The effect of matrix stiffness on mesenchymal stem cell differentiation in a 3D thixotropic gel

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ABSTRACT

Recent studies have demonstrated the effect of matrix stiffness on the phenotype and differentiation pathway of mesenchymal stem cells (MSCs). MSCs differentiated into neural, myogenic or osteogenic phenotypes depending on whether they were cultured on two-dimensional (2D) substrates of elastic moduli in the lower (0.1–1 kPa), intermediate (8–17 kPa) or higher ranges (34 kPa). In this study, MSCs were cultured in thixotropic gels of varying rheological properties, and similar results were found for the three-dimensional (3D) culture as for the previous findings in 2D culture. For the 3D cell cultures in thixotropic gels, the liquefaction stress (τ_y), the minimum shear stress required to liquefy the gel, was used to characterize the matrix stiffness. The highest expressions of neural (ENO2), myogenic (MYOG) and osteogenic (Runx2, OC) transcription factors were obtained for gels with τ_y of 7, 25 and 75 Pa, respectively. Immobilization of the cell-adhesion peptide, RGD, promoted both proliferation and differentiation of MSCs, especially for the case of the stiffer gels (>75 Pa). This study demonstrated the usefulness of thixotropic gels for 3D cell culture studies, as well as the use of τ_y as an effective measure of matrix stiffness that could be correlated to MSC differentiation.

1. Introduction

Bone marrow derived human mesenchymal stem cells (hMSCs) are an important cell source for cell therapy and tissue engineering applications. These stem cells have broad differentiation potential, being able to differentiate into a variety of anchorage-dependent cell types, including neurons, myoblasts and osteoblasts [1]. One of the two stem cell populations in the bone marrow, MSCs are recruited by paracrine factors of tissue repair into the bloodstream, to circulate, engraft and differentiate in response to the microenvironment of the particular tissue concerned. Matrix factors that direct stem cell differentiation may be chemical or mechanical in nature [2,3]. In particular, the proliferation and differentiation of MSCs can be regulated by substrate stiffness [2,4], ligand presentation [5–7] and the presence of soluble factors [2]. It is now known that MSCs commit to neurogenic lineage when cultured on soft brain-like substrates, to myogenic lineage when cultured on stiffer muscle-like substrates, and to osteogenic lineage when cultured on rigid bone-like substrates [2]. As most cells reside within a 3D environment, it would be of great importance to understand the

effect of matrix stiffness on the proliferation and differentiation of MSCs in three dimensions. Additionally, in view of the fact that the biochemical signals that direct cell fate work in conjunction with mechanical signals [8], it can be inferred that the more natural response of a cell towards a biochemical stimuli (e.g. cytokine, drug) would be observed in 3 dimensions. Cukierman et al. have also elucidated a mode of matrix adhesion unique to cells in 3D culture [9], suggesting that the effects of biological signaling due to cell-matrix interactions should not be extrapolated from 2D culture experiments, but should be confirmed or reexamined for the case of the 3D microenvironment.

We have developed a thixotropic polyethylene glycol-silica (PEG-silica) nanocomposite gel for 3D cell culture [10]. This material allows us to independently control the physical and chemical signals presented to the cells. Conventional synthetic hydrogels require enzymatic [11], chemical [12,13] or photo-crosslinking [14–16] processes for gelation. In contrast, the thixotropic PEG-silica gel can be liquefied by simply applying a shear stress; cells can be easily introduced before the material reverts to the gel state. In this paper, we show how the thixotropicity of the gel makes it suitable as a matrix to investigate the effect of mechanical signaling on hMSC differentiation. The matrix stiffness of the gel can be conveniently tuned by controlling the amount of fumed silica (FS) in the gel [17]. As the thixotropic gel is a visco-elastic material that is mechanically characterized via rheological measurements, we

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have defined a suitable parameter, the liquefaction stress τ_y, as a measure of its matrix stiffness. This is in contrast to the use of elastic moduli to define the substrate stiffness of 2D matrices. For the PEG-silica gel, we have measured the dynamic storage modulus G′ (i.e. the amount of stress required to shear the elastic component of the gel by a unit strain) [18,19], the loss modulus G″ (i.e. the amount of stress required to shear the viscous component of the gel by a unit strain) [18,19], and δ (i.e. the phase angle between G′ and G″, given by $\tan^{-1}(G''/G')$) [18,19]. When the gel is in the solidified state, G′ > G″ and $\delta < 45°$. When the gel is in the liquefied state, G′ < G″ and $\delta > 45°$. The gel liquefies when G′ = G″ and $\delta = 45°$; the minimum shear stress required for this to occur is defined as the liquefaction stress τ_y [18,19] (Fig. 1).

