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The spreading, migration and proliferation of mouse mesenchymal stem cells cultured inside hyaluronic acid hydrogels

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Abstract

Synthetic hydrogel scaffolds that can be used as culture systems that mimic the natural stem cell niche are of increased importance for stem cell biology and regenerative medicine. These artificial niches can be utilized to control the stem cell fate and will have potential applications for expanding/differentiating stem cells *in vitro*, delivering stem cells *in vivo*, as well as making tissue constructs. In this study, we synthesized hyaluronic acid (HA) hydrogels that could be degraded through a combination of cell-released enzymes and used them to culture mouse mesenchymal stem cells (mMSC). To form the hydrogels, HA was modified to contain acrylate groups and crosslinked through Michael addition chemistry using non-degradable, plasmin degradable or matrix metalloproteinase (MMP) degradable crosslinkers. Using this hydrogel we found that mMSC proliferation occurred in the absence of cell spreading, that mMSCs could only spread when both RGD and MMP degradation sites were present in the hydrogel and that mMSCs in hydrogels with high density of RGD (1000μM) spread and migrated faster and more extensively than in hydrogels with low density of RGD (100μM).

Introduction

Stem cells have the ability to self-renew and differentiate into a wide range of specialized cell types. Thus, they are very promising for the regeneration of aged, injured and diseased tissues [1–3]. Embryonic stem cells (ESC), induced pluripotent stem cells (iPS), and adult stem cells are currently the primary cell source for research in the lab and clinic. ESCs, which are derived from the inner cell mass of early stage embryos, can be differentiated into most of the cell types found in the body and can be expanded *in vitro* [4–6]. iPS cells are generated by reprogramming specialized cells (eg. fibroblast) to the pluripotent state through the addition of specific transcription factors (eg. Oct-3/4, SOX2, c-Myc, and Klf4). This results in cells that exhibit similar characteristics to ESCs [7–11]. Adult stem cells are extracted from adult tissues and can only be differentiated into limited cell types [12,13]. To successfully apply stem cells in clinics, there are many technical hurdles that need to be solved [14]. Current systems aimed at efficiently expanding and generating pure, pluripotent cells are lacking [15–23]. Furthermore, it is inefficient to differentiate them to a specific

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linage *in vitro*. It is difficult to expand adult stem cells *in vitro* without losing their ability to differentiate [24]. The harsh microenvironments at the disease sites usually leads to low stem cell survival rates and poor integration into the surrounding tissue [25].

Hydrogel-based synthetic 3D culture systems may provide a solution to these well-documented problems [24,26,27]. *In vivo*, stem cells reside in complicated and dynamic 3D microenvironments (stem cell niches) that are composed of extracellular matrix (ECM), soluble/tethered protein factors and supporting cells. This niche provides biophysical and biochemical cues that result in either a self-renewing homeostatic state or a state of cell expansion and differentiation to repair injured tissue [28–30]. Thus synthetic 3D culture systems that mimic stem cell niches can be utilized to control the stem cell fates and will have potential applications for expanding / differentiating stem cells *in vitro*, delivering stem cells *in vivo*, as well as making tissue constructs.

Hyaluronic acid (HA), an anionic, non-sulfated glycoaminoglycan and major component of the ECM, is widely distributed in connective, epithelial and neural tissue [31]. HA interacts with a number of cell surface receptors, such as CD44, RHAMM (receptor for HA-mediated motility) and ICAM-1(intercellular adhesion molecule 1) and contributes to tissue hydrodynamics, cell proliferation and migration [32,33]. Due to its high biocompatibility and low immunogenicity, HA is gaining popularity as a biomaterial for tissue engineering and tissue regeneration [34–37]. Functional groups, such as thiols, acrylates, amines, can be conjugated to the HA backbone through mild chemistries and further used as crosslinking sites to form hydrogels as scaffolds for cells *in vitro* and *in vivo* [38,39]. Several studies have demonstrated that HA-based hydrogels are good candidates for culturing stem cells [40–44]. In this study, we cultured mMSCs in HA hydrogels, which were degradable by hyaluronidases alone or a combination of hyaluronidase and plasmin or hyaluronidases and matrix metalloproteinases. We investigated the role of these different conditions on the ability of mMSCs to spread, migrate and proliferate. Furthermore, we studied the effect of the hydrogel mechanical properties and RGD concentration on migration and proliferation rates as well as the extent of cell spreading.

