which join an increasingly large list of characterized biomaterials that can be employed for delivery of growth factors, particularly within 3D aggregates to facilitate differentiation. Recent advances in complex ESC differentiation strategies towards pancreatic β cells, cardiomyocytes, nephrons, and gastric endocrine cells illustrate the importance of delivering biomolecule cues at precise temporal time frames on both differentiation outcome and cell yield [184–188]. Thus, temporal presentation of biomolecular cues is critical during ESC differentiation, and biomaterials with controlled release properties may contribute to the next generation of differentiation strategies.

These studies also illustrate the potential to engineer the ECM microenvironment and regulate pluripotent stem cell differentiation within aggregates by incorporation of protease-modulating MPs. GMA MPs incorporated within pluripotent stem cell spheroids were able to enhance total MMP levels as well as MMP-2 expression and activity over the time course of differentiation, and the modulation of MMP-2 levels was specific to GMA MP incorporation within a 3D context. Additionally, MMP-2 levels were only increased in spheroids with GMA MPs and not other MPs with similar components, such as methacrylation or other ECM species. Thus, enhancing cellular MMP-2 activity via incorporation of GMA MPs in pluripotent stem cell aggregates is dependent on both manner of presentation and substrate presented. Moreover, MMP-2 expression was localized to the MPs within the spheroids in a non-electrostatically mediated manner.
since MMP-2 has similar net charge to the GMA MPs at neutral pH, thus highlighting the affinity of the protease for its substrate. MMP-2-MP localization implies that protease activity and its resultant effects on differentiation may be localized to the regions of incorporation, further illustrating how MPs can be used to spatially direct differentiation within stem cell aggregates.

Moreover, these studies also explore novel mechanisms for how ECM-based biomolecules regulate cell signaling. These studies additionally suggest a novel MMP-2 mediated pathway involved in stem cell differentiation towards the mesendodermal lineage. MP spheroids had increased mesenchymal and mesendodermal phenotypes, such as increased EMT gene expression and altered ECM expression patterns. Moreover, the differentiation was mediated by MMP-2 activity, since its inhibition abrogated the mesenchymal and mesodermal differentiation response. Thus, although very few reports have documented the roles of MMPs in ESC processes, understanding how MMPs dynamically remodel the ECM in vitro to enable the release of sequestered biomolecules and potent isoforms that can effect downstream lineage specification, will inform strategies to more effectively control differentiation and generate desired cell types for in vivo applications.

In addition, inhibition of SMAD 1/5/8 phosphorylation significantly reduced MMP-2 active forms, indicating that pSMAD 1/5/8 is involved in MMP-2 activity. Since inhibition of pSMAD 1/5/8 can only partially reduce the MP-induced MMP-activity, the MPs may also enhance MMP activity and further mesodermal differentiation by sequestering and concentrating BMPs, which have been demonstrated to readily bind to the MPs in Chapter 3 [131] as well as promote phosphorylation of SMAD 1/5/8
These studies are the first to demonstrate pSMAD 1/5/8 involvement in MMP-2 activity within ESC aggregates, as a previous report that documented pSMAD 1 participation in MMP-2 upregulation was in the context of pancreatic cancer [191]. SMAD 1/5/8 phosphorylation enhances mesodermal differentiation through mediating increased expression of EMT molecules, slug, Mesp1, and Msx1 [101,103,110,183], and at least slug has been implicated in enhanced MMP activity to facilitate mesenchymal morphogenesis [103]. However, these studies were the first to demonstrate a direct link between pSMAD 1/5/8 and MMP activity in ESC aggregates, suggesting the potential of charged MPs to locally concentrate endogenous morphogens for local control of differentiation.

These studies introduced a material platform that have promising potential for delivery of growth factors in a temporal manner for stem cell differentiation, control of which is very reliant on the timing of morphogen presentation. Studies have suggested that stem cells pass through discrete “temporal windows,” during which time they gained and lost responsiveness to exogenous or paracrine signals, such as BMP4, Wnt3a and Activin A, for mesodermal differentiation [173,192,193]. One well-known temporally sensitive differentiation process is the differentiation of PSCs to cardiomyocytes. Activin A and BMP4 delivery to both human and mouse PSCs enable generation of enriched populations of cardiomyocytes [194]. However, the timing of both Activin A and BMP4 presentation impact the efficiency of both mesoderm and subsequent cardiomyocyte differentiation. Thus, multiple studies have successfully induced enriched cardiomyocyte differentiation from human ESCs on monolayer via a protocol delivering Activin A for 24 hours followed by BMP4 for 4 days, yielding at least 30% functional cardiomyocytes.
However, the Activin A/BMP4 differentiation strategy utilizes high concentrations of growth factor and so far has only been performed in monolayer culture, which engrafts poorly compared to aggregates of the same cells in vivo. Highly cross-linked MPs can be utilized to release BMP4 at slower rates compared to soluble delivery (for up to 4 days) from within the spheroid as a strategy to generate high-throughput quantities of cardiomyocyte aggregates for viable clinical therapies with reduced amount of growth factor. Additionally, sequential delivery of different biomolecules has been utilized to differentiate pluripotent stem cells towards neurons [198] and pancreatic endocrine cells [199], and sequential release of molecules such as BMP-2, VEGF and PDGF has even facilitated bone regeneration [200–202]. It is thus anticipated that the MP technology could be adapted to facilitate differentiation and tissue engineering based on sequential release within aggregates.