In the present study, we have cultured hMSCs from a commercial source in 3D thixotropic gels of varying stiffness, and monitored the expression of transcription factors relating to the neurogenic, myogenic and osteogenic phenotypes over a period of two weeks. In addition, we have functionalized one batch of gels with the RGD ligand. This was done by directly conjugating the cysteine-containing peptide GCGYGRGDSPG (which included the cell-adhesive RGD (Arg–Gly–Asp) sequence) to the PEG-silica matrix via cysteine–alginate. The hMSCs were cultured in gels with and without RGD, allowing us to independently study the effects of matrix stiffness and ligand concentration on the hMSC proliferation and differentiation. This enabled us to examine the effect of the integrin-binding ligand in modulating the cellular response to the microenvironment.

2. Materials and methods

2.1. Synthesis of PEG-silica gel

4-arm PEG was sterile filtered through a 0.45 μm filter, and vortexed with FS (Cab-o-sil M5, Sigma, USA) until a homogeneous mixture was obtained. TEOS (Acros Organics, USA) was hydrolyzed by mixing with 0.15 M of acetic acid using a TEOS/acid volume ratio of 1:9 for 1 h, and then sterile filtered. It was added to the multi-arm PEG–FS mixture at a volume ratio of 3:5, and vortexed until a uniform mixture was attained. Sterile ammonia solution was added to the mixture until the final pH was 8. A gel formed overnight, and was washed in sterile deionized (DI) water over several days to remove ethanol. Trituration of the gel with water yielded a particulate suspension that was centrifuged at 5000 rpm for 5 min. The supernatant was removed, and the gel was re-homogenized by vortexing before use.

2.2. Gel functionalization: conjugation of RGD to PEG-silica gel

Cysteine–alginate was synthesized as previously described [20]. The degree of substitution with respect to cysteine was found to be ∼300 μmol/g of product. 155.8 μl of RGD (1 mg/ml in DI water) was added to 191 μl of MAL–PEG–MAL

(2.7 mg/ml in DI water) and vortexed for 0.5 h to form a MAL/RGD mixture. 551 μl of cysteine–alginate (1.84 mg/ml in DI water) were added to 346.8 μl of the MAL/RGD mixture, and vortexed for 1 h to form a MAL/RGD/cysteine–alginate mixture. The resulting mixture was added to the unfunctionalized PEG–FS/hydrolyzed TEOS solution at a volume ratio of 1:10, and used to form the PEG-silica gel according to the procedure described in the previous section.

2.3. Rheological studies of PEG-silica gels

The PEG-silica gels were subjected to rheological tests to determine the minimum shear stress required for gel–liquid transition (τ_y), as well as the liquid–gel transition time (t_{L-G}) upon removal of the shear stress. t_{L-G} was the maximum working time for the addition of cells, ligands, etc. to the liquefied gel before gelation occurred again. G′ value at low shear stress (where $\tau < 0.1\tau_y$) and G″ value at high shear stress (where $\tau > 5\tau_y$) were also determined. Rheological experiments were performed with a Thermo-HAAKE RheoScope1 at 37 °C. A serrated parallel plate system with a diameter of 20 mm and a gap distance of 0.1 mm was used. τ_y was determined by applying a ramping shear stress (τ), and measuring the corresponding G′, G″ and δ (Fig. 1). t_{L-G} was determined by first applying a high shear stress (where $\tau > 5\tau_y$) to liquefy the gel, and then measuring the time taken for re-gelation after the removal of shear stress.

2.4. Cell culture

For cell culture, the gel was first vortexed to a liquid state. Cell suspension in the appropriate medium (up to 30% by volume) was then added to the gel with gentle trituration to uniformly disperse the cells. The mixture of gel and cells was then transferred to the culture vessel, i.e. flask, petri dish or cover slip. After the gel has reset (in <60 s), tissue culture medium was added to the culture vessel and transferred to the incubator. Typically, cells were seeded at a density of 1×10^5 cells/ml of gel. Gel was added to a thickness of 2 mm, and an equal amount of culture medium was added above it.