Methods

Materials

Peptides GCREG-PQGIWGQ-ERCG (HS-MMP-SH), GCRE-NRV-ERCG (HS-Plasmin-SH) and Ac-GCGYG-RGDSPG-NH2 (RGD) were purchased from Genscript (Piscataway, NJ). Bovine plasma thrombin and human fibrinogen (plasminogen depleted) were bought from Sigma-Aldrich (St. Louis, MO) and Enzyme Research Laboratories (South Bend, IN), respectively. Sodium hyaluronan (HA) was a gift from Genzyme Corporation (60 KDa MW, Cambridge, MA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

Cell culture

Mouse bone marrow cloned mesenchymal stem cells (D1, CRL12424) were purchased from ATCC (Manassas, VA) and cultured in DMEM (Sigma-Aldrich) supplemented with 10% bovine growth serum (BGS, Hyclone, Logan, Utah) and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY) at 37 °C and 5% CO2. The cells were passaged using standard protocols.

Modification of hyaluronic acid

Acrylated hyaluronic acid (HA-AC) was prepared using a two-step synthesis (Scheme 1A). Hyaluronic acid (1.0g, 0.017mmole, 60kDa) was reacted with 18.0 g (105.5mmole) adipic

dihydrazide (ADH) at pH 4.75 in the presence of 2.0g (10.41mmole) 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) overnight and purified through dialysis (8000 MWCO) in DI water for 1 week. The purified intermediate (HA-ADH) was lyophilized and stored at −20°C until used. 38.8% of the carboxyl groups were modified with ADH based on the trinitrobenzene sulfonic acid (TNBSA, Pierce, Rockford, Illinois) assay. HA-ADH (1.0g, 0.014mmole) was reacted with N-Acryloxysuccinimide (NHSAC) (0.75g, 4.4mmole) in HEPES buffer (pH 7.2) overnight and purified through dialysis in DI water for 1 week before lyophilization. All the primary amines were acrylated based on the TNBSA assay.

Preparation and characterization of HA hydrogels

HA hydrogels were formed by Michael Addition of bis-cysteine containing peptide crosslinker or DTT onto HA-AC pre-functionalized with cell adhesion peptides (RGD peptides) (Scheme 1B). Lyophilized aliquots of the crosslinker were diluted in 10 μL of 0.3 M TEOA (pH=8.0) buffer immediately before mixing with 90 μL HA-AC solution in 0.3 M TEOA with or without cells. The gel precursor solution was then placed between two Teflon plates for 30-min at 37 °C to allow for gelation. The final gel was swelled in DMEM before being placed inside 96-well plates for long-term culture or other tests.

The storage and loss modulus were measured with a plate-to-plate rheometer (Physica MCR, Anton Paar, Ashland, VA) using a 8mm plate under a constant strain of 0.05 and frequency ranging from 0.1 to 10 rad/s. Hydrogels were made as detailed above and cut to a size of 8.0mm in diameter to fit the plate. A humid hood was used to prevent the hydrogel from drying and the temperature was kept at 25°C.

Encapsulation of cells in 3D HA hydrogels

Cells were encapsulated into the 3D HA hydrogels using two protocols: homogeneous encapsulation, resulting in single cells throughout the hydrogel, and clustered encapsulation, resulting in a single cluster of cells inside a fibrin gel clot. The hydrogels containing homogeneously encapsulated cells were used to study cell proliferation and spreading while the cluster containing hydrogels were used to study cell migration. For the homogeneous encapsulation, 500,000 cells were mixed with 100 μL gel precursor solution before gelation. Cell clusters in fibrin gel clots were made by resuspending 150,000 D1 cells in 5 μL of fibrin and thrombin solution (2 mg/mL fibrinogen and 2 U/mL thrombin). The clusters were made by dropping the suspension onto a Teflon plate and incubating at 37 °C for 20 min. The clusters were incorporated into the HA gel by placing them inside the gel precursor solution. The gel was swelled in DMEM for two hours and cultured in DMEM supplemented with 10% BGS and 1% P/S. At the indicated time points, cells were imaged with an inverted microscope (Observer Z1, Zeiss) to record the phase pictures. These images were analyzed to quantify cell migration out of the fibrin clot. The distance from the edge of the fibrin clot to the leading edge of cell migration was measured for ~10–15 points per clot-containing hydrogel and averaged. Please note that the migration rate also depends on the water content of the fibrin clot. More water removal during the clot formation process results in a tighter clot and a slower migration rate. The clots in each experimental group were made in a similar manner and can be compared. However, only trends can be compared between each experiment and not the absolute values.

