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Systematic engineering of 3D pluripotent stem cell niches to guide blood development

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Abstract

Pluripotent stem cells (PSC) provide insight into development and may underpin new cell therapies, yet controlling PSC differentiation to generate functional cells remains a significant challenge. In this study we explored the concept that mimicking the local in vivo microenvironment during mesoderm specification could promote the emergence of hematopoietic progenitor cells from embryonic stem cells (ESCs). First, we assessed the expression of early phenotypic markers of mesoderm differentiation (E-cadherin, brachyury (T-GFP), PDGFR α , and Flk1: +/-ETPF) to reveal that E-T+P+F+ cells have the highest capacity for hematopoiesis. Second, we determined how initial aggregate size influences the emergence of mesodermal phenotypes (E-T+P+F+, E-T-P+/-F+, and E-T-P+F-) and discovered that colony forming cell (CFC) output was maximal with ~100 cells per PSC aggregate. Finally, we introduced these 100-cell PSC aggregates into a low oxygen environment (5%; to upregulate endogenous VEGF secretion) and delivered two potent blood-inductive molecules, BMP4 and TPO (bone morphogenetic protein-4 and thrombopoietin), locally from microparticles to obtain a more robust differentiation response than soluble delivery methods alone. Approximately 1.7-fold more CFCs were generated with localized delivery in comparison to exogenous delivery, while combined growth factor use was reduced ~14.2-fold. By systematically engineering the complex and

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dynamic environmental signals associated with the in vivo blood developmental niche we demonstrate a significant role for inductive endogenous signaling and introduce a tunable platform for enhancing PSC differentiation efficiency to specific lineages.

Keywords

Stem cells; Blood progenitor cell; Gelatin; Heparin; Microparticles

1. Introduction

Many developing cell therapies and tissue engineering approaches seek to mimic aspects of development to produce therapeutic cells or promote healing within specific microenvironmental contexts. Pluripotent stem cells such as embryonic stem cells (ESCs) are a useful resource for elucidating mechanisms of development and offer tremendous potential for regenerative cell therapies. Although progress has been made in generating many cell types from PSC, the challenge to develop appropriate and scalable inductive processes for targeted cell generation still remains. Differentiation of pluripotent stem cells is commonly induced as 3D cell aggregates, termed embryoid bodies (EBs); a multicellular complex capable of recapitulating various morphogenetic cues from gastrulation and responding to exogenous factors relevant to lineage specification. EBs reproduce many of the temporal and spatial relationships found during normal embryogenesis [1], however, they lack critical developmental factors including biomechanical regulators [2], paracrine signals and the cellular migration that occurs within the murine yolk sac, embryo proper, and placenta [3,4]. Herein we explore the prospective engineering of mesoderm and blood development inductive signals into differentiating aggregates of pluripotent cells, specifically focusing on environmental control of endogenous signaling and the local delivery of exogenous signaling factors.

During mouse gastrulation, morphogenetic movements coupled with cell proliferation and differentiation convert an embryo from two layers (primitive ectoderm and primitive endoderm) to a tri-layered structure [5]. The epiblast cells (embryonic ectoderm) undergo an epithelial-to-mesenchymal transition (EMT), mobilize and migrate through a transient structure called the primitive streak. The primitive streak contains nascent mesoderm that transiently expresses the T-box transcription factor, Brachyury (T) and acts as a specific site of cell ingression, as the three definitive germ layers, endoderm, mesoderm, and ectoderm form [6]. Undifferentiated ESCs express epithelial-cadherin (E-cad) which mediates initial EB formation, and is an indicator of pluripotency that is downregulated during differentiation and EMT events [7–9]. To measure the influence of diverse niche factors on mesodermal differentiation we first set out to determine an early mesoderm/CFC predictive phenotype associated with the primitive streak that could serve to accelerate our niche screening efforts. We investigated the phenotypes generated by combining E-cad staining with the pan mesodermal marker brachyury (T) [10,11], and two receptor tyrosine kinases: platelet derived growth factor receptor- α (PDGFR α) and vascular endothelial growth factor (VEGF) receptor-2 (Flk1), that are expressed by early mesodermal cell types [12] and have been associated with axial, paraxial and lateral plate mesoderm.