2.5. Cell characterization

hMSCs were cultured for 2 weeks in PEG-silica gels. Live–dead assays were performed after 1 week using the Live Dead® Viability/Cytotoxicity Assay Kit (Molecular Probes). Gels were incubated for 30 min with cell culture medium containing 4 μM of calcein AM and 2 μM of ethidium homodimer. Live cells would fluoresce green due to the uptake and hydrolysis of calcein AM, while the nuclei of dead cells were labeled by the red-fluorescent ethidium homodimer. Cell spreading was quantified by obtaining the average cell aspect ratio for all the cells in a selected area. Cell aspect ratio was defined as the longest length across the cell divided by the perpendicular width across the cell. To measure the relative expression of enolase 2 (ENO2), myogenin (MYOG), runt-related transcription factor 2 (Runx2) and osteocalcin (OC) by hMSCs cultured in PEG-silica gels, the gels were disrupted with lysis buffer, and RNA was extracted according to the protocols specified in the RNeasy Mini Kit (Qiagen, USA) and measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). Real-time polymerase chain reaction (PCR) was then conducted using a Bio-Rad iQ5 multicolor real-time PCR detection system (Bio-Rad, USA). The experiments were performed in triplicate, and the results were reported as mean ± standard deviation.

3. Results and discussion

3.1. Effect of FS on gel properties

In the previously reported work on matrix stiffness affecting stem cell lineage commitment, cells were cultured on 2D substrates of varying stiffness and characterized by their elastic moduli [2,4,6,21]. The use of a thixotropic hydrogel, primarily characterized by rheological measurements, called for the use of an alternative parameter as a measure of matrix stiffness. For that, we have employed the liquefaction stress, the minimum stress required to liquefy the gel.

Liquefaction stress of the gel was found to be proportional to the weight percentage of FS incorporated into the gel (Fig. 2). Each FS particle (average size = 200 nm) was composed of an agglomerate of primary particles with a hydroxyl-rich surface [22,23]. As FS was introduced prior to the base-catalyzed condensation of multi-arm PEG and TEOS [10], binding of the FS to the gel particles introduced a large number of free hydroxyl groups to the matrix, and thus increased hydrogen-bonding interactions between the particles [22,23]. These led to a higher liquefaction stress of the final product
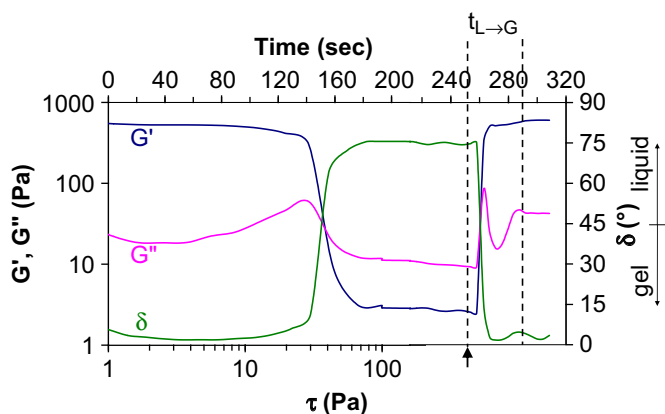

Fig. 1. Typical G′, G″ and δ of PEG-silica gel as a function of increasing shear stress (τ) applied with time. At time = 150 sec, G′ = G″, liquefaction occurs and $\tau = \tau_y$ = liquefaction stress. The time taken for G′, G″ and δ to return to their original levels after the shear stress was removed (at time = 251 sec, as indicated by the arrow) was the liquid–gel transition time (t_{L-G}).
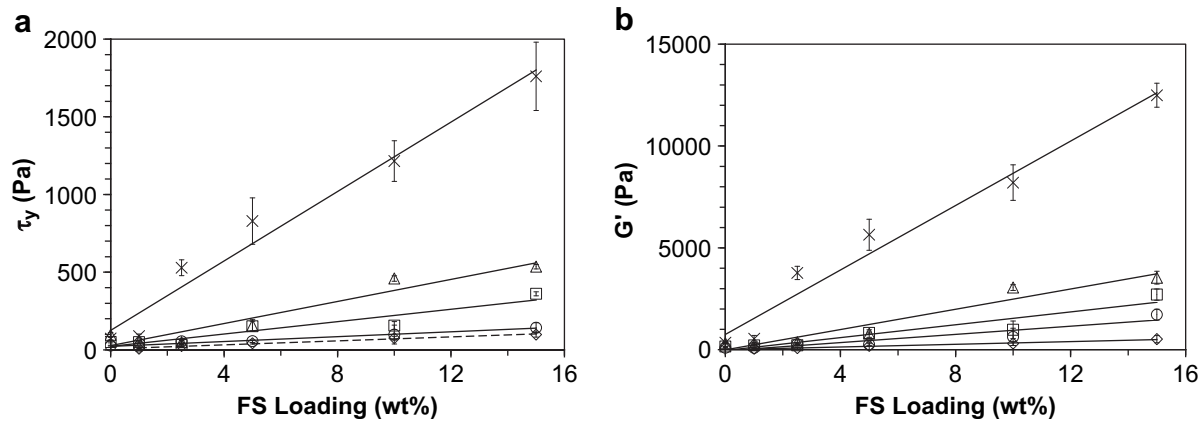
Fig. 2. Mechanical properties of PEG-silica gel. (a) Liquefaction stress τ_y and (b) storage modulus G' as a function of FS loading in the PEG-silica gel with ($\times$) 5, ($\triangle$) 10, ($\square$) 15, ($\circ$) 20 and ($\diamond$) 25 vol% of media.