Cell viability in the HA gels with homogenously encapsulated cells was studied with the LIVE/DEAD® viability/cytotoxicity kit (Molecular Probes, Eugene, OR). Briefly, 1 μL of ethidium homodimer-1 and 0.25 μL of calcein AM from the kit were diluted with 500 μL DMEM without phenol red to. Each gel was stained with 150 μL of this staining solution for 30-min at room temperature in the dark and imaged with a confocal microscope (Leica TCS

SP MP) or an inverted fluorescence microscope (Zeiss Axio Observer). For confocal images 10 pictures were taken for a gel section with 10 μm thickness and the average of the pictures was presented. The Alamar Blue (Invitrogen) assay was used to quantify the cell proliferation rate inside the hydrogels. 20 μL of alamar blue dye was mixed with 100 μL phenol red free DMEM and added to each gel-containing well and incubated at 37 °C for 4 hrs. The proliferation ra tes were presented as fold increase over the value obtain after the first day of culture.

Results

Hydrogel preparation and characterization

Acrylates were conjugated onto the HA backbone through a two-step process (Scheme 1A). TNBSA assay results show that 38.8% of the –COOHs were coupled with ADHs. After reacting the HA-ADH with NHS-AC at pH 7.2 overnight, TNBSA assay showed that no free primary amines were detected for the final products, resulting in approximately 61 acrylates per HA chain.

RGD adhesion peptides were incorporated through Michael addition of the cysteine side chain in the peptide to the acrylate groups on the HA backbone. The cross-linker was then added to form the hydrogels (Scheme 1B). The storage (G') and loss modulus (G") were measured with a plate-to-plate rheometer. Results showing that the G' and G" did not cross at any measured frequency (0.1 to 10 Hz) and frequency independence (Fig. 1A, B) were consistent with typical hydrogel characteristics. The loss tangent values (ratio of G" to G') were lower than 0.06 for all hydrogels tested, indicating that the hydrogels were highly elastic. The mechanical properties of the hydrogels can be controlled by changing the HA concentration or the crosslinking density, “r”, which is defined as moles of –SH from the crosslinkers over moles of –ACs from the HA-ACs (Fig. 1C). The crosslinking density changed the mechanical property moderately. For 3% HA gels, the G' at r = 0.25, 0.4 and 0.5 was 177.1 ± 0.5, 305.4 ± 1.8, and 387.4 ± 1.9 Pa, respectively. The HA concentration had a larger effect on G’. At r = 0.25, the G' for 3, 4 and 5% gels was 177.1 ± 0.5, 817.7 ± 16.5 and 1920.0 ± 12.1 Pa, respectively.

Effect of the crosslinker type

The effect of crosslinker type on mMSC spreading, proliferation and migration was studied using three different crosslinkers: a non-degradable crosslinker, DTT, a plasmin degradable crosslinker, HS-Plasmin-SH [45], and a MMP degradable crosslinler, HS-MMP-SH [27]. Two RGD concentrations (100 and 1000 μM) were tested for each crosslinker. Live/Dead cell staining was performed to assess the cell viability inside the hydrogels immediately after gel formation (data not shown) and 3 days after culturing the cells in the gels (Fig. 2A–F). The sparse number of dead cells indicate that the chemistry and the chemicals for making the gels were non-lethal to cells. Cells in hydrogels crosslinked with DTT (HA/DTT hydrogel) and HS-Plasmin-SH (HA/Plasmin hydrogel) maintained spherical morphologies without spreading through the entire course of the experiment (Fig. 2A–D), while cells in hydrogels formed with HS-MMP-SH (HA/MMP hydrogel) exhibited spindle-like morphologies and extensive spreading (Fig. 2E, F). There were no significant differences in spreading and survival observed between the two RGD concentrations for all the crosslinkers.