We hypothesized that greater spatiotemporal control may allow quantitative contribution of normally convoluted niche parameters and provide insight into how to improve differentiation to desired lineages. The physical size of EBs has been reported to influence the proportion of cells differentiating toward specific lineages [13,14] and impacts diffusion of soluble molecules [15]. We took advantage of recent advances to control EB size through forced centrifugation in micro-pyramidal wells [16] to influence endogenous interactions with the microenvironment. Using a range of mouse ESC aggregate sizes we assessed the predictive value of the identified mesoderm phenotypes (based on E-cad, T-GFP, PDGFR α , Flk1 expression: +/-ETPF) with respect to blood progenitor (CFC) output. We replaced exogenous soluble factors with local delivery within the cell aggregate from microscale biomaterials to mimic factors normally delivered in a more systemic fashion [17,18]. This approach has been used in hESC and mESC aggregates to control the release of small molecules and proteins within the local 3D microenvironment [19–22]. These systems employed a variety of biomaterials, including poly (lactide-co-glycolide) (PLGA) or gelatin microparticles (MPs) capable of sustained release of molecules in a bioactive form. However, none of the previous systems used small aggregates of ESCs (more closely mimicking the developing embryo) or specifically induced cells towards the hematopoietic lineage.

2. Materials and methods

2.1. Cell culture

Brachyury-GFP cells (E14.1, 129/Ola) [23] were maintained on 0.5% gelatin coated flasks in a humidified 5% CO₂ atmosphere, using the modified serum-free maintenance media and base differentiation media previously described [24]. Differentiation was initiated with 5 ng/mL BMP4, 25 ng/mL VEGF (Sigma–Aldrich, St.Louis, MO), and 50 ng/mL TPO (R&D Systems, Inc. Minneapolis, MN) in normoxia (20% O₂) from d0-2/4; only BMP4 and TPO were used in hypoxia (5% O₂).

2.2. Size controlled aggregation

Full or partial microwell inserts [16] were attached to 6- or 24-well plates using polydimethylsiloxane, and allowed to cure overnight at 37 °C. Plates were sterilized with UV and 70% ethanol prior to coating with 5% (w/v) Pluronic F-127 (Sigma). Wells were washed twice with PBS, and allowed to stand in media for a minimum of 30 min at 37 °C prior to seeding. Full-well inserts (6-well plate) were seeded with a single cell suspension in differentiation medium (3.0 mL) at the desired number of cells/microwell and centrifuged for 5 min at 200 g. Partial-well inserts were similarly seeded, however cells were suspended in 4–6 mL DMEM (Invitrogen) to minimize the effect of the uneven surface area of the well bottom. Cells not captured within the micropatterned square-pyramidal wells were carefully removed by aspiration and 3.0 mL of growth media was added. 200 × 200 μ m wells were used to aggregate 1–20 cells (24,000 agg/6-w insert), and 400 × 400 μ m wells for 50–200 cells (6,000 agg/6-w; 1,200 agg/24-w).

2.3. Encapsulation process

To encapsulate 100 cell aggregates, 0.12×10^6 cells were seeded/well in a 24-well plate in a final volume of 1–1.5 mL differentiation media. Approximately 40 h later, for each test condition triplicate wells were collected and settled to 100 μ L. Encapsulation and washing was completed as previously described [24], except a lower vortex speed (7.25, Vortex-Genie2[®], Scientific Industries Inc. Bohemia, NY) and centrifuge setting (350 g) was used. Aggregates were counted pre-and post-encapsulation in a gridded 35 mm petri dish or 24-well plate. Following encapsulation, the sBVT condition was split into one culture without factors and another with sBVT in normoxia or sBT in 5% O₂ for a further two days (d2-4) as indicated. Aggregates with MPs were split between normoxia (with 25 ng/mL VEGF d2-4) and 5% O₂.

2.4. Fluorescent automated cell sorting (FACS)

Flow cytometric analysis expression and cell sorting was carried out as described [25]. Staining of more than 5 samples was completed in a V-bottom 96 well plate with 20 μ L of sample or control cells. Primary antibodies, E-cadherin (R&D) and PDGFR α -biotin (eBioscience Inc. San Diego, CA) were added at 1:100 for 25 min on ice before washing twice with HF (2% FBS in Hank's Buffered Salt Solution). Secondary or conjugated antibodies (BD, Franklin Lakes, NJ) were added at 1:200 for 35 min on ice: goat anti-mouse PECy7 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Stv-APC-Cy7, Flk1-APC or isotype rat IgG2 before washing twice and resuspending in 1 μ g/mL 7-amino-actinomycin D (7AAD, Molecular Probes[®] Invitrogen). Occasionally, PDGFR α -APC, Flk1-PE and their corresponding isotypes were used. Cells were analyzed on a BD FACSCanto (Firmware version 1.14), using BD FACSDiva software (Version 5.0.1) with positive staining defined as fluorescence emission > 99.1% of negative control cells from the same starting population or undifferentiated cells. Cells were sorted on a BD FACSaria and collected in IMDM supplemented with 10% serum, washed and resuspended in serum-free medium without growth factors.

