



The role of stiffness of gelatin–hydroxyphenylpropionic acid hydrogels formed by enzyme-mediated crosslinking on the differentiation of human mesenchymal stem cell

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ABSTRACT

We report the stimulation of neurogenesis and myogenesis of human mesenchymal stem cells (hMSCs) on the surfaces of biodegradable hydrogels with different stiffness. The hydrogels were composed of gelatin–hydroxyphenylpropionic acid (Gtn–HPA) conjugate were formed using the oxidative coupling of phenol moieties catalyzed by hydrogen peroxide (H_2O_2) and horseradish peroxidase (HRP). The storage modulus of the hydrogels was readily tuned from 600 to 12800 Pa. It was found that the stiffness of the hydrogel strongly affected the cell attachment, focal adhesion, migration and proliferation rate of hMSCs. The hMSCs on stiffer surfaces have a larger spreading area, more organized cytoskeletons, more stable focal adhesion, faster migration and a higher proliferation rate. The gene expression related to the extracellular matrix and adhesion molecules also differed when the cells were cultured on hydrogels with different stiffness. The differentiation of hMSCs on the surface of the hydrogel was closely linked to the hydrogel stiffness. The cells on a softer hydrogel (600 Pa) expressed more neurogenic protein markers, while cells on a stiffer hydrogel (12000 Pa) showed a higher up-regulation of myogenic protein markers.

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1. Introduction

It is increasingly recognized that physical parameters are the essential design variables of substrates used in tissue engineering applications besides biochemical ones [1]. Cells adhering to a substrate are able to sense the mechanical stimuli and consequently regulate many important physiological processes including cell morphology [2], adhesion [3,4], migration [2,5], differentiation [6,7], proliferation, apoptosis [8] and gene expression [9]. Substrate mechanics [10,11] and the topography of the extracellular micro-environment [12] can modulate the cell phenotype of the tissue in similar manner to biochemical signals. The effect of substrate stiffness on cell behavior has been intensively investigated. Soft-tissue cells are sensitive to the substrate stiffness of their micro-environment [13], and human mesenchymal stem cells (hMSCs) provide particularly striking evidence that they sense the stiffness of their substrate. Consequently, they differentiate into specific cell lineages according to the substrate's stiffness. For example, hMSCs cultured on collagen-coated polyacrylamide gels of varying

elasticity that correspond to the tissue elasticities of brain (0.1–1 kPa), muscle (8–17 kPa) and nascent bone (>34 kPa) [6] respectively are found to express key markers of early neurogenic, myogenic and osteogenic lineages even though the culture media remains the same. There also appears to be some interplay between the substrate stiffness and the extracellular matrix (ECM) protein as evidenced by the expression of osteogenic and myogenic transcription factors by hMSCs [7]. The potential applications of hMSCs in tissue regeneration would be achieved, if a suitable material system could be designed to allow a more physiologically relevant environment for the growth and differentiation of the cells.

From this perspective, we have developed an injectable hydrogel scaffold system with tunable stiffness for controlling the proliferation rate and differentiation of hMSCs in a three-dimensional (3D) context in normal growth media (Fig. 1a) [14]. Hydrogels composed of gelatin–hydroxyphenylpropionic acid (Gtn–HPA) conjugate were formed by the oxidative coupling of HPA moieties catalyzed by hydrogen peroxide (H_2O_2) and horseradish peroxidase (HRP). A rheological study showed the independent tuning of stiffness and gelation rate of the Gtn–HPA hydrogels. The H_2O_2 and HRP determined the hydrogel stiffness and gelation rate of the injectable hydrogel, respectively. It was also found that the

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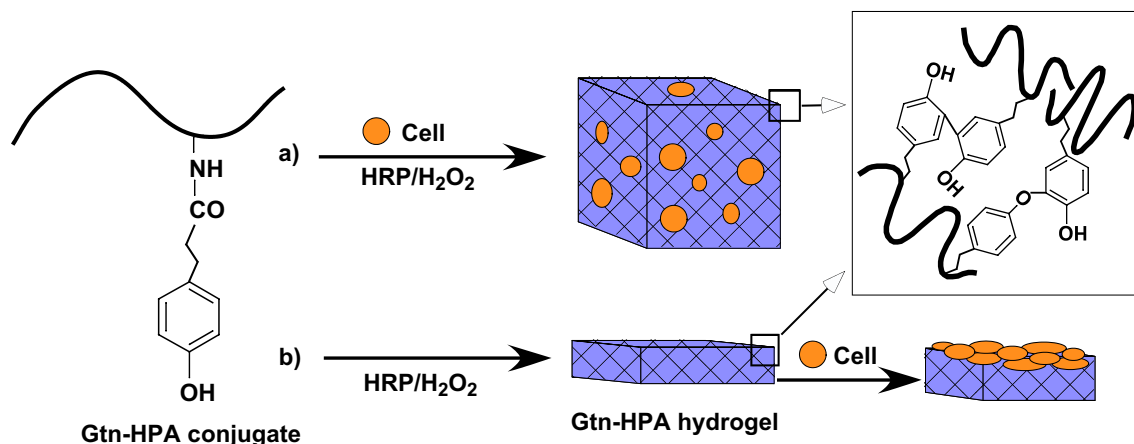


Fig. 1. Formation of Gtn-HPA hydrogels by enzyme-catalyzed oxidation for a) 3D and b) 2D cell growth/differentiation.

proliferation of hMSCs inside the Gtn-HPA hydrogel increased with the decrease in hydrogel stiffness and the neurogenic differentiation was also enhanced when the cells were cultured in hydrogels with lower stiffness [14]. These results indicate that injectable Gtn-HPA hydrogels with tunable mechanical properties for 3D cell culture and differentiation would be an important tool to treat neurological disorders or brain injuries. However, due to the inherent difficulties in 3D culture systems such as poor transportation of nutrients and low degradability of hydrogels as a result of increasing stiffness, the culture and differentiation of hMSCs in hydrogels with storage modulus (G') higher than 1000 Pa appeared to be challenging.

hMSCs are differentiated to different phenotypes by collagen-coated polyacrylamide gels of varying stiffness, as described above. Thus, it is important to design an appropriate system to attain the full benefits of stem cell differentiation for tissue regeneration with our Gtn-HPA hydrogel with tunable mechanical properties. The Gtn-HPA hydrogel would be useful as a hydrogel scaffold system, if hMSCs cultured on Gtn-HPA hydrogels with higher stiffness could be differentiated to different phenotypes and the hydrogels could be degraded after the differentiation of cells. We herein report the proliferation and differentiation of hMSCs on Gtn-HPA hydrogels with a wide range of stiffness (Fig. 1b).