[17]. Liquefaction stress of the gel was also affected by its water content. The final step in gel synthesis involved centrifuging the gel at a fixed speed and duration (5000 rpm for 5 min) to remove most of the water in the gel, while still maintaining its thixotropic properties. Water was then added back into the gel in the form of cell culture medium so as to make it more conducive for cell growth. In the gel compositions tested, up to 25 vol% of culture medium was added to the gel (Fig. 2). For a given gel composition (i.e. FS loading), the liquefaction stress decreased with increasing volumes of media added. Beyond a certain limit (for example, 30 vol% of media for gels with 0% FS), the liquefaction stress could no longer be measured as the gel–media mixture has essentially become a particulate suspension that was no longer thixotropic.

3.2. Effect of matrix stiffness on cell proliferation

Monitoring of RNA concentration over a period of time revealed that hMSCs proliferated more rapidly in matrices containing immobilized RGD (Fig. 3). This was not an unexpected result, as bound RGD has been known to promote proliferation of various cell types [24,25]. Perhaps more importantly, the effect of RGD was greatest in the gels of higher stiffness ($\tau_y \geq 40$ Pa), increasing by 100–1200% for the gels with highest stiffness ($\tau_y = 100$ Pa) when RGD was immobilized. In fact, cells appeared to proliferate poorly in the higher stiffness gels that lacked RGD.

It is currently understood that cells use actomyosin contractility for matrix interactions [3]. In cells, matrix–integrin interactions on the active lamellipodia cause indirect attachment of integrins to actin filaments [26]. As the filaments are moved rearward by myosin motors, they generate a force on the matrix when it resists movement. RGD, a generic cell-adhesion sequence found in fibronectin and other ECM proteins, binds to the integrin $\alpha5\beta1$ receptor on the cell membrane, leading to the formation of focal adhesions that act as the main sites of application of force by the cells [27]. Consequently, hMSCs were observed to exhibit greater cell spreading in thixotropic gels containing higher RGD concentrations after 1 week of *in vitro* culture [10]. It has been calculated that the focal adhesion of a typical fibroblast would be able to exert a stress of ~5500 Pa on the surrounding matrix [27], which is one order of magnitude larger than that required to liquefy the stiffest thixotropic gel in this work ($\tau_y = 100$ Pa). In a stiff gel lacking RGD, cells presumably proliferated poorly due to the inability to generate adequate force to liquefy the gel. In contrast, immobilized RGD allowed more focal adhesions to be generated, providing sufficient stress to liquefy the gel and thus provided for cell motion and division. Such a requirement for focal adhesions would be relaxed

in the more compliant gels, for which the liquefaction stresses were lower. This observation is in agreement with the understanding that cells on soft gels become less contractile [28], with reduced adhesion strength [29] and $\alpha5$ integrin downregulation [30], and thus the absence of additional focal adhesions due to RGD would not greatly affect cell proliferation.