A fibrin/cell clot was placed in the hydrogel to test if the hydrogel was able to support cell migration and which crosslinker resulted in the fastest migration. No migration was observed in HA/DTT and HA/Plasmin hydrogels regardless of the RGD concentration used

(Fig. 2G–J). Cells migration was seen when the mMSCs cultured in HA-MMP hydrogels, with faster migrations observed in the gel with 1000 μM RGD (Fig. 2K, L).

Alamar blue assay was utilized to evaluate if the crosslinkers affect the cell proliferation for cells cultured inside the hydrogels. The proliferation rate of cells in HA/DTT hydrogels was slower than those for HA/plasmin and HA/MMP hydrogels. However, the cells did proliferate through the first 3 days of culture. The proliferation rate of the cells in HA-Plasmin hydrogels climbed from day 1 to day 2 before hitting a plateau, whereas the proliferation rate of the cells in HA-MMP hydrogels increased throughout the culture. HA-MMP hydrogels were chosen for the remaining of the study.

Effect of RGD concentration

RGD peptides, which are ligands for integrin receptors, were introduced as adhesion points in the gels. Though cells were alive throughout the culture as shown by the absence of dead cells through the live/dead cell staining, they were unable to spread and maintained spherical morphologies in the HA-MMP hydrogels without RGD (Fig. S1 and 3A, RGD 0 μM). Though no influence on the cell viability was observed, introducing RGD significantly changed the cell morphology to exhibit spindle-like morphologies (Fig. S1 and 3A, RGD 100 to 1000 μM). The RGD concentration influenced both the starting time and the extent of cell spreading. Few cells were spreading after 1-day of culture (referred as Day 1 for the rest of the paper) and large percentage of cells remained spherical at after 3-days of culture inside the hydrogels with low RGD concentrations (Fig. S1 and 3A, RGD 100, 250 μM). While the majority of cells were spreading at day 1 inside the hydrogels with high RGD concentrations (Fig. S1 and 3A, RGD 750, 1000 μM), it was difficult to quantify the extent of spreading. However, it was generally observed that cells were much longer and extended in hydrogels with more RGD.

RGD concentration affected the cell proliferation as well, with low RGD concentration resulting in enhanced proliferation (Fig. 3B, RGD 100 and 250 μM) than with high RGD concentrations (Fig. 3B, RGD 500, 750 and 1000 μM). Surprisingly, cells in the hydrogel without RGD proliferated as well as the gels with low RGD concentrations (Fig. 3B). We further tested if RGD concentration was an important factor to modulate cell migration in HA hydrogels. Cells failed to migrate without RGD (Fig. 3C, RGD 0 μM), while they migrated in the hydrogels with RGD (Fig. 3C, RGD 100–1000 μM). The migration rates in HA hydrogels (3% HA, r = 0.4) with 0, 100, 250, 500, 750 and 1000 μM RGD were 0, 55, 68, 77, 72 and 87 μm/day, respectively (Fig. 3D). Generally, high RGD concentrations promoted faster cell migration.

Effect of HA concentration

We tested if cells spread, proliferate and migrate differently in hydrogels with different HA concentrations. HA hydrogels with 3, 4 and 5 HA-% and either 100 or 1000 μM RGD were used. Cells were alive in all the hydrogels as shown by the Live/Dead staining (Fig. 4A). With 100 μM RGD, only the cells in the 3% HA spread, as indicated by the spindle-like morphologies inside the 3% hydrogel and the spherical morphologies inside the 4 and 5% gels. With 1000 μM RGD, cells in 3% and 4% HA exhibited spindle-like morphologies, while cells in the 5% HA stayed spherical (Fig. S2 and 4A). The proliferation rates of cells in 3, 4, and 5% HA hydrogels with 100 μM RGD all increased during the culture. However, cells proliferated slower at higher HA concentrations (Fig. 4B).

High HA concentration inhibited cell migration (Fig. 4C) as hydrogels with 100 μM RGD in 3, 4 and 5% HA had migration rates of 95, 50 and 0 μm/day respectively. With 1000 μM

RGD, the migration rates increased to 88 and 35 μm/day in the 4 and 5% HA gels, but only slightly affected the migration rate in the 3% HA gel (99 μm/day) (Fig. 4D).