2. Materials and methods

2.1. Materials

Gelatin (Gtn) ($M_w = 80\text{--}140$ kDa, $pI = 5$) and horseradish peroxidase (HRP) (100 units/mg) were obtained from Wako Pure Chemical Industries (Japan). 3,4-Hydroxyphenylpropionic acid (HPA), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl), type I collagenase (246 units/mg), Triton X-100, bovine serum albumin (BSA), anti- β -tubulin, anti-neurofilament light chain (NFL) and anti-desmin antibodies were purchased from Sigma-Aldrich (Singapore). Anti-myogenic transcription factor (MyoD) antibody was purchased from Chemicon (USA). Human mesenchymal stem cells (hMSCs) were acquired from Cambrex Bio Science (USA). Mesencult human basal medium supplemented with mesencult human supplement was purchased from Stem Cell Technologies (Canada). Fetal bovine serum (FBS), 4',6-diamidino-2-phenylindole (DAPI) and fluorophore-conjugated secondary antibodies were provided by Invitrogen (Singapore). HRP-conjugated secondary antibodies were purchased from GE Healthcare (Singapore). Actin/focal adhesion stain kit was provided by Millipore (Singapore). Phosphate buffer saline (PBS, 150 mM, pH 7.3) solution was supplied by media preparation facility in Biopolis (Singapore).

2.2. Rheological measurement

Gtn-HPA conjugates were synthesized as described previously [14–16] and the percentage of HPA introduced to the amine groups of Gtn was 90% as determined by the conventional 2,4,6-trinitrobenzene sulfonic acid (TNBS) method [17].

Rheological measurements of the hydrogel formation were performed with a HAAKE Rheoscope 1 rheometer (Karlsruhe, Germany) using a cone and plate geometry of 35 mm diameter and 0.945° cone angle. The measurements were taken at 37°C in the dynamic oscillatory mode with a constant deformation of 1% and frequency of 1 Hz. To avoid slippage of samples during the measurement, a roughened glass bottom plate was used. The solutions of HRP and H_2O_2 with different concentrations were added sequentially to an aqueous solution of Gtn-HPA (5 or 10 wt.%, 250 μl in PBS). The solution was vortexed and then immediately applied to the bottom plate. The upper cone was then lowered to a measurement gap of 0.024 mm and a layer of silicon oil was carefully applied around the cone to prevent solvent evaporation during the experiment. The measurement parameters were determined to be within the linear viscoelastic region in preliminary experiments. Rheological measurement was allowed to proceed until the storage modulus (G') reached a plateau.

2.3. Enzymatic degradation of Gtn-HPA hydrogels

Slab-shaped Gtn-HPA hydrogels were prepared as described previously with some modifications. Briefly, lyophilized Gtn-HPA was dissolved in PBS at a concentration of 5 wt.%. 6 μl of HRP was added to 1 ml of Gtn-HPA solution to give a final concentration of 0.15 units/ml. Crosslinking was initiated by adding 6 μl of different concentrations of H_2O_2 solution to give final concentrations of 1.7, 3.4 and 8.5 mM. The mixture was vortexed vigorously before it was injected between two parallel glass plates clamped together with 1 mm spacing. Then, round hydrogel disks with diameters of 1.6 cm were cut out from the hydrogel slab using a circular mold. The hydrogel disks were swollen in PBS for 24 h to reach swelling equilibrium and then sandwiched between plastic nets to facilitate retrieval of the hydrogels during degradation. The hydrogels were immersed in 20 ml of PBS containing 6.7 units/ml of type I collagenase and incubated at 37°C in an orbital shaker at 100 rpm. The degree of degradation of the hydrogels was estimated by measuring the residual hydrogel weight. The hydrogels were removed from the solution, blotted dry and weighed at specific time points to measure their residual weight.

2.4. Time course assay on cell attachment

Gtn-HPA hydrogels were prepared in the 24-well plate in a similar manner as described above. The hydrogels were allowed to settle for 4 h. 500 μl of hMSCs in mesencult human basal medium supplemented with mesencult human supplement (passage number <6) at cell density of 3×10^5 cells/ml was seeded onto the hydrogels. The plates were returned to the incubator for an appropriate period of time ranging from 1 to 6 h. At selected time intervals, the media with unattached cells were aspirated and the wells were washed with PBS. A cell culture well plate without the hydrogel served as a comparison. The cells attached to the hydrogels were harvested by incubating the hydrogels with collagenase solution (6.7 units/ml) to digest the hydrogels, whereas the cells attached to the culture plate were harvested by trypsinization. DNA was quantified to determine the number of attached cells at specific time on the hydrogels or culture well plate. The cell pellets were lysed by a freeze-thaw cycle in 200 μl of DNA-free lysis buffer. Samples were then incubated with 200 μl of PicoGreen working solution. The number of cells attached to the surface was then determined by the fluorescence measurement of the sample solution along with the known concentration of cell suspension for the standard curve. The fluorescence measurement was performed in four replicates using a microplate reader with excitation and emission at 480 and 520 nm, respectively.

2.5. Cell proliferation assay

For 2D cell proliferation on the surface of hydrogels, 250 μ l of hMSCs in mesencult human basal medium supplemented with mesencult human supplement at cell density of 6×10^5 cells/ml was seeded onto the Gtn-HPA hydrogels that had been allowed to set for 4 h. The culture medium was changed every 2–3 days. To evaluate the cell proliferation on hydrogels, DNA was quantified using the PicoGreen assay as described above.

2.6. Cell migration assay

Cell migration assay was performed in a 24-well plate. 20 μ l of hMSCs in mesencult human basal medium supplemented with mesencult human supplement at cell density of 1×10^6 cells/ml were seeded onto the center of Gtn-HPA hydrogels. The cells were allowed to attach onto the surface overnight. Unattached cells were removed by washing with PBS before 250 μ l of culture medium was added to each well. The plate was incubated in a humidified chamber (37 °C, 5% CO₂) to allow cell migration. At selected time intervals, the cells were examined microscopically to record the distance they had travelled from the center of the circle. This experiment was performed in three replicates.

