1. Introduction

Mesenchymal stem cells (MSCs) represent a subpopulation of stromal cells derived from bone marrow that can be differentiated into numerous cell lineages. Friedenstein and co-workers initially observed spindle-shaped fibroblast-like cells within bone marrow that were able to form colonies of fibroblasts (the colony-forming unit-fibroblasts, CFU-F), which could be induced into bone cells [1]. These bone marrow stromal cells (BMSCs) were later termed mesenchymal stem cells [2] and since then, MSCs have now been isolated in nearly all tissues and organs in the body [3–5]. Efforts to define the MSC phenotype have focused on the expression of cell-surface markers; for example, Stro-1 [6], CD105/endoglin[7], integrin α1 [8], and nerve growth factor receptor (NGFR) [9], as well as the absence of hematopoietic markers CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules [10]. In vitro, MSCs are often characterized by their adherence to tissue culture plastic, their expression of key markers including CD105/endoglin, CD73, and CD90, and their ability to differentiate into osteoblasts, adipocytes, and chondroblasts [10].

MSCs have been extensively studied as a potential candidate for various cell and gene therapy treatments. They offer many advantages in that they can be induced into multiple cell types [11], they can be acquired relatively easily from autologous sources, and they are easily expandable in vitro. Autologous sources of MSCs capable of differentiating into many mesenchymal phenotypes are of great therapeutic potential for tissue engineering and regenerative medicine where these cells are well studied as a basis for cartilage, bone, neural, and tissue repair [12–14]. However, one limitation to the use of MSCs in clinical settings is their tendency to lose potency for proliferation and differentiation when cultured in vitro. Donor age, plating density, serum composition, as well as passage time have all been implicated in MSC senescence [15–17]. Methods to maintain the long term multipotency of these cells would thereby significantly enhance their function as reservoir cells for clinical use.

Recent evidence suggests MSCs are a subset of perivascular cells [18,19], reside around sinusoids [20], remain in a quiescent state [21], and maintain a niche for hematopoietic stem cells [22–24]. Material platforms to recapitulate this natural niche environment may offer an engineering approach to prolong the in vitro lifespan of MSCs while still maintaining their multipotency. Funaki and colleagues altered matrix mechanics and demonstrated that MSCs on soft 250-Pa gels become quiescent, but can resume proliferation and differentiation with the addition of chemical or mechanical stimuli [25]. Dalby and colleagues reported that MSCs could be promoted to undergo osteogenic differentiation if cultured on disordered nanostructures, while these same cells retained their mesenchymal phenotypes and multipotency when cultured on ordered square nanostructures [26,27]. In both studies,
mechanotransductive events between the cell and its substrate were key to influencing phenotype.

In this paper we show that retention of the mesenchymal stem cell phenotype is promoted by restricting cell spreading using microcontact printing of self-assembled monolayers on gold. Our results demonstrate that when cultured in patterns, MSCs become quiescent and express higher levels of stem cell markers. Even after removal from patterns—either through trypsinization of the cells or desorption of the monolayer chemistry—high levels of these markers persist and these cells retain the ability to differentiate to osteoblasts and adipocytes.

2. Materials and methods

2.1. Materials

Laboratory chemicals and reagents were purchased from Sigma Aldrich unless otherwise noted. Tissue culture plastic ware was purchased from Fisher Scientific. Cell culture media and reagents were purchased from Gibco. Human MSCs and differentiation media were purchased from Lonza and produced by Osiris Therapeutics, Inc. These cells were derived from bone marrow isolated from the iliac crest. MSCs were tested for purity by Lonza, and were positive for CD105, CD166, CD29, and CD44, negative for CD14, CD34, and CD45 by flow cytometry, and had ability to differentiate into osteogenic, chondrogenic, adipogenic lineages (http://www.lonza.com). The use of human MSCs in this work was reviewed and approved by the University of Illinois at Urbana-Champaign Biological Safety Institutional Review Board.