In a previous study, I have demonstrated that delivery of morphogens from traditional gelatin/GA MP incorporation enables similar differentiation for up to 10 days of culture compared to soluble treatment with 12-fold less growth factor [203]. Moreover, in Chapter 3, I have demonstrated that the GMA MPs at lower cross-linking densities can consistently load more than 5 times the amount of BMP4 than the GA cross-linked MP, which has been widely utilized as a delivery vehicle for a myriad of tissue engineering applications despite the difficulty in varying its degradation rate. It is anticipated that GMA MPs can serve as tunable growth factor delivery vehicles, as varying degree of methacrylation will enable delivery of multiple growth factors with increased temporal control, facilitating differentiation to multiple mesendodermal lineages with possible 5-fold reduction of growth factor necessary. However, a caveat to increasing
methacrylation, and thus prolonging release, is a reduction in the GMA MP loading capacity. Thus, in case greater growth factor loading is required for a prolonged release, conjugation of glycosaminoglycans (GAGs), such as heparin or chondroitin sulfate, to GMA MPs can not only significantly enhance binding, but also prolong the release of growth factors if necessary to obtain the desired delivery trajectories. For example, heparin methacrylate MPs (HMAm) can load 2 fold greater BMP-2 over GMA MPs, and GMA MPs release 30% of loaded BMP-2 compared to less than 10% released by HMAm MPs over 7 days in the same conditions [40,167,203]. Additionally, chondroitin sulfate methacrylate MPs release an almost negligible amount of oppositely charged TGF-β1 (<1 ng) over a 15 day time period, further corroborating studies that GAGs can strongly sequester and maintain growth factors in a bioactive state [204–207]. Conjugation of GAGs to GMA may enable sequestration of potent growth factors until GMA degradation, which can be controlled by cross-linking density modification, allowing for prolonged growth factor release rates, which can be tailored to deliver multiple growth factors for more complicated differentiation strategies. However, little characterization on GAG proteases within PSCs has been performed, thus expression of heparinase and chondroitinase ABC during PSC differentiation should be determined in order to inform design of and predict growth factor release from GAG conjugated MPs in PSC aggregates.

Investigation of MMP activity in ESC spheroids enables engineering of “on demand” stimuli responsive growth factor release systems. As discovered in these studies, ESCs have a natural onset of MMP-2 activity between day 5 and day 7 of differentiation within both serum and some serum-free conditions. This time-point is a
very critical period during differentiation, as it is typically the onset of mesodermal differentiation in the presence of serum or TGFβ/Wnt family growth factors [173]. Cues presented at this stage can drastically affect differentiation trajectory, especially down the mesodermal pathway, to either the hematopoietic or cardiomyogenic lineage among others. For example, previous studies have demonstrated that upon Flk1+ commitment to mesodermal fate, the addition of combinations of Wnt, Activin/nodal, and BMP signaling can drive hematopoietic mesoderm [208]. Furthermore, Wnt has a bisphasic role, since Wnt is procardiac prior to mesoderm induction, but afterwards, it is antagonizing to cardiac differentiation. After mesoderm commitment, inhibition of Wnt/β-catenin signaling can generate cardiac mesoderm [209], thus early induction of Wnt by glycogen synthase kinase 3 (Gsk3) inhibition, followed by later stage inhibition of β-catenin signaling by shRNA was able to generate high yields of cardiomyocytes with 80-98% efficiency [186]. Novel MPs can be formulated with MMP-2 degradable cross-links, and these MPs can be utilized to sequester growth factors or small molecules until mesoderm stage cells secrete activated forms of MMP-2. The activated MMP-2 can then cleave the degradable MMP-2 cross-links and allow for the sequestered biomolecules to facilitate either cardiomyogenesis or hematopoiesis. Molecules that could be delivered after mesoderm induction to induce cardiomyogenesis include the Wnt inhibitors: DKK-1, β-catenin shRNA, Frizzled-8/Fc chimeric protein (Fz8/Fc), or small molecule inhibitors of Wnt ligand production (IWP2 and IWP4a) [186,208–211].

MMP expression and activity during ESC differentiation have not been widely studied, despite considerable characterization in developing embryos, perhaps because in focusing towards attaining specific cell lineages, studies may concentrate on downstream
gene and expression changes [211–213], although ECM profiles of differentiating ESCs are increasingly reported [214,215]. Identification of the MMPs produced with different lineages and at varying stages of differentiation can become a powerful tool for characterizing differentiation status and identifying novel targets for controlled differentiation strategies. MMPs appear to play a role in differentiation through releasing either bioactive fragments or biomolecules within the ECM, as well as even cell-surface and soluble biomolecules that mediate downstream signaling [51], thus understanding specificity of MMP cleavage during differentiation will aid in designing biomaterials that can be dynamically remodeled by MMPs for directed differentiation strategies. Once the roles of MMPs in distinctive differentiation trajectories are further elucidated, MP delivery systems can also be designed to incorporate MMP cleavable peptides corresponding with MMPs secreted that are preferentially secreted during specific differentiation processes to facilitate further lineage commitment. These MMPs can thus release molecules from the MPs that can drive further maturation of the differentiated lineages at later time points during differentiation, during which period the cells may be more primed to respond to such signals. Although much characterization remains on what MMPs cells secrete during differentiation trajectories, currently MMP-3 has been associated with cardiogenesis [90], MMP-9 with neural stem cell commitment [92,216], and MMP-13 for chondrogenesis [217]. For instance, MMP-3 cleavage may be able to release cues sequestered within MPs with MMP-3 cleavable peptides that promote further differentiation towards either of the cardiomyocyte populations. For example, although both nodal or working (atrial or ventricular) cardiomyocytes can be obtained from pluripotent stem cells, nodal-type cardiomyocytes molecules are beneficial for a
biological pacemaker application, while working-type cardiomyocytes are more suitable for other regions of the heart because their implantation may increase the risk of arrhythmias [218]. Additionally, cues presented within MMP-9 cleavable MPs can be released to obtain more specialized subpopulations of neurons, oligodendrocytes, schwann cells, or astrocytes from neural stem cells. However, one challenge to creating MPs with MMP-cleavable cross-linkers is that many MMPs have overlapping substrate specificities [51]. Although MMP degradable sequences can be designed with up to 6 hundred-fold increases in enzymatic specificities (kinetic parameter $k_{cat}/K_M$) over the MMP cleavage site in collagen (GPQGIAGQ) by screening phage display libraries for degradation by specific MMPs, most sequences can still be cleaved by a variety of different MMPs, although perhaps less efficiently [5,219,220]. Thus, these overlapping specificities should be recognized when choosing cross-linkers for MP growth factor delivery in diverse differentiation contexts.