Effect of crosslinking density

We tested if the crosslinking density influenced the D1 cells in the gels. HA-MMP hydrogels (3% HA) at r = 0.25, 0.4 and 0.5 in the presence of 100 and 1000 μM RGD were prepared. Cells were viable in all the hydrogels as shown by the Live/Dead cell staining (Fig. 5A). Increasing the crosslinking density inhibited cell spreading. At 100 μM RGD the majority of cells exhibiting spherical morphologies in the r = 0.4 and 0.5 hydrogels. Raising the RGD concentration to 1000 μM led to cell spreading in all the hydrogels (Fig. S3 and 5A). The proliferation rates of cells in 3% HA at r = 0.25 and 0.4 increased during the culture, while for r = 0.5 proliferation plateau after the second day of culture (Fig. 5B).

The cell migration rate was extensive for all the crosslinking ratios tested (Fig. 5C). The migration rates were higher for the lowest crosslinking ratio used for both 100 μM and 1000 μM with migration rates of 220 μm/day and 240μm/day, respectively (Fig. 5D). For r = 0.4 the migration rates were 220 μm/day and 160 μm/day for 100 and 1000μm/day respectively and for r = 0.5 the migration rates were 210 μm/day and 160 μm/day respectively for 100 and 1000 μM RGD respectively (Fig. 5D).

Discussion

The natural ECM is an extremely dynamic network that consists of protein fibers and polysaccharides that support cell fate and provide biophysical and biochemical cues to cells through cell surface receptors, such as integrins [46,47]. Cells degrade the ECM through proteases during their migration. Synthetic ECM hydrogels that aim to provide an environment to direct stem cell fate should recapitulate key features of the natural ECM such as integrin mediated cell binding, cell migration, and cell proliferation, while also allowing for soluble factor diffusion to the encapsulated cells. Here HA was crosslinked with DTT or peptides using Michael addition chemistry to form hydrogels that were degradable exclusively through hyaluronidases or through a combination of hyaluronidases and plasmin or hyaluronidases and MMPs. In addition, the HA backbone was modified with RGD peptides to introduce integrin binding sites.

HA hydrogels were formed through the reaction of acrylate groups in the HA backbone and thiols at the ends of DTT or peptide crosslinkers. Acrylate groups were introduced to the HA backbone using a two-step synthetic scheme (Scheme 1A), which resulted in approximatedly 61 acrylates per HA chain (38% modification). This large number of acrylates per chain brings two advantages over 4 or 8-arm PEG based systems. First, since only 10% percent of acrylates are needed to form a hydrogel, it allows the other 90% of acrylate groups to be used to incorporate bioactive signals, such as adhesion peptides and protein growth factors without compromising hydrogel formation. Second, it enables a wide range of crosslinking densities without changing the percent of HA used. In these experiments we used 3% HA w/v and incorporated up to 1000μM RGD.

Cell spreading and migration inside hydrogels required that the cells be able to displace the polymer chains or degrade them in order to make space for their movement. Hyaluronic acid hydrogels are attractive tissue engineering scaffolds because the backbone is completely degradable by cell release hyaluronidases. In order to determine if degradation by hyaluronidases was sufficient to allow mMSC spreading, proliferation and migration *in vitro*, HA was crosslinked with DTT, a non-degradable crosslinker (Fig 2). Although cells inside HA/DTT hydrogels fail to spread or migrate, LIVE/DEAD staining and proliferation assays demonstrated that the cells were viable and able to proliferate. These results indicate

that hyaluronidases released by mMSCs alone cannot promote spreading or migration; extensive matrix degradation was not needed for proliferation or keeping the cells alive. These results agree with previous reports that show viable cells in MMP insensitive HA hydrogels with or without RGD peptide [43]. HA crosslinked with a plasmin degradable crosslinker also resulted in no cell spreading or migration, indicating that mMSCs do not produce significant amounts of plasmin to allow cell migration or spreading. However, the cells were viable and able to proliferate. These results differ from those regarding fibroblast culture inside plasmin degradable PEG hydrogels, in which spreading and migration was observed [48]. Our results further confirm that extensive matrix degradation is not required for viability or proliferation. MMP degradable HA hydrogels resulted in significant cell spreading, proliferation and migration for both 100μM and 1000μM RGD concentration. These results agree with previously reports that show mMSC [49] and fibroblast [48] cell spreading and proliferation in MMP degradable HA and PEG hydrogels, respectively.