3.3. Effect of matrix stiffness on hMSC differentiation

The magnitude of traction that is applied by the cell and the extent of its deformation are dependent on the stiffness of the
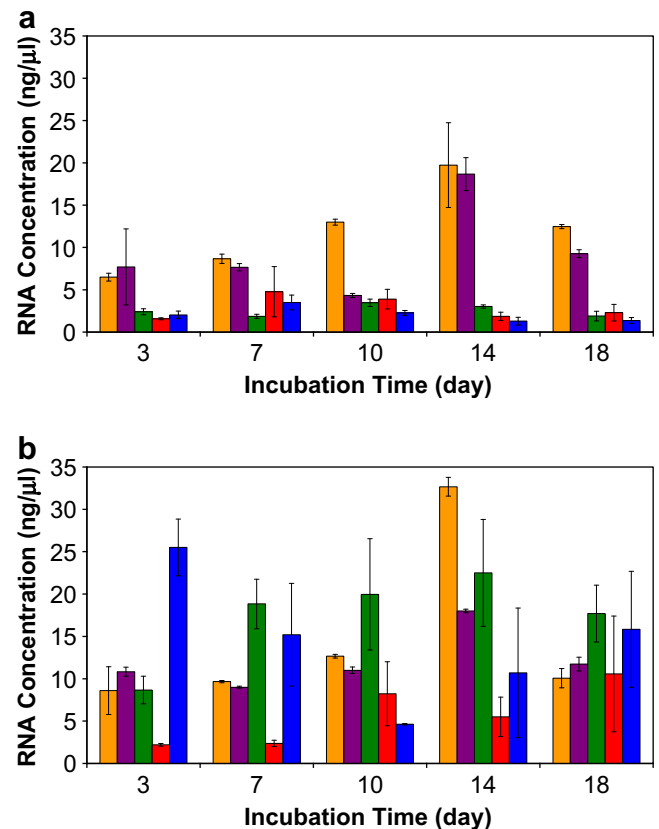