2.5. Hematopoietic cell assays

EBs were dissociated with 0.25% trypsin-EDTA (3 min, 37 °C, Sigma) before seeding the myeloid-erythroid colony forming cell assay (ME-CFC) at 100,000 c/mL in 35 mm duplicate plates (M3434, Stem Cell Technologies, Vancouver, BC) or at 60–100,000 c/mL in 24-well plates (0.5 mL duplicate wells). Previously sorted cells were also seeded in 24-well plates, but at variable densities below 20,000 c/mL (2–3 wells with 0.3 mL). Colonies were enumerated 7–10 days after seeding as previously detailed [26].

2.6. Manufacturing and loading gelatin microparticles

Microparticles of type A (G1890) or B (G9391, Sigma) gelatin were generated using a water-in-oil emulsion method and fluorescently labeled as previously described [27]. Expected electrostatic interactions between the gelatin types and proteins were examined using ExPASy's Compute pI/MW program (www.phosphosite.org/psrSearchAction.do) [28,29]. Heparin sodium salt (CalBiochem, San Diego, CA) was conjugated to gelatin type A MP 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 μ M ratios respectively, relative to heparin dissolved in 800 μ L activation buffer (0.1 M MES, 0.5 M NaCl, pH 6.0) and reacted for 15 min at room temperature to modify the carboxyl groups of heparin to amine reactive S-NHS esters. The EDC/NHS reaction was quenched with 20mM 2-mercaptoethanol and the activated heparin was added to 400 μ L of particles in PBS at a 5:1 M ratio of heparin to gelatin and allowed to react with agitation for 4 h at 37 °C. Prior to cell culture, MPs were treated in 70% ethanol for a minimum of 30 min before washing 3 $\times$ with ddH₂O. Each MP batch was lyophilized and stored at -20 °C. Growth factors were added to MPs at 5–5.5 μ L/mg and kept overnight at 4 °C, MPs were resuspended in differentiation media (500 μ L) and counted on a hemocytometer to estimate the concentration (ng/MP). For use with cells, BMP-MPs were loaded using 10–50 μ g/mL stock solutions, and TPO-MPs were loaded with a 50 μ g/mL stock.

2.7. Generating mixed aggregates: microparticles and cells

A single cell suspension was generated from undifferentiated T-GFP ESC and centrifuged into the microwells as described above; 200 g for 5 min. Resuspended microparticles were added to the wells to obtain the desired factor concentration before centrifuging a second time at 200 g for 5 min. This method of aggregate and MP formation has also been achieved with D3 ESC [27]. Aggregates were maintained with full media exchange in the microwells (daily or daily starting at 48 h) prior to removal of soluble growth factors and transfer to Petri dishes on day 4. Alternatively, aggregates were removed 38–42 h after formation for encapsulation in agarose and/or dilution into 60–100 mm Petri dishes at approximately 300 aggregates/mL.

2.8. Human BMP4 ELISA

A quantitative sandwich enzyme immunoassay technique was used to determine BMP4 concentration after release from MPs suspended in PBS, based on the manufacturer's protocol (R & D, DBP400). Briefly, 2–5 mg of MPs were suspended in 750 μ L of a 0.1% solution of BSA in PBS at 37 °C with rotation; at each sample time point 300 μ L was removed following centrifugation of the microparticles and replaced with an equivalent volume.

2.9. Statistical analysis

Unless indicated, data are reported as mean $\pm$ standard deviation of the mean. Statistical significance was assessed using one-way ANOVA with Tukey's post hoc analysis, student's *t*-test, or nonparametric Mann–Whitney test (Minitab 15/16, State College PA and OriginPro7.5 Northampton, MA). *P*-values of less than 0.05 were considered significant (*n* 3).

3. Results

3.1. Cell population phenotypes

We previously demonstrated that in serum-free conditions the addition of a trio of mesoderm inducing cytokines, BMP4, VEGF, and TPO (BVT) resulted in an induction of myeloid-erythroid colony forming cells (ME-CFC) [30]. In order to quantitatively measure the

impact of our niche engineering efforts on hemogenic mesoderm generation we sought to develop a set of predictive phenotypic markers. Multiple cell lines respond to this differentiation strategy, however, to trace the dynamic process of mesodermal specification in greater detail we employed the Brachyury (T)-GFP line [23]. We postulated that the dynamic upregulation of brachyury and downregulation of E-cadherin, that appear to signal the upregulation of two mesodermal receptors (Flk1 and PDGFR α), could be used in combination to identify the putative hemogenic population for tracking purposes.