Integrin mediated cell-matrix interactions were necessary for cell spreading as showed by the lack of spreading and migration of the cells in HA-MMP gels without RGD (Fig. 3). In general, higher concentration of adhesion peptides in the gels corresponds to stronger cell-matrix interactions [50]. Stronger cell-matrix interactions lead to cells spreading earlier, more extensively and migrating faster as demonstrated by the earlier start spreading time, longer morphologies and faster migration rates in the presence of 1000 μM RGD (Fig. 3C, D). These findings are similar to previously published reports that show extensive spreading for higher concentration RGD in PEG hydrogels [51].

Cell-matrix interactions affected cell proliferation differently than that observed for spreading and migration. Cells cultured inside hydrogels with low RGD concentration proliferated more than cells cultured inside hydrogels with high concentration of RGD (Fig. 3B). These results agree with previous results that showed that bone marrow stroma cells proliferated at the same rate inside PEG or HA hydrogels with or without RGD peptide modification [43,52].

The ability of mMSCs to spread, proliferate and migrate in HA/MMP hydrogels with different mechanical properties was studied using different crosslinking ratios, while keeping the HA concentration constant or by changing the HA concentration, while keeping the crosslinking ratio constant. The resulting hydrogels ranged in storage modulus from 177 to 387 Pa for 3% gels formed with different crosslinking ratios and 177 to 1919 Pa for different HA concentrations, which cover the mechanical properties of the soft tissues in the body (Fig. 1) [54]. Changes in mechanical properties from 177 to 387 Pa did not result in changes in spreading for high concentrations of RGD (1000 μM, Fig. 5). However, cell spreading was inhibited in 387 Pa hydrogels, which contained 100 μM RGD, indicating that the density of integrin binding sites can overcome spreading limitations in stiffer higher crosslinked materials. In general, migration was faster for cells seeded in lower crosslinked hydrogels (softer). Increasing the HA concentration from 3% to 4% in the presence of 100 μM RGD stopped cell spreading, inhibited the cell migration and slowed down proliferation (Fig. 4). Similarly to our findings with different crosslinking ratios, increasing the RGD concentration to 1000 μM facilitated cell spreading and migration for 4% hydrogels (816 Pa). However, for 5% hydrogels (1919 Pa) no spreading or migration was observed and proliferation was significantly lower than for softer hydrogels.

Mechanical properties have been shown to strongly modulate stem cell fate [55]. For cells cultured inside hydrogel scaffolds with elastic modulus of 11–30 kPa and 2.5–5 kPa lead mMSCs commit to osteogenic and adipogenic lineages, respectively [50]. Further, MSCs, seeded in two dimensions, can be differentiated into neuron, myoblast, osteoblast lineages by using substrates with compliances close to the brain, muscle and bone tissues,

respectively [54]. Thus hydrogels with a wide range of mechanical properties, such as the ones reported, are ideal to study stem cell differentiation potential.