These studies are the first to evaluate proteolytic responses to biomaterials within ESC aggregates and the resultant effects of the proteases on pluripotent stem cell extracellular remodeling and differentiation. Understanding the proteolytic effects of materials may yield insights into design and development of biomaterial systems to direct stem cell differentiation. Inducing proteolytic responses may be a novel method to trigger or enhance stem cell differentiation processes, since tissue morphogenesis commonly requires matrix remodeling to facilitate cell migration. Although several studies have explored proteolytic responses to introduced ECM materials or protease-cleavable peptide, this study was the first to monitor proteolytic responses to a substrate with different degradation rates, implying that tuning the degradation rate of the introduced
material can be used to modulate the protease response and subsequent effects on differentiation. These studies have demonstrated that materials with tunable degradations are able to modulate MMP activity in a degradation dependent manner. Only fast degrading MPs were able to enhance MMP-2 expression in the very early stages of differentiation (day 1-4), suggesting that degradation products may be necessary to instigate MMP production. MMP-2 active form appears by day 7, possibly because prior to this time point, ESCs do not express the cellular machinery necessary to activate MMP-2, such as MMP-14 and TIMP-2 [66]. After day 7, the slower degrading MPs have the greatest active form, perhaps elicited by its initial degradation byproducts as it begins to degrade. Thus, tuning physical properties of MPs, including degradability, can modulate temporal onset of MMP activity. Additionally, different ECM-based materials should be evaluated for their ability to elicit specific forms of MMPs that may be able to promote differentiation towards desired cell lineages. It is anticipated that proteolytic inductive materials can be used in tandem with other biomolecular and biophysical signals in a potent bioactive state to direct stem cell differentiation within aggregates.

Furthermore, introduction of proteolytically sensitive materials can offer a biomaterials-based avenue to study how enzymatic activity can modulate stem cell differentiation, since a large majority of tissue development and morphogenesis requires matrix remodeling to facilitate downstream signaling and cell migration. Utilization of materials-based proteolytic responses eliminates the use of recombinant proteases, which based on molecular weight, may face diffusion limitations to reach the interior of the aggregates [31,221].
Embryonic stem cells hold such intense promise for regeneration of diseased organs and tissues, however much greater control over differentiation must be obtained in order to fulfill their clinical potential. Strategies to direct differentiation of stem cells are increasingly moving towards utilizing biomaterial approaches because they can be standardized, low cost, and easily reproducible [35,222–224]. These studies sought to understand the interaction of embryonic stem cells with gelatin, a very common biomaterial applied to a myriad of tissue engineering therapies, and it uncovered responses at the proteolytic level yielding strong effects on resultant extracellular remodeling and differentiation trajectory. This study suggests that inducing stem cell proteolytic responses may be an innovative avenue to modulate differentiation processes, since tissue development and morphogenesis often require advanced matrix remodeling and cell migration. This study also demonstrates that analyzing the proteolytic effects of materials may generate insights into design and development of biomaterial systems to direct stem cell differentiation. Future studies employing these proteolytic inductive materials in tandem with other cues, such as soluble factors, can be utilized to efficiently direct stem cell differentiation within aggregates.
APPENDIX A

MICROPARTICLE MEDIATED MORPHOGEN DELIVERY
WITHIN PLURIPOTENT STEM CELL AGGREGATES

Introduction

Transfer of pluripotent stem cells from adherent monolayer to three-dimensional suspension culture of cell aggregates, referred to as embryoid bodies (EBs), is frequently used to promote differentiation towards cell types of all three germ layers [35,225,226]. In many instances, limiting differentiation within aggregate cultures to a particular cell type has proven difficult, but the addition of specific morphogenic growth factors to the culture medium often enhances the production efficiency of desired cell types. For example, the addition of bone morphogenetic protein 4 (BMP4) to the culture medium at early time points after aggregate formation enhances mesoderm differentiation by activation of the transcription factor Brachyury-T through SMAD 1/5/8 signaling, a mechanism conserved in mouse and human development [15,227]. BMP4 signaling can also be inhibited to promote ectoderm differentiation by the addition of noggin, which directly binds BMP4, or other small molecule inhibitors of the SMAD pathway [198,228].

Due to the three-dimensional nature of multicellular aggregates, inherent barriers exist to the free diffusion of molecules throughout the aggregate and we, in addition to several other labs, have demonstrated diffusion limitations encountered with several different types of molecules [31,32,221]_ENREF_4. Therefore, concentration gradients

Modified from:
of molecules created throughout cell spheroids may be, at least in part, responsible for the
general difficulty in controlling the homogeneity of differentiation within three-
dimensional aggregates. The challenge of precise dosing control is further compounded
by the fact that, in terms of scalability, growth factor delivery methods have not kept pace
with recent advances in stem cell technologies allowing for scalable formation and
culture of homogeneous pluripotent stem cell aggregates [22–24,229].