Monitoring the expression of E-cadherin, brachyury, PDGFR α , and Flk1 during differentiation distinguishes 16 possible phenotypes (Fig. 1A). Once differentiation was initiated with BVT, E-cadherin expressing cells (E+T-P-F-) progressively downregulated that adhesive molecule while brachyury and both surface receptors were upregulated (Fig. 1B). The presence of either one or both of the tracked receptors in the absence of brachyury was only observed after the initial peak of E-T+P+F+/- cells and may correspond to more differentiated cells (day 5, Fig. 1B). Due to the rarity of many of the phenotypic populations it is likely that they represent transient expression states during lineage specification.

We grouped the expression patterns into populations that could broadly be classified as having mesendoderm (ME), mesoderm (M), endoderm (E), or unknown potential, and differences in their gene expression profiles demonstrate this (Suppl. Fig. 1). We sorted the most abundant day 3.75 phenotypes associated with hemogenic mesoderm and assessed their colony forming capacity after 3 more days of suspension culture (the standard time to assess CFC). We found that the E-T+P+F+ population had the greatest hemogenic capacity, and was significantly enriched compared to the unsorted population and all other fractions (Fig. 1C). The total number of colonies generated from the unsorted population was equivalent to the sum produced by the individual sorted fractions once the initial frequency of these phenotypes was taken into account. This analysis defined the starting population necessary to further track and optimize parameters of hemogenic mesoderm differentiation.

3.2. Aggregate size and mesodermal phenotype

Endogenous signals can impact differentiation [31] and it has been established with both 2- and 3-D systems [32,33] that the number of neighboring cells impacts autocrine and paracrine factors within the immediate media surrounding the cells [34,35]. Thus, we examined how the initial number of cells per aggregate influenced mesoderm differentiation due to the interplay of endogenous stimulatory or inhibitory signals and exogenous factors (Fig. 2A) utilizing a centrifugal forced-aggregation strategy [16] and assessing the resultant phenotypes and functional cell types. Total cell density was controlled by seeding different cell numbers into 200 or 400 micron square-pyramidal well inserts that covered an eighth-, quarter-, half-, or full-well within 6-well plates (Fig. 2B) to normalize the levels of nutrients and growth factors in the bulk media. The conditioning effect that occurs with larger cell aggregates during microwell differentiation was demonstrated by exchanging media between 10- and 100-cell aggregates. Media conditioned for two days by 100-cell aggregates boosted the CFC output of the smaller aggregates while no striking effect was observed with the reverse media exchange (Suppl. Fig. 2).

Mesoderm phenotypes associated with CFC potential were modestly enhanced with increasing aggregate size (Fig. 2C). To insure that similar differentiation kinetics occurred across the different aggregate sizes we evaluated the CFC output of day 7–9 EBs (Suppl. Fig. 3); the greatest output occurred on day 7 for each size (Fig. 2D). The hemogenic capacity of aggregates mirrored the trends of the combined phenotypic response and was maximal with 100-cell aggregates (1 in 145 ± 8 d7 cells). Uniformly sized aggregates initially seeded at 50–200 cells produced significantly more CFC ($p < 0.05$) than non-uniform LSC aggregates. Without inductive factors (BVT), spontaneous differentiation accounts for less than 0.5% of the CFC produced (data not shown). Thus, the addition of soluble factors is necessary to initiate hemogenic induction within the 100-cell aggregates. Based on the results of these studies, aggregate size was fixed at 100 cells for further investigations.

3.3. Microparticle growth factor delivery

Pluripotent populations demonstrate heterogeneity and plasticity in vitro and these properties can be modulated by extrinsic signalling [36], thus we sought to provide exogenous factors locally using growth factor delivery vehicles incorporated within the cell aggregates. Local delivery within multicellular aggregates may enhance the efficiency of differentiation by increasing the effective growth factor concentration and limiting the formation of gradients. We first confirmed that the physical incorporation of gelatin microparticles (MPs) within the extracellular space of 100 cell aggregates (Fig. 3Ai) did not adversely influence aggregate formation, brachyury expression, or CD45 output (Suppl. Fig. 4A,B). By varying the seed ratio of MP to cells, we noted that 1 MP to 3 or more cells produced stable aggregates with incorporated particles distributed throughout the volume of the 3D spheroids (Fig. 3B, Suppl. Fig. 4C).