Conclusion

HA hydrogels were developed for 3D culture of stem cells. The effects of the hydrogel properties on mMSC proliferation, spreading and migration were studied. Variable parameters include: crosslinker type, concentration of adhesion peptide (RGD), HA concentration, and crosslinking density. Degradation of the scaffold via MMPs was required for cell spreading and migration, but not for cell proliferation. Introducing the RGD adhesion peptides into the hydrogel enabled the cells to interact with the scaffold and was crucial for cell spreading and migration. In general, cells spread earlier, more extensively and migrated faster in the presence of high concentration of RGD. However, cells in hydrogels with low concentration of RGD exhibited high proliferation rates. The mechanical properties of the hydrogels, which could be controlled through changing the HA concentration or crosslinking density, influenced the cell behavior as well. Cells in stiffer hydrogels showed less spreading, migration and slower proliferation rates. The information gained can provide valuable insight into the designing of hydrogels for 3D stem culture. Additionally, the hydrogel developed in this study can be used as a platform for developing synthetic niches for various stem cells in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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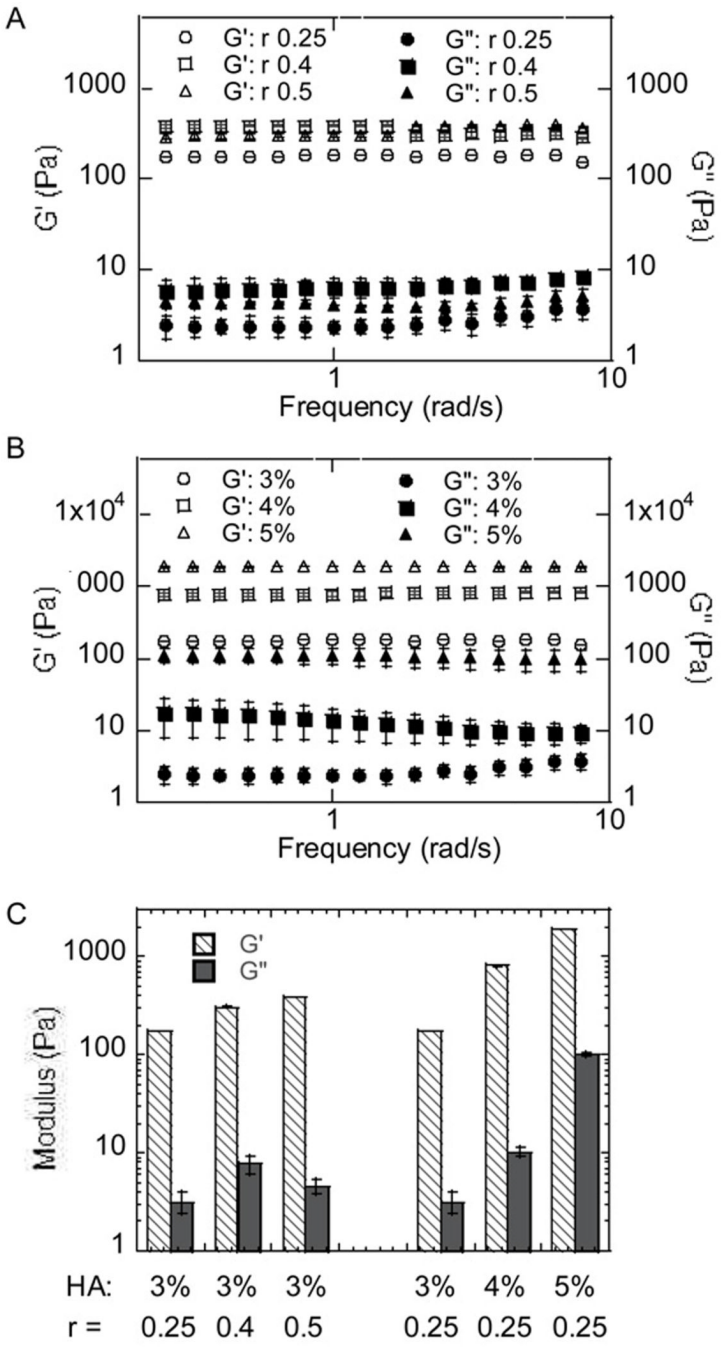


Figure 1.

The storage (G') and loss modulus (G") of HA-MMP hydrogels. (A) 3% HA hydrogels with various crosslinking densities (r = 0.25, 0.4, 0.5). (B) 3%, 4%, 5% HA hydrogels at r = 0.25. The modulus were measured with a rheometer at constant strain of 0.05 with frequency ranging from 0.1 to 10 rad/s. The average of G' and G" over the frequency range were shown in (C)

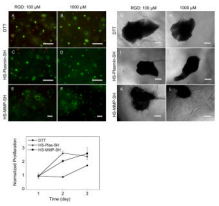


Figure 2.

Effect of crosslinker identity on D1 cells in HA hydrogels. (A–F) Cell viability at day 3 (green: live cell; red: dead cell). (G–L) Cell migration from fibrin/cell clot into hydrogels. Snapshots were taken at day 3. The dash lines highlight the edges of the clots. “C” refers to fibrin/cell clot. (M) Proliferation rates of cells in hydrogels with 100 μM RGD. Crosslinker: DTT for (A, B, G, H); HS-Plasmin-SH for (C, D, I, J); HS-MMP-SH for (E, F, K, L). RGD concentration: 100 μM for (A, C, E, G, I, K); 1000 μM for (B, D, F, H, J, L). Scale bar: 100 μm. 3.5% HA and r = 0.4 for all gels