We present here a scalable method for integrating biomaterial, microparticle
(MP)-based growth factor delivery vehicles that, because the growth factor is delivered
from within the aggregate itself, is independent of the volume of the medium in the
culture vessel and can circumvent barriers to diffusion of molecules from the culture
medium throughout the aggregate. Delivery of BMP4 and noggin from gelatin-based
MPs was separately tested to direct early pluripotent lineage commitment in three-
dimensional aggregates. The ability to localize MPs laden with different morphogens
within a particular hemisphere of multicellular aggregates was also investigated as a
method to spatially control differentiation within a model of mammalian development.
MP delivery of growth factors does not require medium manipulation for directed
differentiation and is an important step towards scalable differentiation of pluripotent
stem cells for cell biomanufacturing and tissue engineering purposes.
Methods

Fabrication and loading of gelatin microparticles

Microparticles (MPs) of gelatin type B (G9391, Sigma Aldrich, St. Louis, MO) were generated using a water-in-oil emulsion method and fluorescently labeled as previously described [35]. Heparin sodium salt (CalBiochem, San Diego, CA) was conjugated to MPs after MP formulation in the following manner: EDC and S-NHS (Thermo Scientific, Waltham, MA) were added to heparin at 10:1 and 25:1 molar ratios respectively, relative to heparin dissolved in 800 µL activation buffer (0.1M MES, 0.5M NaCl, pH 6.0) and allowed to react for 15 minutes at room temperature to modify the carboxyl groups of heparin to amine reactive S-NHS esters. The EDC/NHS reaction was quenched with 20 mM 2-mercaptoethanol and the activated heparin was added to 400 µL of MP in PBS at a 5:1 molar ratio of heparin to gelatin and agitated for 4 hours at 37ºC. Prior to cell culture, MPs were treated in 70% ethanol for a minimum of 30 minutes before washing 3x with ddH₂O. Each MP batch was lyophilized and stored at -20 C until further use. Growth factor solutions were added to lyophilized MPs at 5 µL/mg overnight at 4ºC to allow for rehydration of the MPs and uptake of the growth factor. Growth factors were added at either 50 or 125 ng/mg of MPs. After growth factor loading, all of the loaded MPs were suspended in differentiation media (500 µL) and then 10 µL of the solution was counted on a hemocytometer to determine the concentration of MPs.
Albumin release from MPs

Bovine serum albumin (Sigma Aldrich, St. Louis, MO) was labeled with AlexaFluor 555 using EDC/S-NHS chemistry. Free dye was removed from the protein solution using an Amicon Ultra-15 centrifugal filter unit with a 30 kDa cutoff (Millipore, Billerica, MA). Labeled BSA was loaded into gelatin MPs as described above at a 1 mg/mL loading concentration.

Cell culture

Undifferentiated D3 ESCs were maintained on 0.1% gelatin-coated tissue culture dishes in DMEM media supplemented with 15% fetal bovine serum, 2 mM L-glutamine (Mediatech), 1X MEM non-essential amino acid solution (Mediatech), antibiotic/antimycotics (Mediatech), 0.1 mM β-mercaptoethanol (MP Biomedicals, LLC), and 10^3 U/ml leukemia inhibitory factor (LIF) (Millipore, Billerica, MA). Mouse Brachyury-T GFP cells (E14.1, 129/Ola) [230] were maintained on 0.5 % gelatin coated petri dishes in a humidified 5 % CO₂ atmosphere using modified serum-free maintenance media and base differentiation media. The defined media was composed of a DMEM/F12 (50/50) (Thermo Scientific) media supplemented with N2 (Gibco) and 50 µg/mL BSA (Millipore) in a 1:1 combination with B27 (Gibco) supplemented Neurobasal™ medium (Gibco) with 100 U/ml penicillin, 100 g/mL streptomycin, 0.25 g/ml amphotericin (Mediatech) and 2mM L-glutamine (Mediatech) [231]. Media was routinely exchanged in ESC cultures every 1-2 days, and cells were passaged every 2-3 days as needed before reaching 80% confluency. ESGRO complete basal medium (Millipore) was used for all differentiation cultures.
Aggregate formation and culture

ESCs were trypsinized into a single cell suspension and aggregates were formed by forced aggregation in AggreWell™ 400 inserts (Stem Cell Technologies, Vancouver, CA) [13]. Briefly, 1.2 x 10^6 cells in 0.5 mL of ESGRO complete basal medium were added to each insert, containing approximately 1200 wells, and centrifuged at 200 x g for 5 minutes to cluster cells in the wells. Gelatin MPs were incorporated within EBs using a second centrifugation of the culture plates after addition of 200 µL of a MP solution. In all cases, the MP:cell seed ratio was 1:3. After 24 hours of culture, cell aggregates were removed from the wells using a wide-bore pipette and transferred to suspension culture on a rotary orbital shaker (40 RPM) to maintain the homogeneity of the aggregate population and prevent EB agglomeration. In the case of soluble growth factor addition, BMP4 (10 ng/mL) or noggin (50 ng/mL) was added during the initial 24 hours of formation, again when transferred to suspension culture, and then supplemented every other day when the spent medium was exchanged until day 4 of EB culture. In some cases, EBs were plated onto 0.1% gelatin-coated culture vessels at day 7 of differentiation to allow attachment and EB cell spreading. After attachment, spent medium was exchanged every other day.

For EB merging studies, after 24 hours of initial aggregate formation, one population of EBs (population A) was added to a second distinct EB population (population B) formed in a separate microwell insert. After an additional 24 hours of culture, EBs from the two populations would merge to form single larger aggregates in the individual microwells. EBs from population A were added at a 1:2 ratio (A:B) to
decrease the probability of adding more than one EB from population A to microwells containing fully formed aggregates from population B.