2.2. Surface preparation

Surfaces were fabricated by electron beam evaporation of 5 nm of Ti followed by 20 nm of Au onto cleaned glass coverslips. To create patterned surfaces, poly(dimethylsiloxane) (PDMS, Polysciences, Inc.) stamps were fabricated by polymerization upon a patterned master of photoresist (SU-8, MicroChem) created using UV photolithography through a laser printed mask. Stamps featuring circular patterns of 1000 μm² were used. Stamps were inked with 10 μm octadecanethiol in ethanol, dried under air, and applied to the surface. Surfaces were then incubated overnight with 3 μM tri(ethylene glycol) undecanethiol in ethanol to prevent protein adsorption and cell adhesion to non-patterned regions. Next, 50 μg/ml fibronectin was applied to surface for 1 h at room temperature. For non-patterned surfaces, 50 μg/ml fibronectin was applied to gold coverslips for 1 h. Surfaces were rinsed with PBS and stored in PBS until use.

2.3. Cell source and culture

Human mesenchymal stem cells (MSCs) were thawed from cryopreservation (10% DMSO) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) low glucose (1 g/mL) media supplemented with 10% fetal bovine serum (MSC approved FBS; Invitrogen), 1% penicillin/streptomycin (p/s), media changed every 3 days and passaged at ~80% confluency using Trypsin:EDTA (Gibco). Passage 4–8 MSCs were seeded on patterned and non-patterned surfaces at a cell density ~5000 cells/cm². Patterned surfaces were visually inspected to ensure cells were only localized to small circular islands. Non-patterned surfaces contained cells spread in a random arrangement.

2.4. Cell differentiation

After one week of culture on patterned or non-patterned substrates, the surfaces were gently transferred to new 12-well plates. Cells were trypsinized and reseeded into 24-well plates with basal media (DMEM supplemented with 10% FBS, 1% p/s). After 1 day, cells were either fixed and assessed for markers, or further subjected to basal media, osteogenic media (DMEM supplemented with 10% FBS, 1% p/s), media changed every 3–4 days and passaged at ~80% confluency using Trypsin:EDTA (Gibco). Passage 4–8 MSCs were seeded on patterned and non-patterned surfaces at a cell density ~5000 cells/cm². Patterned surfaces were visually inspected to ensure cells were only localized to small circular islands. Non-patterned surfaces contained cells spread in a random arrangement.

2.5. Characterization of human MSCs

Mesenchymal phenotype was assessed by staining for mesenchymal markers endoglin/CD105 and Strom-1. Cell proliferation was assessed by BrdU staining. Osteogenic and Adipogenic differentiation was assessed via alkaline phosphatase and Oil Red O staining respectively. All imaging was performed on an IN Cell Analyzer 2000 (GE). 20 fields of view were taken for every sample condition. Quantification was performed using ImageJ.

2.6. BrdU staining

Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 30 min, and blocked with 1% BSA for 15 min. Primary antibody labeling was performed in 5% goat serum containing 1% BSA in PBS overnight at 4 °C with rabbit anti-endoglin (Sigma, 1:200 dilution) and mouse anti-Strom-1 (BD Systems, 1:200 dilution). Secondary antibody labeling was performed similarly with Tetramethylrhodamine-conjugated anti-rabbit IgG antibody and Alexa Fluor 647-conjugated anti-mouse IgG antibody (1:200 dilution) along with Alexa Fluor 488-phalloidin (1:200 dilution) and DAPI (1:5000 dilution) for 1 h at room temperature. Immunofluorescent images were analyzed using ImageJ. Regions of interest were selected by outlining actin filaments for non-patterned surfaces, or by manually selecting patterned cells. For the regions of interest, images were thresholded to select positively stained areas and integrated density (representing mean grayscale times feature area) was calculated. DAPI staining was used to count nuclei. Integrated density was totaled for each condition and normalized to cell number to give intensity per cell (arbitrary units). At least two independent experiments each with duplicate samples were performed to verify results. Each condition analyzed represented 100–3000 cells.

3. Results

3.1. Assessment of DNA synthesis in mesenchymal stem cells

We used microcontact printing of alkane thiols on gold as a model system because this platform shows high fidelity of pattern formation and stability under physiological conditions for up to a week [28,29]. After microcontact printing of octadecanethiol and overnight immersion in the tri(ethylene glycol) undecanethiol solution we can visually observe regions of differential wetting characteristics indicative of patterning (data not shown). X-ray Photoelectron Spectroscopy (XPS) analysis of our surfaces demonstrated formation of tri(ethylene glycol) self-assembled monolayers (Fig. S1). Previous studies have demonstrated the reproducible physisorption of matrix protein to the micropatterned octadecanethiol regions using this microcontact printing method [30]. A study by Langer et al. on the stability of undecanethiol monolayers in PBS and serum conditions also found that although desorption was the most likely cause of monolayer failure, this loss was not significant until about 2 weeks in serum conditions [31].
studies which showed pattern loss after about 2 weeks [32]. We seeded our patterned surfaces immediately after fabrication, and noticed no significant change in patterned cell morphology after 6 days in culture.