2.7. Cell focal adhesion study

For 2D cell focal adhesion study, hMSCs were seeded onto Gtn-HPA hydrogels and maintained for 2 weeks before being immunostained using actin/focal adhesion stain kit. Prior to the immunostaining, the hydrogels together with the cells were fixed with 4% formaldehyde solution at room temperature for 20 min. After washing, the cells were permeabilized using 0.5% Triton X-100 in PBS solution at room temperature for 5 min. The cells were then blocked in 0.05% Triton X-100 containing 1% bovine serum albumin at room temperature for 1 h. The samples were then incubated with an anti-vinculin antibody in blocking buffer solution at 4 °C overnight. The cells were washed and then incubated with a FITC-conjugated secondary antibody in the dark for 30 min. For double labeling, TRITC-conjugated phalloidin was incubated simultaneously with the secondary antibody. The cell nuclei were counterstained with DAPI (1:15,000 in water of 5 mg/ml stock). Confocal images were acquired using confocal laser scanning microscopy (Olympus FV300, Japan).

2.8. RNA preparation

Gtn-HPA hydrogels were prepared in the 6-well plate in the similar manner as described above. 1 ml of hMSCs in mesencult human basal medium supplemented with mesencult human supplement at cell density of 2×10^6 cells/ml was seeded onto the hydrogels. The cells on Gtn-HPA hydrogels and a culture plate were both harvested by trypsinization after the samples were incubated at 37 °C for 2 days. The total RNA was extracted from the samples with TRIzol reagent (Invitrogen) according to the manufacturer's protocol, based on an estimate of 1×10^7 cells. After washing the RNA with 75% ethanol, the samples were cleaned using the RNeasy Minikit (Qiagen), according to the manufacturer's protocol. The RNA yield, purity and concentration were determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer.

2.9. PCR array analysis

Screening for the expression of 84 genes associated with cell adhesion and ECM molecules in hMSCs cultured on Gtn-HPA hydrogels was performed in triplicate using the human ECM and adhesion molecules RT² Profiler™ PCR array (SABiosciences™, USA) according to manufacturer's instructions. In brief, cDNA was prepared from 1 μ g total RNA by using a RT² PCR array first strand kit. PCR amplification was conducted with an initial 10-min step at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min on a MyiQ2 Real Time PCR machine (Bio-Rad, USA). Melting curves were obtained using the following conditions: 95 °C, 1 min; 65 °C, 2 min (OPTICS OFF); 65 °C–95 °C at 2 °C/min (OPTICS ON). Data was imported into an Excel worksheet and analyzed using the Web-based PCR Array Data Analysis from SABiosciences with normalization of the raw data to β -actin.

2.10. 2D cell differentiation

For studies involving hMSC differentiation on hydrogels, the cells were pre-treated with mitomycin C (10 μ g/ml) for 2 h to inhibit their proliferation and washed three times with culture medium. The mitomycin C treated hMSCs at final density of 1×10^5 cells/ml were seeded on the Gtn-HPA hydrogels. The cultures were maintained for 3 weeks. Prior to confocal imaging, the cells were fixed, permeabilized and blocked using the same protocol as described above. The samples were then incubated with the appropriate primary antibody in a blocking buffer solution at 4 °C overnight. After washing, the cells were incubated with the fluorophore-conjugated secondary antibodies in the dark for 30 min. The cell nuclei were counterstained with DAPI (1:15,000 in water of 5 mg/ml stock). Confocal images were taken using confocal laser scanning microscopy (Olympus FV300, Japan). For western blotting, the cells were harvested as described above. The cells in the buffer (4% SDS, 20%

glycerol and 0.02% bromophenol blue in Tris-HCl (0.125 M, pH 6.8)) were sonicated for 30 s. The cell lysate was boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane, blocked, and labeled with HRP-conjugated secondary antibodies. A blot for β -actin served as the control to ensure an equal protein loading level. All western blottings were performed in duplicate.

3. Results and discussion

3.1. Preparation and degradation of Gtn-HPA hydrogels with a wide range of stiffness

We have previously reported that hydrogel composed of Gtn-HPA is formed using the oxidative coupling of HPA moieties catalyzed by H₂O₂ and HRP [14]. It is well known that phenols crosslink through either a more common C–C linkage between the *ortho*-carbons of the aromatic ring or a C–O linkage between the *ortho*-carbon and the phenolic oxygen [18,19]. The stiffness of Gtn-HPA hydrogels could be controlled by varying the H₂O₂ concentration without affecting the gelation rate. The storage modulus (G') of hydrogel ranged from around 20–1000 Pa when 2 wt.% of the Gtn-HPA conjugate was utilized. In this study, the Gtn-HPA hydrogels were prepared by using 5 wt.% of Gtn-HPA conjugate to provide an even wider range of stiffness. Table 1 summarizes the rheological properties of Gtn-HPA hydrogels formed with different H₂O₂ concentrations. The G' of the hydrogels was significantly raised by increasing the H₂O₂ concentration; and when 1.7, 3.4 and 8.5 mM of H₂O₂ concentrations were used, the values of G' were 629 ± 71 , 2529 ± 290 , and 8172 ± 1338 Pa, respectively. The hydrogels with different stiffness (629, 2529 and 8172 Pa) are abbreviated as Gtn-HPA-5-soft, Gtn-HPA-5-medium and Gtn-HPA-5 stiff, respectively. Although a wide range of G' could be tuned by H₂O₂ concentration, little effect was observed on the gel point, defined as the crossover of G' and loss modulus (G''). A slight rise in the gel point, observed when the H₂O₂ concentration was increased from 3.4 to 8.5 mM, might be due to the deactivation of HRP by an excess amount of H₂O₂ [20]. In addition, the time required for G' to reach a plateau increased with the increase in H₂O₂ concentration. These results are in good agreement with previous reports on the independent tuning of hydrogel mechanical properties and gelation rates [14,21–23]. These results indicate that the mechanical strength (i.e. crosslinking density) of the hydrogel could be tuned by H₂O₂ concentration without compromising the rapid rate of gelation. H₂O₂ decomposes to water after oxidizing HRP which in turn oxidizes the HPA. Thus, the percentage of HPA moieties that actually participated in the crosslinking reaction would depend on the amount of H₂O₂ available.

Table 1
Rheological properties of Gtn-HPA hydrogels used in the 2D cell proliferation and differentiation study.^a

Sample	Gtn-HPA (wt.%)	HRP (units/ml)	H ₂ O ₂ (mM)	G' (Pa)	Gel point (s) ^b	Time needed for G' to reach plateau (s)
Gtn-HPA-5-soft	5	0.15	1.7	629 ± 71	<30	79 ± 13
Gtn-HPA-5-medium	5	0.15	3.4	2529 ± 290	<30	172 ± 10
Gtn-HPA-5-stiff	5	0.15	8.5	8172 ± 1338	36 ± 10	1282 ± 129
Gtn-HPA-10-stiff	10	0.16	10.2	12780 ± 1540	<30	109 ± 25

^a Measurement was taken with constant deformation of 1% at 1 Hz and 37 °C ($n = 4$). Results are shown as the average values \pm standard deviation.