The electrostatic affinity between charged biomolecules and gelatin species has been the impetus behind its use as a matrix for the controlled-release of bioactive molecules (reviewed in [37]). The characteristic isoelectric point (pI) of gelatin depends on the manufacturing conditions and it is expected that protein retention would be enhanced in gelatin of the opposite charge (Fig. 3Aii). We tested the bioactivity of growth factor release from gelatin A or B MP with 100-cell aggregates by seeding different amounts of BMP4-loaded MPs (B-MP, Fig. 3B). To examine the inductive effect and release kinetics of BMP4, cells were grown in the wells until T-GFP was measured on day 4.5 with media exchange (ME) occurring daily or daily after an initial 48 h of culture (d2ME). Brachyury expressing cells reached a plateau of ~70% with 2–15 ng/mL soluble BMP4 (sB) treatment in the presence of either gelatin A or B unloaded MPs (U-MP, Fig. 3B). Brachyury induction from both type A and B MPs was equivalent to soluble delivery if media was left standing for 2 days, however, with daily media exchange T-GFP and hence BMP4 release was significantly reduced ($*p < 0.05$). However, this robust response was only seen if media exchange began 48 h after seeding, suggesting that BMP4 release kinetics were not optimal. Compared to continuous delivery, 24 h soluble BMP4 reduced the percent of brachyury positive cells by ~10% ($**p < 0.05$), and a similar proportion of T-GFP cells was observed with gelatin A MPs (Fig. 3B). BMP4 release from type A gelatin induced similar levels of T-GFP as 24 h delivery of soluble BMP4, in contrast, type B gelatin was unable to induce

Brachyury with daily media exchange, and T-GFP expression was significantly lower compared to both type A MPs with daily exchange and 24 h ($\dagger p < 0.05$, Fig. 3B), suggesting that not enough BMP4 was released due to stronger electrostatic interactions with the negatively charged gelatin. These dose responses indicate that MP delivery induced mesoderm differentiation similarly to bulk delivery. We next explored the capacity to increase cellular responses by tuning the release kinetics of our growth factor delivery vehicle through molecular engineering.

3.4. Growth factor release

To further investigate the interaction of BMP4 with gelatin A or B MPs we varied the stock concentration from 5 to 25 $\mu\text{g/mL}$ and determined the characteristic release profiles by ELISA. Very rapid and full release from gelatin A MPs occurred within 24 h when loaded with a 5 $\mu\text{g/mL}$ BMP4 stock solution (Fig. 3C). In contrast, only 35% BMP4 was released over the same time from gelatin B MPs. Neither of these release kinetic profiles would be capable of sustained-release on the order of 4 days and allow the cell aggregates to be transferred to bulk conditions for extended culture. Doubling the stock concentration of BMP4 doubled its release from gelatin B, however, using a 25 $\mu\text{g/mL}$ stock resulted in a more variable and intermediate extent of release ($\sim 50\%$) (Fig. 3C). The heparin binding domain of BMP4 has recently been identified [38], thus, we conjugated heparin to type A gelatin MPs to take advantage of the total release by enhancing the loading capacity via high affinity binding sites. We found that 40–60% less BMP4 was detected in solution with heparinized type A gelatin MPs than untreated gelatin A MPs and this was on the same order of release as gelatin B MPs (Fig. 3C). We hypothesized that using the heparinized type A MPs loaded with BMP4 from stocks $\sim 10 \mu\text{g/mL}$ would enhance the overall delivery and bioactivity of the heparin-bound presentation of the morphogenic factor.

We successfully replaced soluble BMP4 with local delivery or presentation of affinity-bound BMP4 using the heparinized gelatin A MPs in combination with sVT. The MPs were seeded with 100 cells to form aggregates that were transferred to bulk suspension culture on day two (Fig. 3D). We next sought to integrate local factor delivery with other cell inductive parameters in a manner that would lead to robust blood cell development.