Recently, we showed how MSCs can be cultured on microislands fabricated with soft lithography for over a week without dividing and that these cells could undergo differentiation when exposed to soluble lineage guidance cues [33]. To determine if micropatterned cells become quiescent, we performed a bromodeoxyuridine incorporation assay. 1000 \( \mu \text{m}^2 \) islands of alkanethiols were microcontact printed onto gold coated coverslips and adsorbed with fibronectin to provide adhesion sites for cells (Fig. 1A). MSCs were cultured in DMEM low glucose media containing a BrdU labeling reagent for both 4 h and 24 h. Even at the extended time point of 24 h, only 0.9% ± 1.3% of cells cultured in patterns were stained positive for BrdU compared to 21.6% ± 3.8% of cells cultured on fibronectin adsorbed gold (Fig. 1B).

3.2. Expression of mesenchymal stem cell markers Stro-1 and endoglin

The results of the BrdU incorporation assay suggest that MSCs are in a quiescent state when confined to microislands. To determine if these patterned cultures show any differences in expression of MSC markers, we fixed and immunostained MSCs for Stro-1 and endoglin after 24 h in patterned and non-patterned culture. On the patterned surfaces only cells that were confined to patterned regions were counted. Cells within patterned regions displayed markedly higher intensities for Stro-1 (Fig. 2A) and endoglin (Fig. 2E) than cells cultured on non-patterned surfaces (Fig. 2B, F). To ensure this higher intensity was not an imaging artifact due to the constrained cell body, after 1 week in culture MSCs were trypsinized from patterned and non-patterned surfaces and reseeded into fresh 24-well tissue culture plates. 1 day after reseeding, subsets of cells that had been previously cultured in patterned and non-patterned surfaces were fixed and stained. MSCs that had previously been confined to 1000 \( \mu \text{m}^2 \) islands displayed higher intensities of Stro-1 (Fig. 2C) and endoglin (Fig. 2G) than MSCs previously cultured on non-patterned surfaces (Fig. 2D, H). 6 days post reseeding, MSCs that had previously been confined to patterned islands continued to display higher levels of Stro-1 (Fig. 2I) and endoglin (Fig. 2K) than MSCs cultured on non-patterned islands (Fig. 2J, L).

3.3. In vitro differentiation of MSCs

Mesenchymal stem cells are known to spontaneously differentiate into osteogenic lineages when cultured on rigid substrates [34]. Since differentiation of stem cells involves asymmetric division and our patterned cells do not divide when captured in microislands, we reasoned that patterning may restrict spontaneous differentiation. To determine if patterning influences the expression of markers associated with osteogenesis we stained MSCs for alkaline phosphatase production after reseeding from initial patterned or non-patterned surfaces. Analysis of the fraction of MSCs expressing alkaline phosphatase reveals that the non-patterned culture contains greater than twice the number of cells expressing this early osteogenic marker compared to cells that were initially cultured in patterns (Fig. 3).

To assess whether cells initially cultured in patterned and non-patterned surfaces still maintained multipotency, a subset was further cultured in osteogenic and adipogenic induction media for 10 days. Cells cultured in media containing the osteogenic supplement dexamethasone, ascorbic acid and \( \beta \)-glycerolphosphate displayed enhanced alkaline phosphatase production compared to DMEM basal media (Fig. 4). While both previously patterned and non-patterned cells maintained the ability to differentiate, MSCs that had been cultured in patterns showed lower levels of alkaline phosphatase staining compared to MSCs from non-patterned surfaces after 1 week in culture. This trend is consistent with that observed in basal media. When cultured in media formulated to promote adipogenesis (containing dexamethasone, indomethacin and insulin) both previously patterned and non-patterned MSCs show comparable numbers of lipid vacuoles after 1 week (Fig. 4).