Fig. 3. RNA concentrations of hMSCs cultured in PEG-silica gels over time. PEG-silica gels (a) without and (b) with 2×10^{-5} mol of RGD/ml of gel, and of different stiffness: (■) 7 Pa, (■) 25 Pa, (■) 40 Pa, (■) 75 Pa and (■) 100 Pa. Error bars = ±1 standard deviation. Addition of RGD results in higher RNA concentrations ($P < 0.05$), as compared with cells cultured in gels of the same stiffness without RGD.
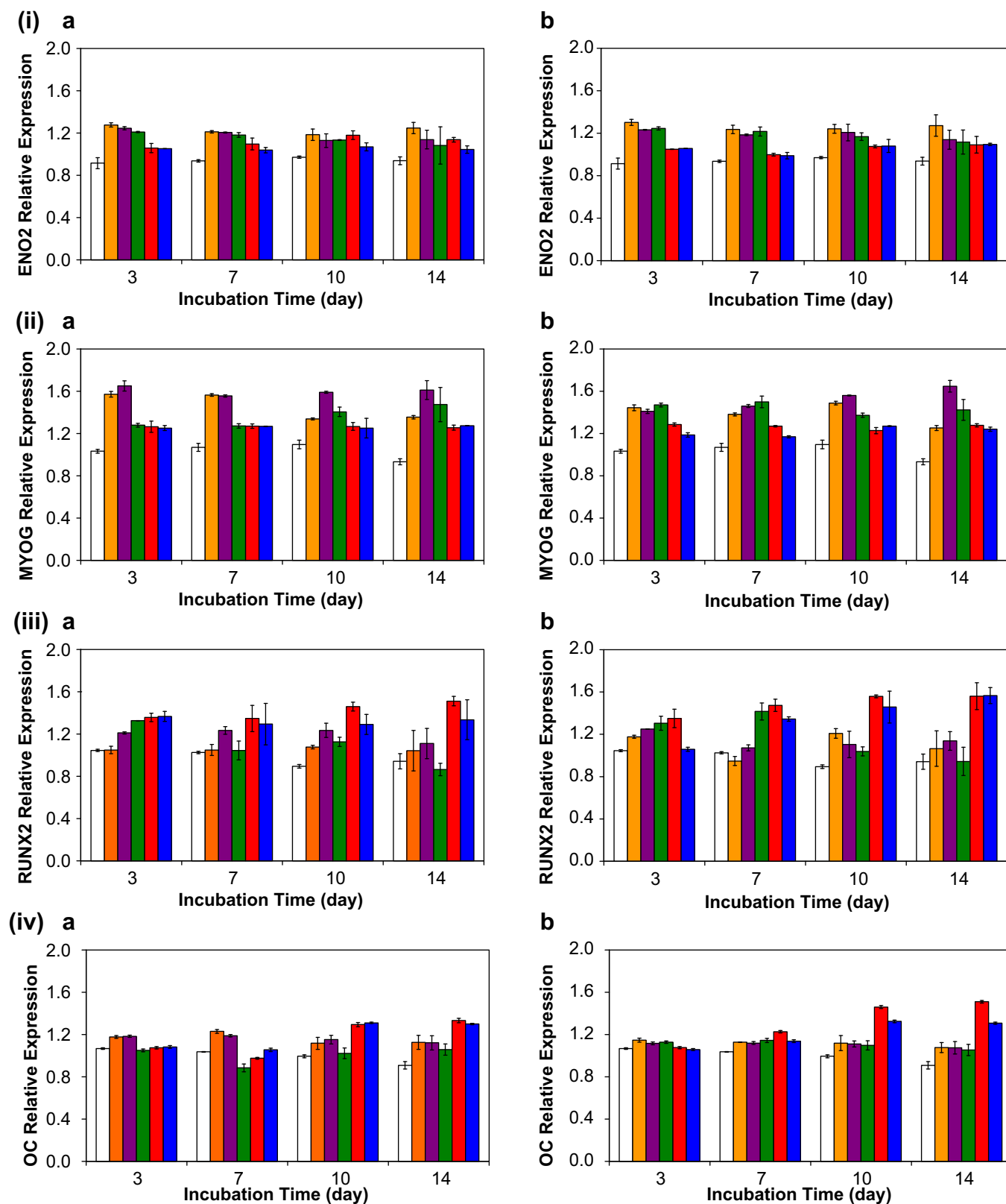
Fig. 4. Gene expression of hMSCs cultured in PEG-silica gels over time. Relative expression levels of (i) ENO2, (ii) MYOG, (iii) Runx2 and (iv) OC for hMSCs cultured in gels (a) without and (b) with 2×10^{-5} mol of RGD/ml of gel, and of different stiffness: (█) 7 Pa, (█) 25 Pa, (█) 40 Pa, (█) 75 Pa and (█) 100 Pa. (□) Control: 2D TCP. Error bars = ±1 standard deviation.
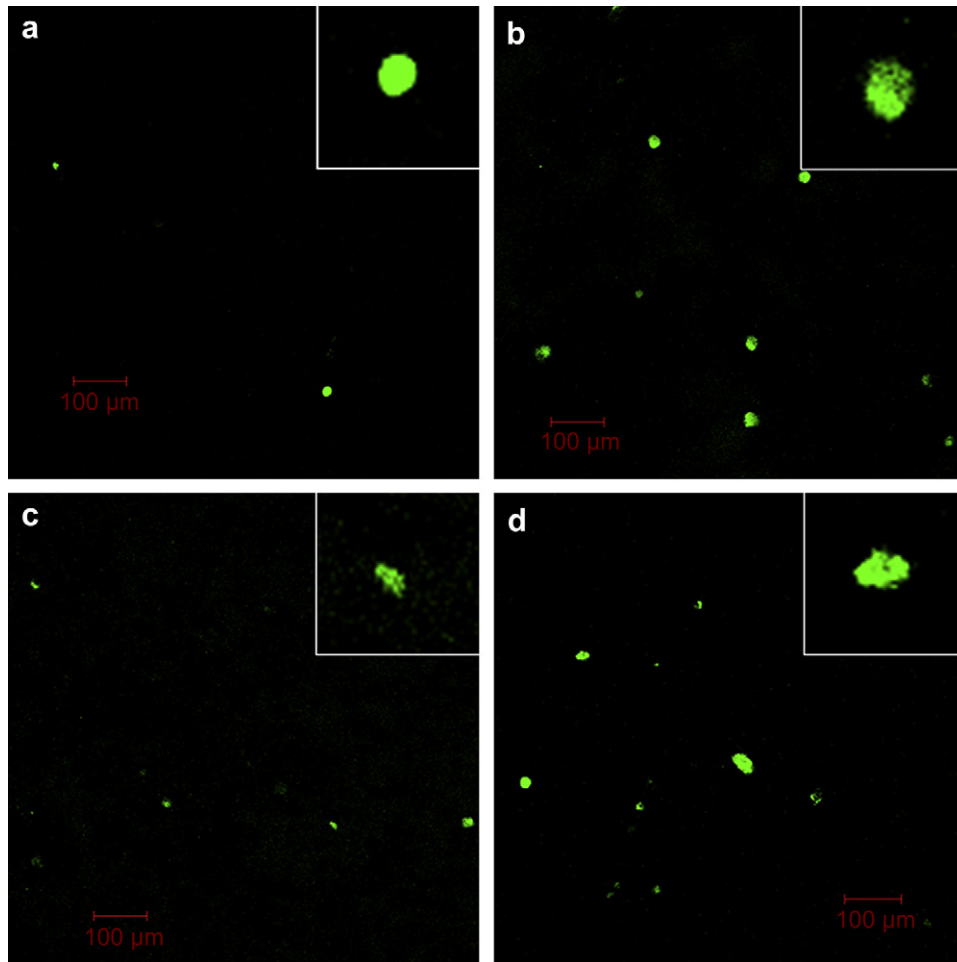
Fig. 5. Morphology of hMSCs cultured in PEG-silica gels after 1 week. Morphology of hMSCs in gels of (a,b) high matrix stiffness (100 Pa) and (c,d) low matrix stiffness (7 Pa), (a,c) without and (b,d) with 2×10^{-5} mol of RGD/ml of gel. Inset shows 4× magnified view of individual cells.