^b Gel point is defined as the time at which the crossover of storage modulus (G') and loss modulus (G'') occurred. Herein, it is used as an indicator of the rate of gelation.

In addition, a Gtn–HPA hydrogel was prepared using 10 wt.% of Gtn–HPA conjugate to increase the G' further. The G' was increased to around 12800 Pa. This hydrogel is abbreviated as Gtn–HPA-10-stiff. The increase in G' achieved by using a higher concentration of Gtn–HPA conjugate indicates that a higher number of HPA moieties participated in the crosslinking reaction.

It is well known that the stiffness of a hydrogel affects its enzymatic degradation. We assessed the enzymatic degradation of Gtn–HPA hydrogels using type-1 collagenase, a member of the matrix metalloproteases (MMP) family. MMP degrades the extracellular matrix, leading to cell migration and growth in the body [24]. Accordingly, they can digest proteolysis-sensitive hydrogels [25]. Fig. 2 shows that the rate of hydrogel degradation decreased with increased hydrogel stiffness. This result suggests that the enzymatic degradability of Gtn–HPA hydrogels can be well-controlled by hydrogel stiffness.

3.2. Cell attachment, migration and proliferation

The role of substrate stiffness on cell functions such as adhesion, migration, differentiation, or spreading has been demonstrated for various cell types that have been cultured on different substrates [2–8,10,11]. Although there is cell-to-cell variability, it is generally found that cells growing on stiffer surfaces have a larger spreading area, more organized cytoskeletons and more stable focal adhesions. Across the range of stiffness studied in this report, Gtn–HPA hydrogels have provided an excellent support for cell attachment. As shown in Fig. 3, the number of hMSCs attached to hydrogels with different stiffness was significantly higher than the hMSCs attached to a NUNC® culture well plate during the initial 6 h of incubation. The excellent cell adhesiveness of Gtn–HPA hydrogels is considered to be due to the positively charged residues and RGD peptide sequences of Gtn [26]. Also, the cell anchorage of Gtn–HPA hydrogels was enhanced by increasing the stiffness of the hydrogels.

Next, we analyzed the proliferation rate of hMSCs grown on Gtn–HPA hydrogels. We found a good correlation between the proliferation rate and hydrogel stiffness (Fig. 4). The hMSCs cultured on Gtn–HPA-5-medium and Gtn–HPA-5-stiff hydrogels grew much faster than the ones grown on Gtn–HPA-5-soft. We attribute this observation to firmer adhesion, decreased apoptosis and a greater proportion of phosphorylated focal adhesion kinases

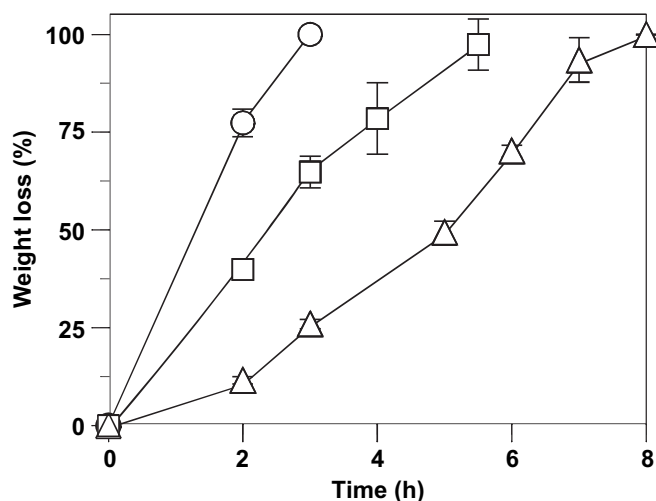


Fig. 2. Enzymatic degradation of Gtn–HPA-5-soft (○), Gtn–HPA-5-medium (□) and Gtn–HPA-5-stiff (△) hydrogels in the presence of 6.7 units/ml of type 1 collagenase at 37 °C. Results are shown as the average values ± standard deviation ($n = 3$).

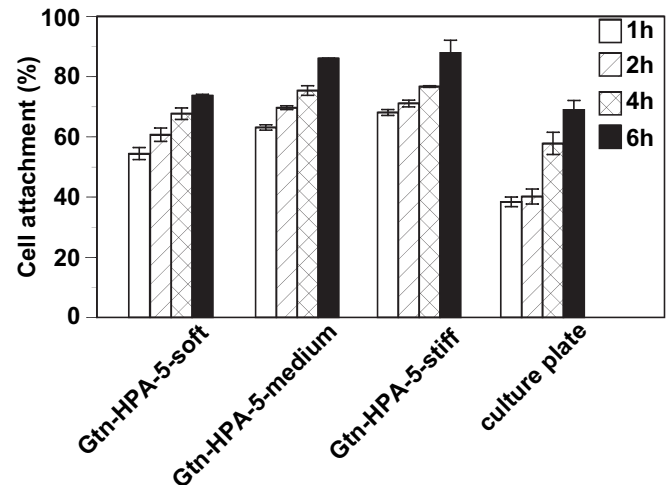


Fig. 3. hMSC attachment on the surface of Gtn–HPA hydrogels after different incubation periods. Results are shown as the average values ± standard deviation ($n = 3$).

(FAK) as a result of higher spreading [27–30]. To confirm our hypothesis, we performed immunostaining on the hMSCs to detect their focal adhesion actin cytoskeleton. Confocal fluorescence microscopy of the stained cells revealed focal contacts in green using an anti-vinculin monoclonal antibody and a FITC-conjugated secondary antibody (Fig. 5). F-Actin was stained in red, and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and appear in blue. The cells appeared to be more spread out when they were cultured on a stiffer hydrogel. The F-actin was much more organized when the cells were in contact with a stiff hydrogel. Both the focal contact and F-actin organization showed a progressive trend, from being diffused when in contact with soft hydrogels to having a more structured arrangement when the cells were attached to the stiffer ones. These observations are in good agreement with the earlier reports of stem cells responding to matrix elasticity on both synthetic and naturally-derived material [6,7,14,30]. These various responses to substrate stiffness through the focal contact patterns and the F-actin arrangement of hMSCs may induce their differentiation as discussed in the following experiment.