3.5. Low oxygen environment with dual growth factor delivery

We and others have shown that low oxygen tension (5% O_2) is beneficial in generating hemogenic mesoderm [39,40] via a mechanism that dynamically tunes VEGF signaling. We combined 5% oxygen tension with local factor delivery to enhance blood development in the absence of exogenous VEGF. Gelatin microparticles were incorporated in 100-cell aggregates to support mesoderm development either loaded with BMP4 or TPO (previously shown to enhance CFC production), or as unloaded controls (Fig. 4Ai). Cells were allowed to form stable aggregates for 32–42 h before they were removed from the microwells and encapsulated in agarose to allow our engineered niche to be incorporated into suspension culture processes (Fig. 4Aii). Brachyury induction was apparent after 3 days (Fig. 4Aiii) and in addition to the soluble growth factor controls (BMP4, TPO), 25 ng/mL VEGF was provided for 4 days in normoxia (20% O_2) in order to match the CFC induction due to VEGF upregulation in 5% O_2 .

Although thrombopoietin (pI 9.4) has a slightly higher pI than BMP4, we hypothesized that it would interact with the heparinized gelatin MPs in a similar fashion. We found that increasing the dose of BMP4 delivered by the MPs, while holding the dose of TPO relatively constant (range 9–15 ng), yielded an increasing number of CFCs (Fig. 4B). The cells were split between normoxic and hypoxic conditions following encapsulation and similar trends were observed in both the phenotypic induction and CFC output in response to the B-MP and TPO-MP delivery regardless of oxygen tension, indicating that cells in low oxygen effectively upregulated endogenous VEGF to replace the exogenous delivery in normoxia.

In normoxic conditions 2–5 ng/well B-MP with TPO-MP delivery generated an increasing trend in CFC output but did not significantly differ from the soluble BVT (d0-2) control. Providing 8 ng/well B-MP provided a significant improvement over two days of soluble factors and was similar to the sBVT (d0-4) control. The highest CFC output occurred with 12 ng/well of B-MP delivery. This indicates that depending on the dose provided the factors were active following encapsulation (i.e. 8 ng, d0-4 and 12 ng, > 4 days). The early mesodermal phenotypes observed at day 4 (E–T+P+F+ and E–T–P+/–F+) correlated to the CFC producing cells present at day 7 (Fig. 4C). Representative erythroid, myeloid and mixed colonies are shown from the 5 ng/well B-MP normoxic condition (Fig. 4D). Altering the presentation of the growth factors, either from MP delivery or 5% O₂ induction, did not significantly alter the proportion of colony types produced by the majority of the different treatment conditions (Suppl. Fig. 5A–C; Mann–Whitney test).

Seeding 8 or 12 ng BMP4 and ~12 ng TPO within the spatial context of 100-cell aggregates (1,200 total) appears to induce comparable, if not greater, mesoderm differentiation than 5 ng/mL exogenous BMP4 and 50 ng/mL TPO. When the total growth factor loaded into the MPs and the additional media required for the soluble control following encapsulation (300 agg/mL) are accounted for, roughly 14× less BMP4 and TPO was required for local MP delivery/presentation than for bulk delivery.

In all, we have demonstrated that tracking the emergence of E–T+P+F+, E–T–P+/–F+, and E–T–P+F– phenotypes can predict the general capacity of a culture to produce CFC when seeded 3 days later, and aid in understanding the various microenvironmental factors capable of impacting CFC production. Aggregate size can be used to enhance CFC with soluble factors and manipulating the microenvironmental niche through controlled aggregation with microparticles that deliver BMP4 and TPO can generate greater numbers of CFCs than soluble delivery (~1.7×) of factors in both low oxygen (sBT) or normoxic (sBVT) environments. These results demonstrate that local delivery by affinity-bound presentation of growth factors from biomaterials can effectively induce mesoderm differentiation.

4. Discussion

Modulating cell–cell interactions and the effects of autocrine, paracrine, and exogenous factors through initial aggregate size, oxygen tension, and local growth factor delivery, has provided insights into directed differentiation by monitoring both cellular phenotypes and

functional responses. We first explored the differential expression of mesodermal cell phenotypes and the functional CFC response to exogenous growth factors in a serum-free media. We used a serum-free culture system that maintains the self-renewal of undifferentiated ESCs [30,41] and the embryoid body system to model blood development as it induces differentiation similar to embryonic gastrulation. In an aim to better define and characterize subsets of nascent mesodermal cells for tracking and culture optimization purposes we monitored E-cadherin, brachyury, PDGFR α and Flk1.