matrix. In a thixotropic environment, we postulate that either a constant amount of stress (exceeding the liquefaction stress) or gradually increasing amount of stress would be applied by the cell on its environment, resulting in a lowering of the local storage modulus (G') and a corresponding increase in loss modulus (G''). At the point when the gel liquefies, no more traction would be possible. The liquefaction stress (τ_y) would therefore reflect the maximum traction available to the cell. As this traction allows cells to exert force on its actin cytoskeleton via focal adhesions and transduce the appropriate signals for differentiation, the liquefaction stress of the matrix would be a relevant measure of matrix stiffness affecting hMSC differentiation.

The highest expressions of neural (ENO2), myogenic (MYOG) and osteogenic (Runx2, OC) transcription factors were observed for gels of low ($\tau_y = 7$ Pa), intermediate ($\tau_y = 25$ Pa) and high ($\tau_y = 75$ Pa) liquefaction stresses, respectively (Fig. 4). The highest expression level of the early bone differentiation transcription factor, Runx2, was exhibited for the higher stiffness gels ($\tau_y \geq 75$ Pa) after 7 days in culture, whereas osteocalcin only became the predominant osteogenic message for the same gels at a later time of 10 days in culture. Overall, a much higher expression for all four markers was observed for the 3D thixotropic gel, as compared to the 2D tissue culture plate (TCP). This implied that the hMSCs cultured on tissue culture polystyrene were in a less differentiated state, as compared to the cells cultured in 3D. This agrees with studies that show a higher

differentiation effect of a 3D matrix, as MSCs are reported to remain undifferentiated in a 3D fiber encapsulation environment for only 1 week, after which they begin to express markers of the neural lineage [31]. On the other hand, in the absence of the relevant biochemical or mechanical signals, MSCs can be cultured for up to 4 passages on TCP without any observable differentiation [32].

RGD immobilization had a more pronounced effect for the matrices of higher stiffness ($\tau_y = 75$ Pa), whereby the osteogenic message was predominant. This was especially true for the case of osteocalcin expression on Days 10 and 14, where RGD immobilization in the gel of $\tau_y = 75$ Pa promoted $\sim 13\%$ higher expression of the osteogenic transcription factor. It has been hypothesized that adhesion receptors such as integrins act as mechanoreceptors, being able to sense mechanical stress and transmit these signals across the cell surface over a specific molecular pathway [33]. Seen from this viewpoint, the RGD ligand was acting as a handle on the ECM through which the cell was able to feel the matrix stiffness and translate it to the cytoskeleton. RGD immobilization made a greater impact on cell proliferation for stiffer matrices because a higher degree of cytoskeletal tension could be generated for the stiffer matrix, and it is this tension that transduced the signals for differentiation.

The above observation clarified the role of the cell-adhesive ligand in presenting the effect of matrix stiffness to the cells. In previous work, a thin coating of ECM protein was employed to

achieve MSC adhesion to an underlying polyacrylamide substrate, whose stiffness was varied. By using an inhibitor of non muscle myosin II (NMM II), Engler et al. were able to show that the MSC differentiating effect of the substrate plus its ECM coating was a primarily mechanical effect [2]. However, Rowlands et al. demonstrated an interplay of stiffness and adhesive ligand presentation, exemplified by the observation that osteogenic differentiation of MSCs occurred significantly only on the collagen Type I coated substrate for the highest substrate stiffness tested, implying that the other ECM proteins used (collagen IV, fibronectin and laminin) had provided chemical signals to modulate the differentiating effect of matrix stiffness [6]. By using a synthetic RGD cell-adhesion ligand presented on an otherwise inert matrix, we have shown that in the absence of other biochemical factors, mechanical signals alone could control lineage specification of the hMSCs in 3D. While one might argue for a proliferative effect of RGD on the hMSCs that in turn affected their differentiation pathway, this study showed that lineage specification was largely independent of the degree of cell proliferation. This is in agreement with Engler et al.'s observations of mitomycin-C inhibiting the proliferation of MSCs [2].