To further investigate the effect of hydrogels stiffness on cell function, we sought to analyze the migration of hMSCs. A previous

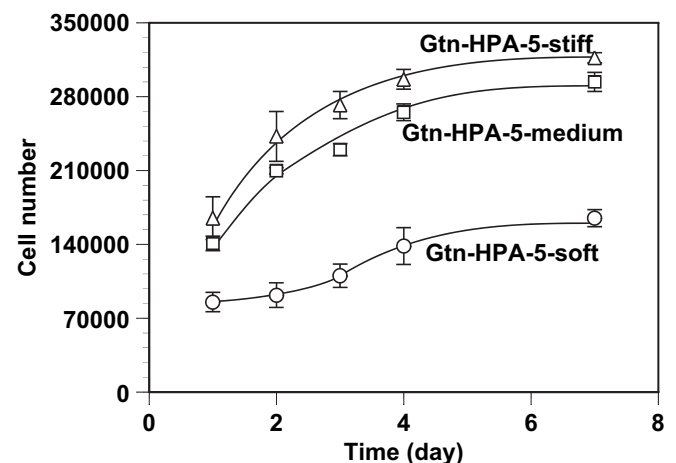


Fig. 4. 2D hMSC proliferation on Gtn–HPA hydrogels with different stiffness. Results are shown as the average values ± standard deviation ($n = 4$).

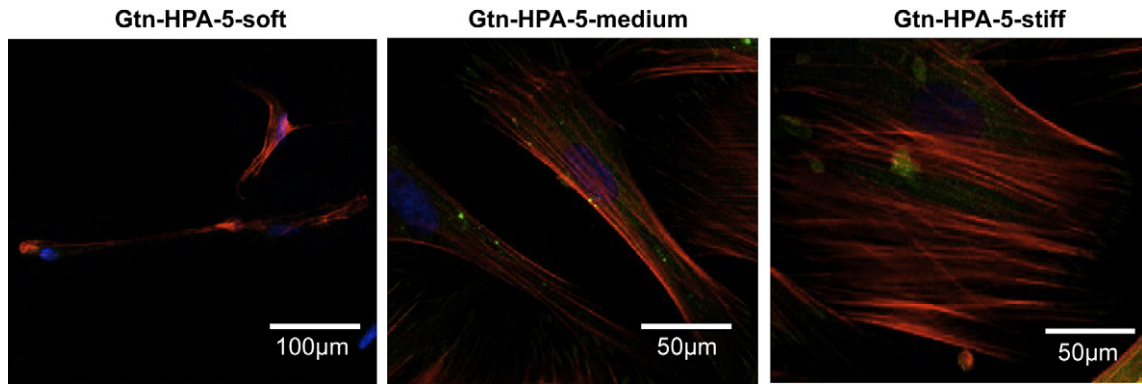


Fig. 5. Confocal fluorescence microscopy of focal adhesion and actin cytoskeleton in hMSCs cultured on Gtn-HPA hydrogels.

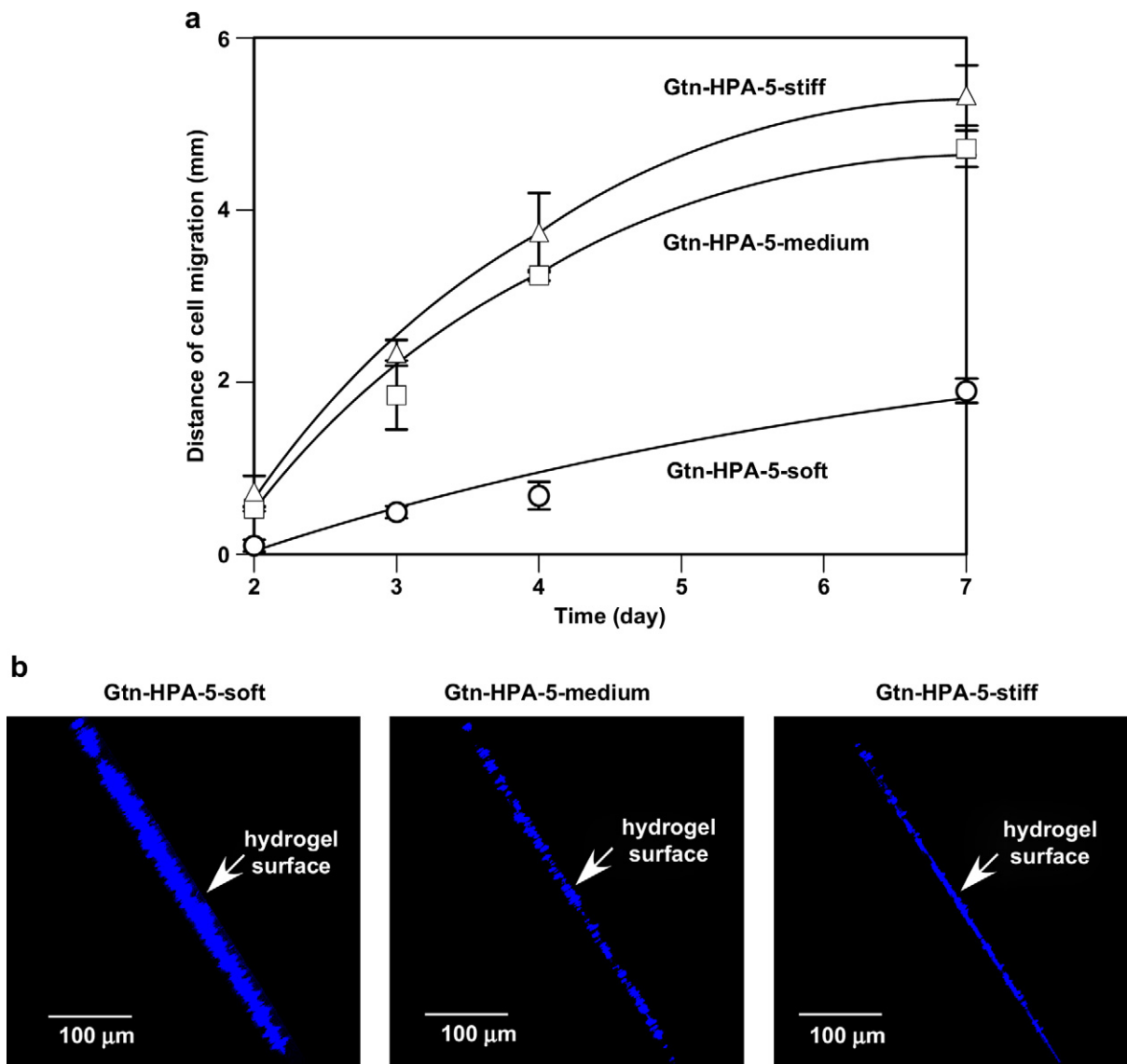


Fig. 6. (a) The distance that hMSC migrated on Gtn-HPA hydrogels with different stiffness. (b) Cross-sectional image of the hydrogels. Results are shown as the average values \pm standard deviation ($n = 4$).