3.4. Inhibition of myosin II and ROCK

Previous studies have suggested that cellular microenvironments that promote low actomyosin contractility, promote MSC quiescence [25,27]. Small micropatterns have been shown to foster a low degree of cytoskeletal tension in patterned MSCs; thus we sought to determine whether the increase in Stro-1 and endoglin intensity of cells cultured in 1000 \( \mu \text{m}^2 \) islands is due to a mecha-notransduction event corresponding to decreased cell contractility. Blebbistatin, an inhibitor of myosin II, and Y-27632, an inhibitor of Rho-associated protein kinase (ROCK), have been shown to reduce cell contractility and decrease the fraction of cells undergoing...
Fig. 2. Pre-patterning MSCs promotes expression of stem cell markers. MSCs were stained for Stro-1 and endoglin while cultured in patterns (A,E) compared to MSCs cultured on Fn adsorbed slides [NP] (B,F). After being released from patterns (C,G) MSCs still displayed higher intensities of Stro-1 and endoglin than their non-patterned counterparts (D,H). MSCs initially cultured in patterns showed higher levels of Stro-1 and endoglin (I,K) after 6 days compared to cells on non-patterned surfaces (J,L). Scale ¼ 200 µm. Mean values shown ± standard deviation (n = 4); *p < 0.05. **p < 0.01 by one-way ANOVA.
osteogenesis in patterned shapes [33]. We cultured cells on either patterned surfaces in DMEM basal media, or non-patterned surfaces in DMEM basal media supplemented with 1 μM blebbistatin or 2 μM Y-27632 for one week before being reseeded into 24-well plates and cultured in DMEM basal media. After 1, 6, and 16 days following reseeding, cells were fixed and stained for Stro-1 and endoglin. After 1 day following reseeding, MSCs originally cultured on patterned surfaces display higher intensity of Stro-1 and endoglin than MSCs cultured on non-patterned surfaces with or without the addition of blebbistatin or Y-27632. Stro-1 and endoglin intensity between cells with or without blebbistatin and Y-27632 remain similar (Data not shown). After 6 and 16 days, we observe elevation of Stro-1 and endoglin for cells that were previously treated with blebbistatin and Y-27632 compared to non-patterned cells without drug treatment (Fig. 5).

4. Discussion

In this study, we demonstrate that by capturing MSCs to small islands we restrict the inappropriate lineage commitment often observed in culture and promote the multipotent stem cell phenotype. From earlier work, we noted that MSCs do not divide when confined to small area islands [33]. This observation is in line with previous work by Chen et al. where they demonstrated that restricting cell spreading decreases DNA synthesis [35]. We reasoned that arresting cellular division may also influence the expression of mesenchymal stem cell markers. MSCs are routinely characterized by positive expression of markers such as CD31, CD44, CD90, Stro-1, endoglin, CD106, and CD166 [36,37]. In particular, the expression of endoglin [38,39] and Stro-1 [27,40,41] have been extensively used as phenotypic markers of mesenchymal stromal cell multipotency. MSCs have been shown to express similar levels of endoglin in early and late passage MSCs [42,43]; however, other reports suggest higher expression during early passages compared to senescent passages [44,45]. Similarly, Stro-1 has been shown to be downregulated in prolonged culture [6]. We show that MSCs confined to small 1000 μm² islands express higher levels of endoglin and Stro-1 compared to cells cultured on non-patterned surfaces. Expression of these markers persists even after the cells are removed from the islands after a week, and cultured on tissue culture plastic for up to 16 days. In addition, MSCs cultured without confinement show higher levels of osteogenesis markers suggesting that inappropriate differentiation occurs when they are allowed to proliferate under standard cell culture conditions. MSCs that were previously patterned retain their multipotency as assayed by in vitro differentiation in adipogenic and osteogenic media.