An interesting insight is made through this line of argument. RGD has been known to promote osteogenesis, and is in fact a popular adhesive ligand used in conjunction with biomaterials for bone therapy [34]. We postulate that the mechanism by which RGD promoted osteogenesis is via its role as a ligand to the integrin mechanoreceptor, allowing stem cells to detect the high matrix stiffness required for bone differentiation.

3.4. Comparison with other non-thixotropic synthetic hydrogels

To study the effect of matrix stiffness on lineage specification, hydrogels that allow unrestricted deformation and spreading in a 3D environment are desirable. In this aspect, the typical PEG-based synthetic hydrogels are somewhat unsuitable, presenting themselves to cells as an isotropic continuum with mesh sizes of <0.1 μm, and thus preventing cell deformation/migration in the absence of any degradative mechanism [35,36]. While larger mesh sizes that allow cell migration are achievable, these would invariably involve a hydrogel with poor mechanical characteristics. Synthetic hydrogels that degrade by hydrolytic mechanisms enable cell deformation and migration to occur. However, these degradative processes typically occur only in the longer term (>1 week), when matrix stiffness would no longer be expected to determine stem cell fate. Studies have indicated that beyond the first week in culture, hMSCs are no longer reprogrammable once they have committed to the lineage specified by matrix elasticity [2]. On the other hand, a rapidly degrading matrix would entail a rapidly changing matrix stiffness, therefore complicating the effect on cell phenotype. While the stiffness or liquefaction stress of the thixotropic gel also changes with time as a result of ECM deposited by the cells [10], these changes are minimal in the case of hMSCs, which do not typically generate large amounts of ECM.

In contrast, biopolymer gels such as collagen and fibronectin possess a porous fibrillar structure on the order of cell dimensions, which allow for cell migration. In addition, a significant viscous component is present, and cells are also able to migrate through the gels by pure fiber dislocation [36]. The drawbacks of using biopolymers are safety, source and batch variation issues, added to the fact that each type of biomolecule is usually associated with biological activity. The latter feature complicates their use for systematic investigations since the effects of matrix stiffness and biochemical signals cannot be independently examined.

Our nanocomposite PEG-silica hydrogel may present itself as an alternative to the existing systems [10]. The visco-elastic properties of this thixotropic gel appear to be more similar to those of the biopolymers (e.g. collagen, fibrin). As demonstrated in this work, cells are also able to deform and spread within a thixotropic matrix with the provision of a cell-adhesion sequence. Fig. 5 shows the morphology of cells in the thixotropic gel after 1 week of culture. Cells in the higher stiffness gels, both without and with RGD, were generally more rounded (Fig. 5 a and b). When RGD was presented (Fig. 5 b), the cells appeared to populate the gel at a higher density. In contrast, hMSCs in the gels of lower stiffness (Fig. 5 c and d) were more irregularly shaped, and the difference in cell density was less obvious between the non-modified and RGD-modified gels. The differences in cell morphology might reflect the differences in lineage commitment of the cells in the matrices of varying stiffnesses (osteoblasts being more rounded that neural cells), but the fact that different morphologies and proliferative abilities were observed also attested favorably to the deformable nature of the gel.

4. Conclusions

This study has validated the effect of matrix stiffness on the differentiation of hMSCs in 3D, an effect that was reported previously for the culture of stem cells on a 2D substrate. A thixotropic gel was used as the 3D cell culture matrix in this study, some features of which contributed to the positive outcome of the experiment. Firstly, the gel provided an inert, synthetic environment for hMSC culture, which avoided the complication of biological signaling that would have been present for a biologically derived matrix. Secondly, the thixotropic nature of the gel and the RGD presentation in the gel matrix allowed us to make several observations on cell proliferation and differentiation, to reinforce the postulate of matrix stiffness as the primary effect driving hMSC differentiation. Although the value of 3D culture has been demonstrated repeatedly recently, 3D culture has not been widely practiced as yet due to difficulties associated with it in comparison to 2D culture. The development of suitable matrices and protocols, such as that presented in this work, would greatly facilitate more studies in 3D to validate the observations on cell behavior and phenotype that had been made on 2D culture.

Appendix

Figures with essential colour discrimination. Most of the figures in this article have parts that may be difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.09.057.

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