**Spheroid morphology analysis**

At days 4 and 7 of differentiation, EBs were collected from rotary culture, fixed in 10% formalin for 30 minutes, and suspended in Histogel (Richard–Allan Scientific, Kalamazoo, MI). The samples were then embedded in paraffin and cut into 5 µm-thick sections (MICROM HM 310, Global Medical Instrumentation Inc., Ramsey, MN). After the sections were deparaffinized, they were stained with hematoxylin and eosin (H&E). Histological samples were imaged via a Nikon 80i upright microscope equipped with a SPOT Flex camera (15.2 64 MP Shifting Pixel, Diagnostic Instruments).

**Confocal microscopy**

The presence of GFP expressing cells within EBs was analyzed using a LSM 510 NLO confocal microscope (Zeiss, Thornwood, NY). EBs were removed from suspension culture, fixed in 4% paraformaldehyde, and stained with Hoechst (1:100) before imaging on glass slides. Visualization of GFP signal was performed using an argon laser with a 488 nm excitation filter and a 510 emission filter.

**Gene expression analysis**

RNA was extracted from spheroids after 4 days of differentiation with the RNeasy Mini kit (Qiagen Inc, Valencia, CA). RNA was converted to complementary DNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and analyzed using real time PCR (MyIQ cycler, BioRad). Forward and reverse primers for *Oct4*, *Brachyury-T*, *Pax6*, *Flk1*, *
and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were designed with Beacon Designer software and purchased from Invitrogen (Table 1). Gene expression was calculated with respect to expression levels of EBs without MPs using the Pfaffl method [170].

**Table A.1. Primer sequences and conditions for qPCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Melt Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GCCTTCCGTGGTCTCTCC</td>
<td>GCCTGCTTCCACCCCTTC</td>
<td>55</td>
</tr>
<tr>
<td>Oct-4</td>
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<td>GCGATGTGAATGTCTGAG</td>
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</tr>
<tr>
<td>Pax-6</td>
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<td>GCTGAAGTCGATCAG</td>
<td>58</td>
</tr>
<tr>
<td>Brachyury-T</td>
<td>CACACCAGTACGGCAGC</td>
<td>GAGGTATGAGAGGCTTTG</td>
<td>55</td>
</tr>
<tr>
<td>Flk-1</td>
<td>GCCGCCGCTGGAGAGTATC</td>
<td>TGACAGGGCGATGATGG</td>
<td>64.3</td>
</tr>
</tbody>
</table>

**Flow cytometry**

Spheroids were rinsed thoroughly with PBS and dissociated to a single cell suspension with 0.25% trypsin-EDTA and trituration for 10 minutes. The single-cell suspension was rinsed 3 times in PBS and pelleted at 1000 rpm for 5 minutes between rinses. Flow cytometry was performed with an Accuri C6 cytometer (Accuri Cytometers, Ann Arbor, MI), with a minimum of 20,000 events per sample collected within the FSC/SSC gate for live cell populations (n = 3 independent experimental samples per condition). Heparin-gelatin MPs, gelatin MPs, and undifferentiated Brachyury-T GFP cells alone were used for to establish appropriate gates and compensation. Within the FSC/SSC gate, polygonal gating was used on the (FSC)/FL-1 (480 nm excitation; 530 ± 15 nm emission) plots to limit 1% of undifferentiated negative control population via
FlowJo software (Tree Star, Inc., Ashland, OR). The whole cell population was gated to include less than 2% heparin-gelatin and 5% gelatin MPs (Figure 6).

**Statistical analysis**

Unless otherwise indicated, all data are reported as mean ± standard error for a minimum of triplicate experimental samples. All data was normalized to a Gaussian distribution using a Box-Cox power transformation before statistical analysis. Statistical significance was assessed using student’s t-test or one-way ANOVA with Tukey’s post hoc analysis after verifying variance equality from Levene’s equality of variances test. P-values of less than 0.05 were considered statistically significant.
Results

Morphogen delivery via incorporated MPs

Gelatin MPs were incorporated within embryonic stem cell aggregates using forced aggregation within microwells at a 1:3 (MP:cell) seeding ratio, based on previous studies [35]. Fluorescently labeled MPs were easily identifiable within the aggregate during formation and after transfer to suspension culture (Figure A.1A). In order to visualize protein release within the aggregates, fluorescently labeled albumin was loaded within the gelatin MPs prior to incorporation within ESC aggregates (Figure A.1B). Punctate fluorescence patterns within EBs were observed through the first 4 days of differentiation, indicating protein association primarily with the MPs during this time period. However, punctuate fluorescence was replaced by a more diffuse fluorescent pattern suggesting protein release from the MPs over a total period of 7 days of examination.
Gelatin microparticle incorporation and albumin release. A) Alexa Fluor® 488-labeled gelatin MPs were incorporated within stem cell aggregates using forced aggregation. After 24 hours of culture, the MPs remained visible within the aggregates in the microwells and after transfer to suspension culture. B) Alexa Fluor® 546-labeled albumin was incorporated within gelatin MPs to model protein release within EBs. Punctate fluorescent signals demonstrated protein localization within the MPs up to 4 days, after which a diffuse fluorescence signal suggestive of protein release was observed. Scale bar = 200 µm.

Gelatin MPs were next used to deliver either BMP4 or noggin within EBs for directed differentiation. Efficacy of MP-based delivery was tested at two loading concentrations of each molecule, 50 or 125 ng of growth factor per mg of MPs. The corresponding total amounts of growth factor delivered via MPs to ~1200 EBs per 10 cm plate of were 6 and 17 ng respectively; more than an order of magnitude less than the total amount of 210 ng of growth factor added over 5 days of soluble supplementation with a 10 ng/mL growth factor solution. After 4 days of culture, gene expression of early lineage commitment markers, Brachyury-T, Flk1 and Pax6, was analyzed (Figure A.2).
EBs containing both concentrations of BMP4-loaded particles exhibited increased expression of early mesoderm markers Brachyury-T and Flk1 compared to untreated and noggin treated samples. Pax6, a marker for neural ectoderm, was increased in noggin MP samples and decreased in BMP4 MP samples compared to untreated EBs. No significant differences were observed between noggin loading conditions; however, loading of 125 ng BMP4 per mg MP resulted in significantly greater gene expression of Brachyury-T and less Pax6 compared to the lower loading concentration (50 ng/mg MP). Therefore, for all subsequent studies of morphogen delivery from microparticles, a loading concentration of 125 ng of BMP4/mg MP was used, whereas 50 ng/mg MP was used for noggin delivery.