report by Lo et al. has revealed that cells move with a higher average migration rate when grown on stiffer surfaces [31]. We performed a migration assay and we found that the migration distance calculated from the seeding site of hMSCs was twice than that of Gtn–HPA-5-soft when cells were grown on Gtn–HPA-5-stiff (Fig. 6a). The difference in migration rate between Gtn–HPA-5-medium and Gtn–HPA-5-stiff was insignificant. However, an analysis of the cross-sectional distribution of the cells revealed that the cell migration depended on cell culture conditions. The hMSCs growing on Gtn–HPA-5-soft migrated in a 3D plane (Fig. 6b). That is, in conjunction to the horizontal displacement on the surface of the hydrogels, the cells also moved in a Z direction into the hydrogel. However, the majority of the hMSCs seeded on Gtn–HPA-5-medium and Gtn–HPA-5-stiff remained on their surfaces. The high stiffness restricted the cells from moving into the hydrogel. As the enzymatic degradability of Gtn–HPA hydrogel increased with decreasing the stiffness of hydrogel (Fig. 2), we consider that the cell migration in a Z direction was affected by the degradation of the hydrogel.

To understand the behavior of cells cultured on Gtn–HPA hydrogels with different stiffness, the gene expression profiles of the cells were examined by using human ECM and adhesion molecules RT² Profiler™ PCR array [32]. There was a set of primers for 84 genes related to ECM and adhesion molecules in the assay plate. There were 67 genes detected in the culture well plate, 69 in the Gtn–HPA-5-soft, 68 in the Gtn–HPA-5-medium and 68 in the Gtn–HPA-5 stiff, respectively. Out of the 84 genes, 66 (79%) were detectable in all 4 samples.

Table 2 summarizes gene's expression with a greater than 2-fold change in hMSCs cultured on the hydrogels compared to those cultured on the well plate. We found that 11, 9 and 5 genes were up-regulated at least 2-fold when hMSCs were cultured on Gtn–HPA-5-soft, Gtn–HPA-5-medium and Gtn–HPA-5-stiff hydrogels, respectively. MMP1, SPP1, ITGB4, ADAMTS13 and CLEC3B were among those genes whose expression was up-regulated at least 2-fold, and were commonly up-regulated on all the Gtn–HPA hydrogels regardless of their stiffness. Four genes (TNC, ICAM1, VCAM1 and LAMA1) were commonly up-regulated in Gtn–HPA-5-soft and Gtn–HPA-5-medium. Only two genes (MMP-8 and MMP-

13) were uniquely up-regulated on Gtn–HPA-5-soft. SPP1, ITGB4, TNC and ICAM1 are reported to mediate cell–matrix and cell–cell interactions [33–35]. Interestingly, the change in expression of the genes was enhanced as the stiffness of Gtn–HPA hydrogels was decreased. As described earlier, cell attachment on Gtn–HPA hydrogel dropped with decreasing stiffness. Therefore, it is considered that such an up-regulation of gene's expression might be affected by hydrogel stiffness. Consequently, cell attachment on the hydrogels with lower stiffness may be encouraged with enhanced adhesive protein molecules. It is known that MMPs are not constitutively expressed by cells *in vivo* [36]. However, alterations in the cell–matrix interactions regulate MMP expression [37]. Furthermore, MMPs degrade ECM proteins to expose the RGD sequence in the protein and enhance cell–matrix interactions [36]. We found that the change in the expression of MMPs increased with the decrease in hydrogel stiffness. In particular, MMP1 was highly up-regulated by a hydrogel with lower stiffness. These results indicate that the stiffness of Gtn–HPA hydrogel could affect cell attachment and migration due to the regulation of adhesive protein and MMPs.

Our results present further evidence that cells can sense and respond to the environment of their substrate's stiffness, by regulating their spreading, attachment, migration and proliferation. The extent to which cells can sense their substrate stiffness and how cells respond to changes in their substrate stiffness can have profound implications for tissue engineering and regeneration.

3.3. hMSC differentiation on Gtn–HPA hydrogels

The differentiation of hMSCs in response to stiffness of Gtn–HPA hydrogel was evaluated. For this purpose, the cells were pre-treated with mitomycin C to inhibit proliferation and they were cultured on hydrogels with different stiffness in the presence of a normal culture medium for 3 weeks. Mitomycin C is proven to have little impact on average cell shape and morphology [6]. Cells on the hydrogels with different stiffness were then stained with a group of neuron-specific antibodies consisting of neurofilament light chain (NFL), late neuronal marker neurofilament heavy chain (NFH) and neuron-specific marker β 3 tubulin, or with a group of myogenic markers consisting of myogenesis differentiation protein 1 (MyoD1) and desmin. Nuclei were counterstained with DAPI and images were collected by immunofluorescent microscopy. Cells grown on both Gtn–HPA-5-soft and Gtn–HPA-5-medium revealed the expression of neuron-specific proteins (Fig. 7a). On the contrary, cells cultured on Gtn–HPA-5-stiff did not express any of the neuron-specific proteins over the same period of time (data not shown). These results indicate that Gtn–HPA hydrogels with a stiffness of less than 2500 Pa stimulated the neurogenesis of the hMSCs. Western blotting was used to confirm expression of the same group of protein markers. hMSCs cultured on the Gtn–HPA-5-soft and Gtn–HPA-5-medium for 3 weeks expressed neuronal markers, whereas cells cultured on Gtn–HPA-5-stiff for the same period of time showed a much reduced amount of neuronal protein marker expression (Fig. 7b). The expression level of these proteins was normalized to that of β -actin. These quantitative results strongly suggest that the commitment of hMSCs differentiation to a given lineage is influenced by the stiffness of the hydrogel. Indeed, these findings are in agreement with previous results that showed that hMSCs underwent neurogenesis when grown on 2D collagen-coated polyacrylamide gel.