One important aspect of MSC culture that has been shown to promote differentiation in vitro is the physical properties of the substrate and how this influences cell spreading. For instance, MSCs cultured on stiff substrates are well spread, with high cytoskeletal tension, and express high levels of bone cell markers [46]. In contrast, cells on soft substrates are less spread, have low cytoskeletal tension and become quiescent [25]. Chen and colleagues...
further explored cell spreading during osteogenesis by micro-patterned single cells in islands of different adhesive area [47]. When exposed to a mixed media of soluble differentiation cues, MSCs with high spreading in large islands preferred to adopt an osteoblast outcome while cells with low spreading in small islands preferred to undergo adipogenesis. In the subsequent years, researchers have varied cell geometry [33], micro and nanotopography [48,49] and adhesion ligand affinity [50] to demonstrate that conditions that increase cytoskeletal tension all tend to promote osteogenesis. Here we show that under normal MSC culture conditions, there is an increase in the fraction of cells that express early osteogenesis markers. We hypothesize that a subset of highly spread MSCs have a high degree of cytoskeletal tension that promotes differentiation. In contrast, when MSCs are cultured in small microislands they exhibit low cytoskeletal tension, become quiescent, and increase the expression of mesenchymal stem cell markers Stro-1 and endoglin. Although we did not measure cytoskeletal tension directly, previous reports have demonstrated high cytoskeletal tension is linked to cell spreading [33,47]. Mesenchymal stem cells cultured in the presence of a myosin II and ROCK inhibitor (blebbistatin and Y-27632 respectively), both shown to reduce actin cytoskeleton organization [51], show a similar trend in elevated MSC marker expression. Taken together, these results suggest that reduced actomyosin contractility is important for maintenance of the MSC phenotype.

Previous studies using a number of stem cell systems supports our hypothesis that cell shape and cytoskeletal tension are important parameters in promoting multipotency. Prockop and colleagues have demonstrated that subpopulations of small and rapidly self-renewing MSCs have higher multipotentiality than their larger and more spread counterparts [52,53]. Likewise, studies on human embryonic stem cells have shown that compact, rounded hESCs exhibit higher expression of pluripotent transcription factors than flattened ESCs [54]. In addition, embryonic stem cell self-renewal is promoted by soft substrates [55] or by using the ROCK inhibitor Y-27632 [56,57]. Dalby and colleagues observed lower levels of myosin expression on their nanostructured substrates that promote multipotency, further supplementing our findings that lower cytoskeletal tension is required to maintain stemness in MSCs [41]. These results all indicate the importance of cell spreading and low cytoskeletal tension in maintaining multipotency. Since cells cultured in small islands have considerably lower cytoskeletal tension compared to spread cells on the non-patterned substrates, we speculate that low actomyosin contractility will not only influence differentiation—as demonstrated previously using micropatterned MSCs [33,47]—but may also preserve or even enrich the stem cell phenotype.

Design of novel biomaterial platforms that may enhance in vitro lifetime of MSCs offers promising applications for both cell culture and tissue engineering. Conventional culture methods allow for quick expansion, but at the cost of altering phenotype. Evidence that stiff substrates directly alter stem cell fate [46] highlights the importance of tailoring the cell culture substrate for a desired application. We demonstrate that restricting cell shape may be a key factor in maintaining multipotency, and one can imagine that culture conditions restricting cell spreading either through substrate stiffness, chemistry, or topography may be used to prolong the MSC phenotype during ex vivo culture. Similarly, these studies will serve to guide the design of 3D biomaterials where control of cell adhesion and spreading may prove important for the success of regenerative therapies and tissue engineering.

5. Conclusion

Cell shape and actomyosin contractility have been demonstrated to play a key role during MSC fate decisions. Here we show that MSCs cultured under conditions that promote low cytoskeletal tension—either through culture in small microislands or with small molecule inhibitors of actomyosin contractility—display elevated expression of stem cell markers compared to standard culture conditions (cell culture plastic ware). After release from the patterns, MSCs proliferate and maintain multipotency to differentiate to adherent cell lineages. It is tempting to speculate that geometric constraints that promote low actomyosin contractility recapitulate aspects of the MSC niche architecture for conserving the stem cell phenotype. Nevertheless, this study demonstrates the importance of cell shape in maintaining “stemness” and provides a new strategy to maintaining MSC multipotency via the use of microengineered substrates. Considering the inappropriate differentiation and loss of multipotency commonly observed during ex vivo expansion of MSCs in the clinic, there is a need for new platforms that promote the stem cell phenotype in vitro. The approach presented here adds to the suite of materials-based tools to facilitate the ex vivo culture of autologous cells for regenerative medicine and tissue engineering.

Disclosures

The authors indicate no potential conflicts of interest.
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Appendix A

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiomaterials.2013.02.029.

References