![Graph showing dose-response to BMP4 and Noggin](image)

**Figure A.2. Dose-response to BMP4 and Noggin delivered from gelatin MPs.** Increasing the concentration of growth factor of the loading solution for gelatin microparticles from 10 µg/mL of growth factor to 50 µg/mL growth factor resulted in variable differences in gene expression. While increasing the levels of Noggin had little effect, increased BMP4 loading resulted in increased Brachyury T expression and decreased Pax6 expression. No effects were observed in the response of Flk1 or FoxA2.
Directed differentiation by MP delivery of BMP4 and noggin was then compared to soluble delivery of the same molecules based upon gene expression analysis after 4 days of treatment (Figure A.3). As expected, soluble delivery of BMP4 resulted in increased Brachyury-T expression and decreased Pax6 expression, while the opposite was true for soluble delivery of noggin. MP-based delivery of BMP4 and noggin resulted in similar changes in Brachyury-T and Pax6, with no significant differences in gene expression between soluble or MP-based delivery of growth factors. The presence of unloaded gelatin MPs within EBs did not significantly influence the expression of the specific genes examined compared to aggregates lacking MPs. Similarly, none of the experimental groups differed significantly from untreated controls in the decreased expression of the pluripotent transcription factor Oct4.

Figure A.3. Comparable gene expression of spheroids with growth factor-laden MPs and soluble treatment. Directed differentiation by MP delivery of BMP4 and noggin induced comparable levels of lineage-specific gene expression compared to soluble delivery of the same molecules. Gene expression analysis indicated no change in the decrease of OCT4 in any of the groups. BMP4 delivered from MPs or added to the medium induced Brachyury-T (mesoderm) expression while noggin delivered by MPs or by soluble addition induced Pax6 (neuroectoderm) expression in day 4 EBs. * = significantly different from untreated samples p < 0.05.
Spheroid morphology

Although no differences in spheroid cross-section morphology were observed after 4 days of differentiation, gelatin MPs were visible in fixed sections of MP-containing EBs stained with hematoxylin and eosin (Figure A.4A). However, by 7 days of differentiation the differentially treated groups developed distinguishing morphological traits (Figure A.4B). In particular, EBs treated with noggin possessed numerous cavitations, while EBs treated with BMP4 displayed areas of mesenchymal-like phenotypes, with a lower cell density organization compared to the non-cavitated regions of noggin-treated EBs. No obvious differences in morphology were observed between EBs treated with soluble or MP-based growth factor delivery for either noggin or BMP4. Early differences in lineage commitment resulted in differences in aggregate morphology after 10 days of suspension culture (Figure A.5A). In both BMP4 MP and soluble BMP4 treated spheroids, optically translucent, bubble shaped outgrowths were observed in the majority of spheroids; these morphological features were not observed in untreated or noggin treated EBs. Similar differences in morphology due to BMP4 and noggin treatment were also observed in day 10 spheroids plated onto gelatin-coated substrates (Figure A.5B). Hollow, ring-like structures were commonly observed in plated BMP4 treated EBs with either MP or soluble delivery, but these structures were not observed in untreated or noggin treated EBs, where neurite outgrowths were frequently observed instead (Figure A.5B). Neurite outgrowths were consistent with early gene expression results for Pax6, a neuroectoderm marker, that was increased in noggin samples and decreased in BMP4 samples (Figure A.3, Figure A.5B). The stark differences in EB and cell morphology after 10 days of differentiation suggested that MP-
based growth factor delivery impacted the trajectory of ES cell differentiation from an earlier period of time.

Figure A.4. Comparable morphologies of spheroids with growth factor-laden MPs and soluble treatment. A) Spheroids treated with BMP-4 and Noggin solubly or via microparticles were formalin fixed on day 4 of suspension culture and stained with hematoxylin and eosin. No differences are seen at the early time point between treatment groups. Scale bar: 100 µm. B) EBs treated with noggin possessed numerous cavitations (denoted by black full arrows), visible in histological analysis of day 7 H&E stained sections. EBs treated with BMP4 displayed areas of mesenchymal-like phenotypes, with a lower cell density organization compared to the non-cavitated regions of noggin treated EBs. No significant differences in morphology were observed between soluble or MP-based delivery. Scale bar = 100 µm.
Figure A.5. Morphological differences of aggregates at later time points of differentiation after differentiation cues are removed or spent. A) Morphological changes in day 10, suspension-culture EBs were observed. BMP4 treated EBs contained translucent, dome-like outgrowths (denoted by white arrowheads), absent in the other groups. In contrast, thin, cellular protrusions were observed in noggin treated EBs. MPs are indicated with black arrowheads. Scale bar = 500 µm. B) Plated aggregates demonstrated markedly different morphologies corresponding to morphogens treatment. In spheroids with BMP4 delivered either solubly or by microparticles, ring-like structures were commonly observed. In addition, neurite outgrowths were common in Noggin and untreated spheroids, but were absent in BMP4 treated spheroids.
Mesoderm induction