Recently, we have reported on the neurogenic differentiation of hMSCs in 3D culture [14]. The cells which were cultured in the Gtn–HPA hydrogel ($G' = 280$ Pa) expressed much more neuronal protein markers compared to those cultured in a hydrogel where $G' = 840$ Pa. In contrast, the neurogenic differentiation of hMSCs

Table 2
Gene expression profiling in hMSCs cultured on Gtn–HPA hydrogels with different stiffness.

Gene description	Gene symbol	Fold change in gene expression ^a		
		Gtn–HPA-5-soft	Gtn–HPA-5-medium	Gtn–HPA-5-stiff
Matrix metalloproteinase 1 (interstitial collagenase)	MMP1	10.5	7.1	2.3
Secreted phosphoprotein 1	SPP1	9.8	6.3	3.3
Integrin, beta 4	ITGB4	3.5	2.2	2.1
Matrix metalloproteinase 8 (neutrophil collagenase)	MMP8	3.3	1.6	1.0
Tenascin C	TNC	3.2	2.0	1.6
Intercellular adhesion molecule 1	ICAM1	3.0	2.2	1.7
Vascular cell adhesion molecule 1	VCAM1	2.8	2.2	1.7
ADAM metalloproteinase with thrombospondin type 1 motif, 13	ADAMTS13	2.6	2.7	3.2
C-type lectin domain family 3, member B	CLEC3B	2.4	2.8	2.6
Laminin, alpha 1	LAMA1	2.3	2.3	1.9
Matrix metalloproteinase 13 (collagenase 3)	MMP13	2.0	1.3	–2.2

^a The fold change for each gene is calculated as the average expression using hydrogels divided by the average expression using cell culture plates.

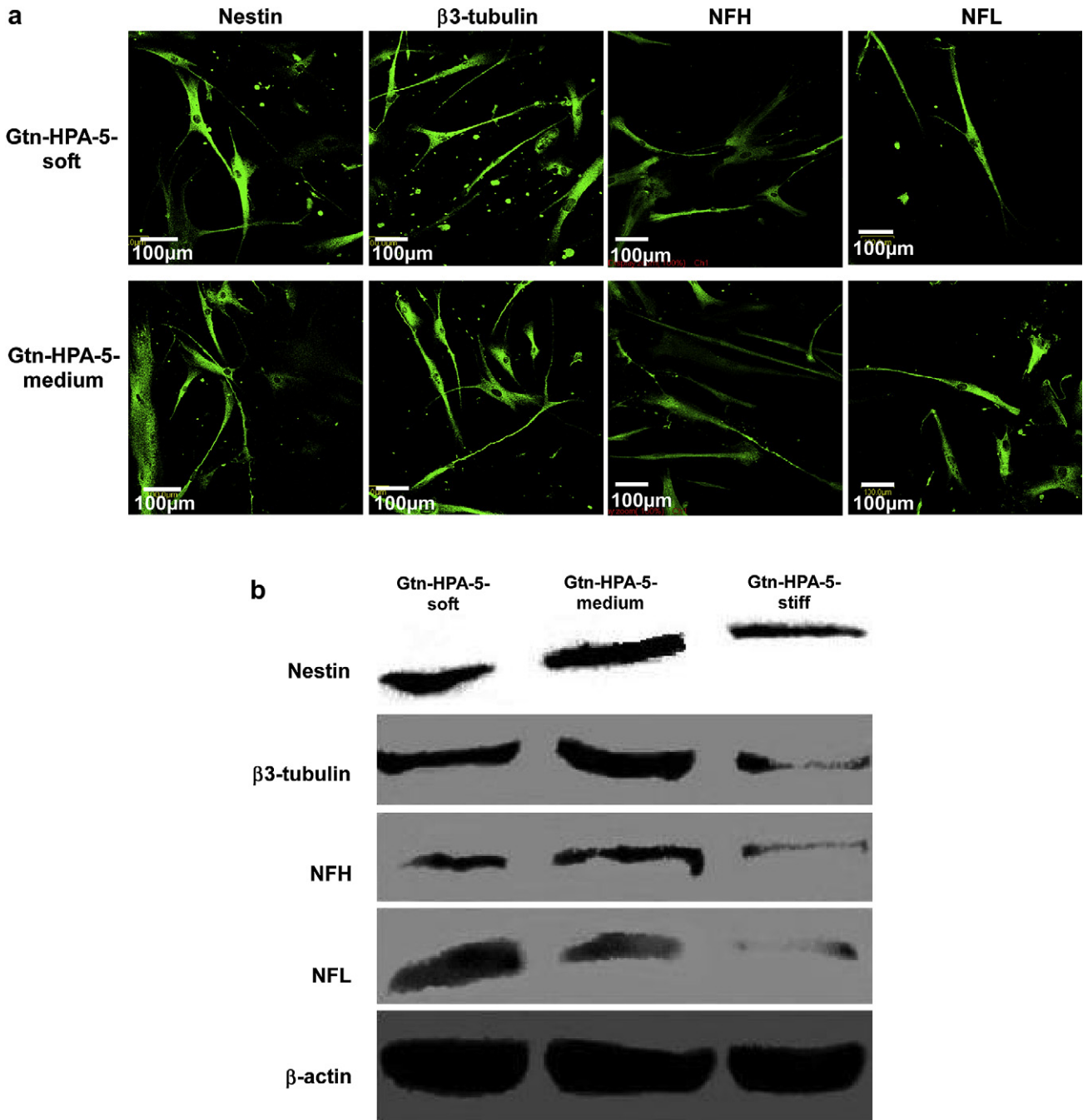


Fig. 7. (a) Immunofluorescence images of neurogenic protein markers and (b) western blotting of proteins expressed in hMSCs cultured on Gtn-HPA hydrogels with different stiffness.

was observed when the cells were cultured on the surface of Gtn-HPA-5-medium ($G' = 2529$ Pa). These results indicate that the range of stiffness of Gtn-HPA hydrogels that induces neurogenesis depends on the cell culture mode (2D or 3D), although the commitment of hMSCs to a neuronal phenotype is generally affected by hydrogel stiffness. It is worthwhile to note that in a 3D culture, the degradability and nutrient transportation in hydrogels, that are often linked to changes in hydrogel stiffness, also affect cell behavior. Thus, we consider that the ranges of hydrogel stiffness that induce neurogenesis are different between 2D and 3D cultures.