Flk1 positive cells have been associated with hematopoiesis and vasculogenesis [23,42,43], as well as other mesodermal cell types [44–46]. Two-marker cell sorting strategies have demonstrated that P+F[−] cells have substantial muscle regeneration potential [47], while P+F⁺ cells demonstrate enhanced cardiac potential [48,49], and P[−]F⁺ cells retain hematopoietic potential [49]. Monitoring E-cadherin and T-GFP [23] expression provided an indication that mesodermal differentiation was proceeding in our cultures and we combined these markers with PDGFR α and Flk1 expression to delineate potential hematopoietic progenitors. For the first time, we demonstrated that cells co-expressing brachyury, Flk1, and PDGFR α had the highest frequency of CFC formation when they did not express E-cadherin. Presumably the CFC output represents the cell autonomous capacity of the sorted phenotypes, as the cells were allowed to mature for an additional three days without the addition of exogenous growth factors. Furthermore, the close link between the cardiac potential (P+F⁺) and hematopoietic potential (E[−]T+P+F⁺) raises the question whether a bi-potent cell population remains at the time we sorted. Insight from these phenotypes can be used to aid future developments in cell generation processes.

Focusing on parameters that may easily be incorporated into engineered systems to enhance hematopoietic specification, we next investigated the influence of local cell density. Examining the initial developmental stages leading up to the assessment of phenotypic expression, we showed an increasing proportion of hemogenic cells with increasing aggregate size (1–200 cells), with a maximum at 100-cell aggregates (Fig. 2B). Aggregates seeded with 5–200 cells with or without growth factors continued to expand at similar growth rates, such that there were no significant differences in population doublings between the conditions. As the rate of aggregate growth was not strongly influenced by initial cell numbers, controlling both aggregate size and the total aggregates per well assured similar numbers of the bioactive molecules (BMP4, VEGF, or TPO) were available on a per cell basis in the bulk media or macroenvironment.

Taking advantage of the capacity for microenvironmental control from within the aggregate itself we incorporated gelatin MPs as a delivery vehicle to locally present BMP4 [38] and TPO [50], which have known and presumed heparin binding domains and hemogenic induction capacity. Heparin has previously been used with scaffolds to sustain the release/presentation of heparin binding growth factors (HBGF) [51] and its use with particulate systems for controlled delivery of HBGF is an emerging area in tissue engineering [52]. In addition, although the specific heparin binding consensus sequence to various hematopoietic cytokines have not been established, cytokine-immobilization using low molecular weight heparin was comparable for TPO, SCF and Flt-3 (both basic and acidic proteins) and ~32%

remained after 6 days [50], suggesting that heparinized gelatin MPs are able to deliver a variety of morphogenic factors in a similar manner.

Finally, we established a system incorporating heparinized microparticle delivery of two factors, BMP4 and TPO, into 100-cell aggregates, which supports the generation of hematopoietic progenitors equivalently, if not to a greater extent (~1.7-fold more CFCs) than bulk exogenous delivery. Similar cell numbers were generated with or without the provision of growth factors and in the presence or absence of microparticles, thus differences in colony numbers and enhanced CFC output represent better process yields from the initial ESC input. In all, integration of a microparticle approach for bioactive molecule delivery within EBs provides the opportunity to deconvolute the complex biological signals which cells receive during blood development.

In the future, time-lapse imaging studies may be used to investigate the localization of the developing mesoderm with enriched CFC activity or the relation between the hemogenic and non-hemogenic endothelium produced by the mesodermal precursors. Furthermore with the establishment of this system, studies may now be designed to explore asymmetrical signals within the aggregate (by providing hemispherical delivery) and further mimic embryonic development or polar axis definitions. Overall, this approach based on engineered combinations of physical and biochemical signaling serves as a model to guide differentiation of pluripotent stem cells to specific mesoderm phenotypes, such as blood, as well as to quantitatively investigate the contribution of normally convoluted niche parameters on pluripotent cell developmental fate decisions.

5. Conclusion

We have provided a model system that used mesodermal phenotype characterization with a forced-aggregation technique to control aggregate size and to embed MPs to serve as local delivery vehicles. Under serum-free conditions, heparinized MPs incorporated prior to aggregate encapsulation were able to induce differentiation to levels that were similar or exceeded bulk delivery methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at [doi:10.1016/j.biomaterials.2011.10.051](https://doi.org/10.1016/j.biomaterials.2011.10.051).

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Fig. 1. Monitoring mesodermal specification. (A): Four-colour FACS employing a T-GFP cell line sheds light onto pan mesoderm development by tracking the surface expression of E-cadherin, PDGFR α and Flk1. (B): Comparison of undifferentiated cells to cells after 3.75 or 5 days in serum free differentiation media with or without BVT highlights the dynamic progression of these lineage markers and demonstrates that not all phenotypic combinations occur. (C): Populations were sorted singly or in combination as indicated; the average efficiency of colony formation from E-T+P+F+ cells (no. of colonies/no. of seeded cells $\times$

100) was significantly (*) higher than all other sorted fractions ($n = 4$, except E-T-P+/-F+ $n = 2$; ANOVA with Tukey's post hoc analysis $\alpha = 0.05$).