ESCs genetically engineered to express green fluorescent protein (GFP) under the control of the Brachyury-T promoter have been previously used to investigate hemogenic mesoderm specification during EB differentiation [39,230]. The Brachyury T-GFP reporter ES cells serve as a convenient tool to observe the spatial patterns of differentiation in response to a growth factor such as BMP4, that is known to promote early mesoderm lineage commitment in both human and mouse pluripotent cell lines [39,193,232–234]. BMP4 was delivered to Brachyury-T GFP EBs through MP-based delivery and soluble delivery, and GFP expression was analyzed by flow cytometry (Figure A.8A,B). Detectable numbers of GFP+ cells were first observed on day 4 of differentiation and reached a maximum level after 5 days, after which the % of GFP+ cells declined (Figure A.7). A low percentage of GFP+ cells were detected in untreated EBs (9.6±0.3%) after 5 days, as well as EBs with unloaded heparin-gelatin (6.9±1.0%) or gelatin particles (7.7±0.7%) (Figure 8D). Soluble addition of BMP4 resulted in the greatest number of GFP+ cells (35.9±1.0%) at day 5 of differentiation. EBs with BMP4-loaded heparin-gelatin MPs had significantly higher Brachyury-T GFP+ cells (20.5±2.5%) compared to untreated and unloaded MP spheroids, but not compared to spheroids containing gelatin MPs loaded with BMP4. Interestingly, the percentage of GFP+ cells was significantly lower when BMP4 was added to the culture medium of EBs containing unloaded heparin-gelatin MPs compared to soluble BMP4 added to untreated EBs or EBs with unmodified gelatin MPs, suggesting a possible interaction of the heparin-modified particles with exogenously added growth factor. The percentage of GFP+ cells within BMP4-loaded gelatin MP aggregates (16.4±5.3%) was the most
variable, with no significant difference in the number of GFP+ cells compared to untreated and unloaded MP spheroids, as well as EBs with BMP4-loaded heparin-gelatin MPs.

The same set of experimental conditions were also examined after 5 days of differentiation by confocal microscopy to visualize patterns of GFP expressing cells to determine the spatial effects of soluble versus MP-based delivery of growth factors (Figure A.8C). Few or no GFP expressing cells were observed in untreated EBs or in EBs containing unloaded gelatin or heparin gelatin MPs. When soluble BMP4 (10 ng/ml) was added to the culture medium, GFP+ cells were observed throughout EBs. Similarly, when BMP4 was added solubly to EBs containing unloaded MPs, GFP expression was again detected throughout EBs. On the other hand, incorporation of BMP4-laden MPs resulted in increased GFP expression in the vicinity of the observed MPs, with heparin-gelatin MPs having a more pronounced effect than simply gelatin MPs. Overall, the relative abundance of GFP+ cells in different EB experimental groups was consistent with the flow cytometry data. These results demonstrate that MP-based delivery of morphogens can induce differentiation in a more localized manner within multicellular aggregates compared to conventional soluble delivery of the same molecules.
Figure A.6. Flow cytometry experimental gates. The flow signals from MPs were removed prior to cellular flow analysis by gating on the cell population that encompasses a very low percentage of both heparan and gelatin MPs (A-C). Brachyury-GFP of all experimental groups in figure 3 and supplementary figure 4 was determined based on gating undifferentiated Brachyury-GFP cells as seen in D.

Figure A.7. Brachyury-T GFP expression reaches maximum at day 5 of differentiation. The percentage of Brachyury-GFP cells within spheroids with soluble BMP4 delivery was measured over the first eight days of differentiation to determine GFP expression kinetics. After 5 days of differentiation, GFP expression reached a peak of approximately 40% positive cells after which expression decreased to below 22% positive cells.
**Figure A.8.** MP delivery of BMP4 induces efficient mesoderm differentiation. A) Brachyury-T driven GFP expression was compared between MP and soluble delivery of BMP4 to EBs. MPs laden with BMP4 were added during formation, whereas soluble BMP4 was supplemented up to day 5. B) Few GFP+ cells were observed in untreated spheroids or in spheroids with unloaded MPs. Compared to unmodified MPs, heparin modification of gelatin MPs resulted in increased numbers of GFP+ cells surrounding the incorporated particles. C,D) Flow cytometry for GFP+ cells was performed on dissociated aggregates on day 5. Spheroids treated with soluble BMP4 had the highest percentage of GFP+ cells (27.6 ± 1.1%), however BMP loaded MP groups had greater Brachyury-T GFP+ cells (20.5 ± 2.5% for Hep MP and 16.4 ± 5.3% for Gel MP) than the untreated and unloaded MP control groups (9.6 ± 0.3%). p < 0.05 denotes significance. Scale bar = 100 mm.
Aggregate co-culture for localized mesoderm induction