When the cells were stained with myogenic markers, they also showed a stiffness-dependent pattern of expression. The hMSCs

cultured on Gtn-HPA-5 stiff showed positive staining for myogenic markers such as MyoD1 and desmin (Fig. 8a). MyoD1 stained in green co-localized with the nuclei stained in blue. Co-staining the cells with MyoD1 and DAPI reveals their expression in light blue, and desmin was stained in red. In an attempt to further define the range of stiffness that promotes myogenesis of hMSCs, we utilized a hydrogel of higher stiffness (Gtn-HPA-10-stiff). Western blot results corroborated the up-regulation of myogenic protein markers when the hMSCs were cultured on these hydrogels (Fig. 8b). Conversely, cells that were cultured on the Gtn-HPA-5-medium did not show any protein.

The result of cell proliferation and differentiation on Gtn-HPA hydrogels indicates that the design of a hydrogel scaffold with well-

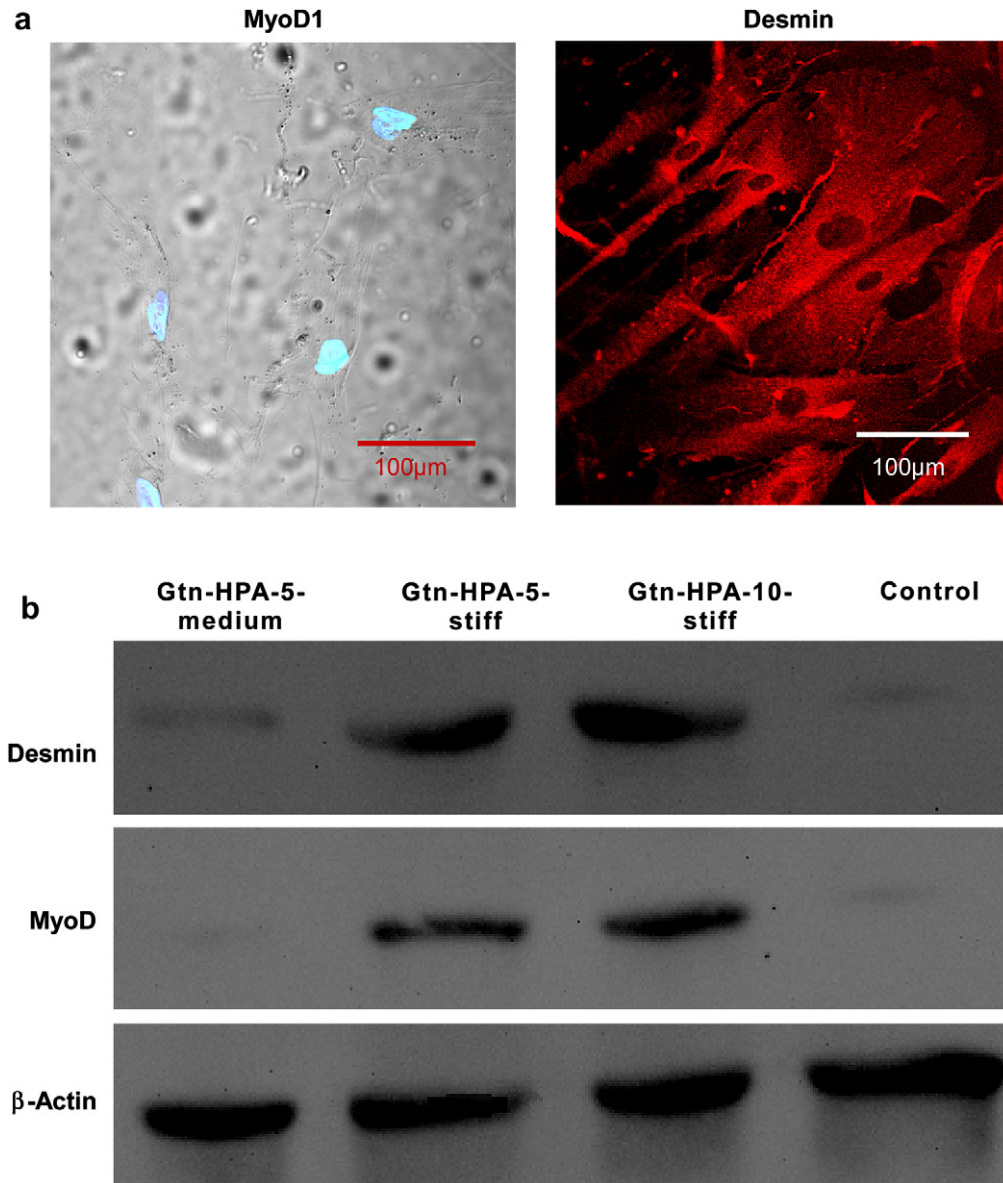


Fig. 8. (a) Immunofluorescence images of myogenic protein markers and (b) western blotting of proteins expressed in hMSCs cultured on Gtn-HPA hydrogels with different stiffness.

controlled mechanical properties is crucial for tissue engineering applications. Physical parameters are as important as biological and chemical parameters, to effectively repair, regenerate or engineer tissues.

4. Conclusion

Biodegradable Gtn-HPA hydrogels were formed by the oxidative coupling reaction of HPA moiety in the presence of H_2O_2 and HRP. The stiffness of the hydrogel was well-controlled by the H_2O_2 concentration without changing the concentration of polymer precursor solution. The hydrogels supported cell attachment and cell proliferation in a stiffness-dependent manner. It was found that hMSCs on stiffer hydrogels have a higher proliferation rate, larger spreading area, more organized cytoskeletons, more stable focal adhesions and faster migration rate. The stimulation of both neurogenic and myogenic differentiation of hMSCs on Gtn-HPA

hydrogels exhibited a stiffness-dependence without the additional use of any biochemical signal. Neurogenesis of hMSCs was observed when the hydrogel stiffness was in the range of 600–2500 Pa. The cells on a softer hydrogel (600 Pa) expressed more neurogenic protein markers. The myogenesis of hMSCs was achieved instead when the hydrogel stiffness is greater than 8000 Pa. We have demonstrated that Gtn-HPA hydrogels with tunable mechanical properties offer a promising system for cell therapy. Their excellent cell adhesion without coating with additional adhesive ligands, biodegradability and optical transparency are the added advantages for the use of Gtn-HPA hydrogels in tissue engineering.

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Appendix

Figures with essential color discrimination. Figs. 1, 5–8 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at [doi:10.1016/j.biomaterials.2010.07.075](https://doi.org/10.1016/j.biomaterials.2010.07.075).

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