Fig. 2.

Controlling initial cell aggregate size influences mesodermal specification. (A): The overall effect of endogenously produced factors would depend on the balance of stimulatory or inhibitory regulators that are secreted by the mixture of cell types. A higher local cell density would condition the microenvironment with more endogenous factors than lower density conditions, as demonstrated in Suppl. Figure 2. (B): Two sizes of micropatterned square-pyramidal wells and partial coverage allows similar overall cell densities with constant volume to be compared. Initial 10- or 100-cell aggregates are shown in 200 and 400

µm inserts respectively, immediately after spinning down the cells and following four days of growth. (C): The mesodermal phenotypes associated most closely with CFC, are shown as a stacked percentage of expression for aggregates that were initially 1–200 cells or non-uniform aggregates, formed from liquid suspension culture (LSC). (D): The phenotypic expression increased with larger aggregate sizes, however, CFC are maximal with 100 cell aggregates. The average number of CFC $\pm$ standard error of the mean are shown. Means that do not share a letter are significantly different ($n = 6-8$; ANOVA with Tukey's post hoc analysis $\alpha = 0.05$).

Fig. 3.

Gelatin microparticles do not interfere with cell aggregation and exogenous factors can be delivered locally. (Ai): A schematic of the aggregate formation process with BMP4 loaded gelatin MPs. (Aii): Both type A and type B gelatin MPs with characteristic pI were tested within the cell aggregates as it was expected BMP4 would have different inductive capacities depending on the electrostatic interaction between the gelatin and protein. (B): A range of BMP4 concentrations were tested by calculating the theoretical amount of BMP4 contained/MP. Brachyury (T-GFP) was assessed on day 4.5 ($n = 3$; one-way ANOVA with

Tukey's post hoc analysis, $\alpha = 0.05$). Open symbols: daily media exchange. Abbreviations: MP, microparticle; B-MP, BMP4-MP; U-MP, unloaded MP; sB, soluble BMP4; d2ME, daily media exchange from day two. $P < 0.05$ for comparison of: *daily exchange (open diamond) to sB (filled square) or d2ME of type A/B (filled diamond) gelatin respectively; **24 h sB (filled grey circle) to continuous BMP4 (filled square/diamond); †daily exchange type B (open diamond) to both type A MPs with daily exchange and 24 h sBMP4 (filled grey circle). The MP:cell ratio used to deliver 1–20 ng BMP4 are shown below the axis; representative fluorescent images were taken 24 h after seeding. (C): BMP4 was loaded into type A (square), type B (triangle), and heparinized type A (open square) MPs from 5, 10 or 25 $\mu\text{g/mL}$ stock solutions as indicated and its release into PBS was monitored by ELISA ($n = 3$). (D): Delivery of 5 ng/well BMP4 with heparinized MPs was as effective as soluble delivery for 100 cell aggregates ($n = 4$, student's t -test; 1200 aggregates, 24 well plate, 400 μm). (For better interpretation of the fluorescent images in this figure, the reader is referred to the web version of this article.)

Fig. 4.

Combining local growth factor delivery with low oxygen supports mesodermal development. (Ai): Aggregates formed with blue and red microparticles can be removed and (Aii): encapsulated for further culture. (Aiii): The aggregates respond to media nutrients and soluble factors as this control aggregate seeded with unloaded MPs differentiated with BVT and expressed brachyury (T-GFP). The agarose shell is highlighted with a dashed white ring. (B): BMP4 and TPO were delivered locally within 100 cell aggregates using heparinized type A gelatin MPs, while VEGF was provided in solution (d0-2) in normoxia.

Means that do not share a letter are significantly different ($n = 4-8$; one-way ANOVA with Tukey's post hoc analysis $\alpha = 0.05$). ND- not detected. (C): Assessing the phenotypes of the developing mesoderm at d3.75 as described earlier (ETPF), accurately predicts the general trend in CFC output from day seven cells in both 20% O₂ (black bars) and 5% O₂ conditions (grey bars). (D): Representative myeloid and erythroid colonies from dual BMP4 and TPO microparticle delivery are shown. Suppl. Figure 5 shows the distribution of colony types for each condition. All scale bars are 200 μm .