Having established that incorporation of growth factor-laden MPs within stem cell spheroids was capable of inducing efficient localized differentiation, we investigated the potential of using MP incorporation within single aggregates to spatially control differentiation within merged spheroids. This study was motivated by previous observations that MPs incorporated within opposite hemispheres of ESC aggregates maintained spatial separation throughout several days of differentiation [43]. We previously demonstrated that magnetic control of multicellular aggregate merging can be achieved to spatially pattern complex 3D structures; however, this method required tedious merging of single spheroids one-at-a-time. By simply adding a second spheroid population to microwells containing a pre-formed population of ESC aggregates after 24 hours (Figure A.9Ai), we observed that the two populations of EBs would readily merge after an additional day of culture (Figure A.9Aii,iii). The second spheroid population was added at half the number of the first population (preformed in the microwells) to decrease the probability of multiple merging events in single microwells. This method allowed for the creation of single aggregates containing MPs localized within one hemisphere of the newly merged spheroid (Figure A.9B). EBs merged in the absence (Figure A.9C) or presence of BMP4 (Figure A.9D) resulted in similar GFP expression patterns to the respective single EB studies described above. When unloaded heparin-gelatin MPs were incorporated on one hemisphere and BMP4 was added to the culture medium, Brachyury-T GFP+ cells were observed throughout the aggregate, although the density of GFP+ cells appeared greater in the hemisphere containing the MPs (Figure A.9E). However, when BMP4 was delivered only from heparin-gelatin incorporated
MPs, GFP+ cells were located predominantly within the hemisphere containing the originally incorporated MPs (Figure A.9F), suggesting the ability of morphogen-laden MPs to spatially direct differentiation within specific regions of multicellular stem cell aggregates.
Figure A.9. Spheroid merging enables local control of mesoderm induction. A) One day after aggregate formation, a second spheroid population was added containing fluorescently labeled MPs. After an additional day of culture, spheroids had merged in the microwells (arrows). B) A merged spheroid in suspension fabricated from an untreated spheroid and a spheroid containing optically dense MPs. C) The merging of untreated spheroids resulted in few dispersed GFP+ cells, whereas D) addition of soluble BMP4 promoted GFP+ cells throughout merged aggregates. E) Soluble BMP4 addition to spheroids containing unloaded heparin-gelatin MPs resulted in some GFP localization mostly within the hemisphere containing the MPs. F) The merging of untreated aggregates with aggregates containing BMP4 loaded heparin-gelatin MPs yielded GFP+ cells localized predominantly within the hemisphere of the spheroid containing the MPs. Scale bars = 100 mm (A,B), 200 mm (C,F).
Discussion

In this study, we demonstrated that MP-mediated delivery can be used as an efficient alternative to soluble addition of growth factors for directed differentiation of pluripotent stem cell aggregates. The morphology of aggregates containing BMP4- or noggin-loaded MPs was markedly different between treatment groups after 7 days of EB differentiation and differences persisted throughout 10 days of culture, suggesting that the differentiation trajectory of ESCs can be controlled by early presentation of morphogens using MPs. Compared to soluble treatments, MP delivery of BMP4 and noggin within EBs resulted in similar induction of mesoderm and ectoderm gene expression, respectively, without significantly affecting loss of pluripotency, as denoted by decreased Oct4 expression. Brachyury-T gene expression was increased in EBs treated with soluble BMP4 or MPs loaded with BMP4 and the percentage of Brachyury-T-GFP positive cells was similarly increased by either method. In contrast to conventional soluble delivery methods, MP delivery concentrates the amount of delivered growth factor within the volume of the multicellular aggregates such that even over short periods of growth factor treatment (i.e. 5 days), an order of magnitude less total growth factor is needed to elicit a comparable phenotypic response. Therefore, MP-based growth factor delivery approaches are a particularly attractive alternative for more efficient scale-up production of differentiated cells from ESCs in suspension culture.

Interestingly, when BMP4 was delivered from gelatin MPs conjugated with heparin, Brachyury-T induction was localized near the MPs. This observation indicated that alterations in the affinity of the material to the delivered growth factor can result in changes to the microenvironment surrounding the MPs. For instance, our previous study
on the release of BMP4 from MPs fabricated from gelatin types A and B confirmed the important role of the polyion complexation between the growth factor and the MP base materials[39]. BMP4, with an isoelectric point of approximately 9.0, carries a net positive charge at neutral pH and was almost entirely released from similar positively-charged, gelatin type A particles after just 6 hours. Heparin modification of gelatin type A particles resulted in release kinetics mirroring those of negatively charged type B particles with most release occurring over a 4 day period and leaving residual growth factor associated with the MPs. Growth factor-MP affinity is likely responsible for stable localization of growth factor and subsequent mesoderm induction of the cells surrounding the MPs. Based on the ability of heparin to sequester exogenously delivered BMP4 [93,95], the low percentage of GFP+ cells in spheroids with unloaded heparin-gelatin MPs suggests a lack of endogenous BMP4 activity within EBs during the first several days of differentiation. Along the same line of thought, biomaterials with engineered affinity to molecules secreted endogenously during differentiation may present an opportunity to sequester and harness autocrine and paracrine signaling within three-dimensional aggregates for directed differentiation without the use of exogenous factors. Such an approach can target a specific molecule, such as vascular endothelial growth factor [235], or a group of growth factors with similar affinity, such as heparin-binding growth factors, for example [205,236]. Thus, engineering of MP properties and desired molecular affinities may be aided by studies of endogenous extracellular matrix production and growth factor expression profiles by ESC cells undergoing differentiation [214].
We hypothesized that BMP4 delivered either by incorporation of loaded heparin-gelatin MPs or delivered solubly to spheroids with unloaded heparin-gelatin MPs may be sequestered by the MPs and locally concentrated. Sequestration of BMP4 by heparin-modified MPs, would, at least partially, account for the reduced frequency of Brachyury-T GFP+ cells using MP-based delivery compared to soluble delivery alone. This hypothesis prompted investigation of pairing morphogen and material interactions to spatially control morphogen delivery within ES cell aggregates. When BMP4-laden, heparin-gelatin MPs were localized to one hemisphere of a single EB, mesoderm differentiation, visualized by Brachyury-T GFP expression, was localized to the same hemisphere. The ability to spatially control delivered morphogens via MP-mediated delivery of growth factors addresses an inherent limitation and unavoidable shortcoming of traditional soluble treatment methods. The process of mammalian development is heavily dependent on spatially and temporally controlled presentation of morphogenic factors, and while EBs are frequently used to study early developmental events, such as gastrulation, primitive streak, and epithelial-mesenchymal transitions [7,237,238], study of more complicated phenomena, including tissue morphogenesis, requires finer spatiotemporal control of morphogen presentation, as demonstrated here. Overall, flexibility in the properties of MP-base materials permits control over the temporal release profile as well as the amount of growth factor delivered, two parameters, which are difficult to control in classical mammalian developmental models.
REFERENCES