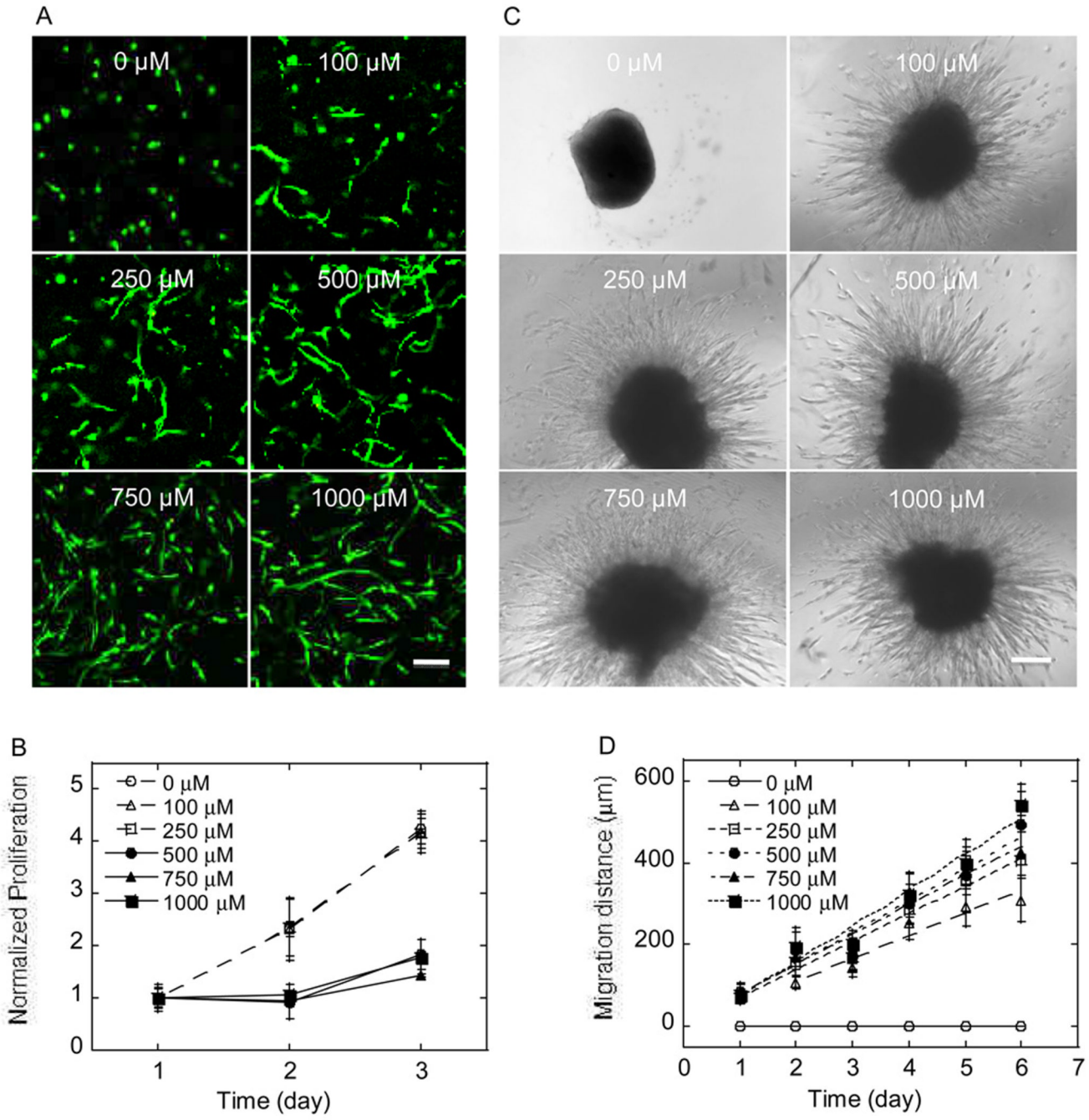


Figure 3.

Effect of RGD concentration on D1 cells in HA-MMP hydrogels. (A) Cell viability and spreading at day 3 (green: live cell; red: dead cell). (B) Proliferation rates. (C) Cell migration at day 5. The dash lines highlight the edges of the clots. “C” refers to fibrin/cell clot. (D) Migration rates were 0, 55, 68, 77, 72 and 87 μm/day for cells in the gels with 0, 100, 250, 500, 750 and 1000 μM RGD respectively. Scale bars for (A) and (C) are 200 and 100 μm, respectively. 3% HA and r = 0.4 for all gels

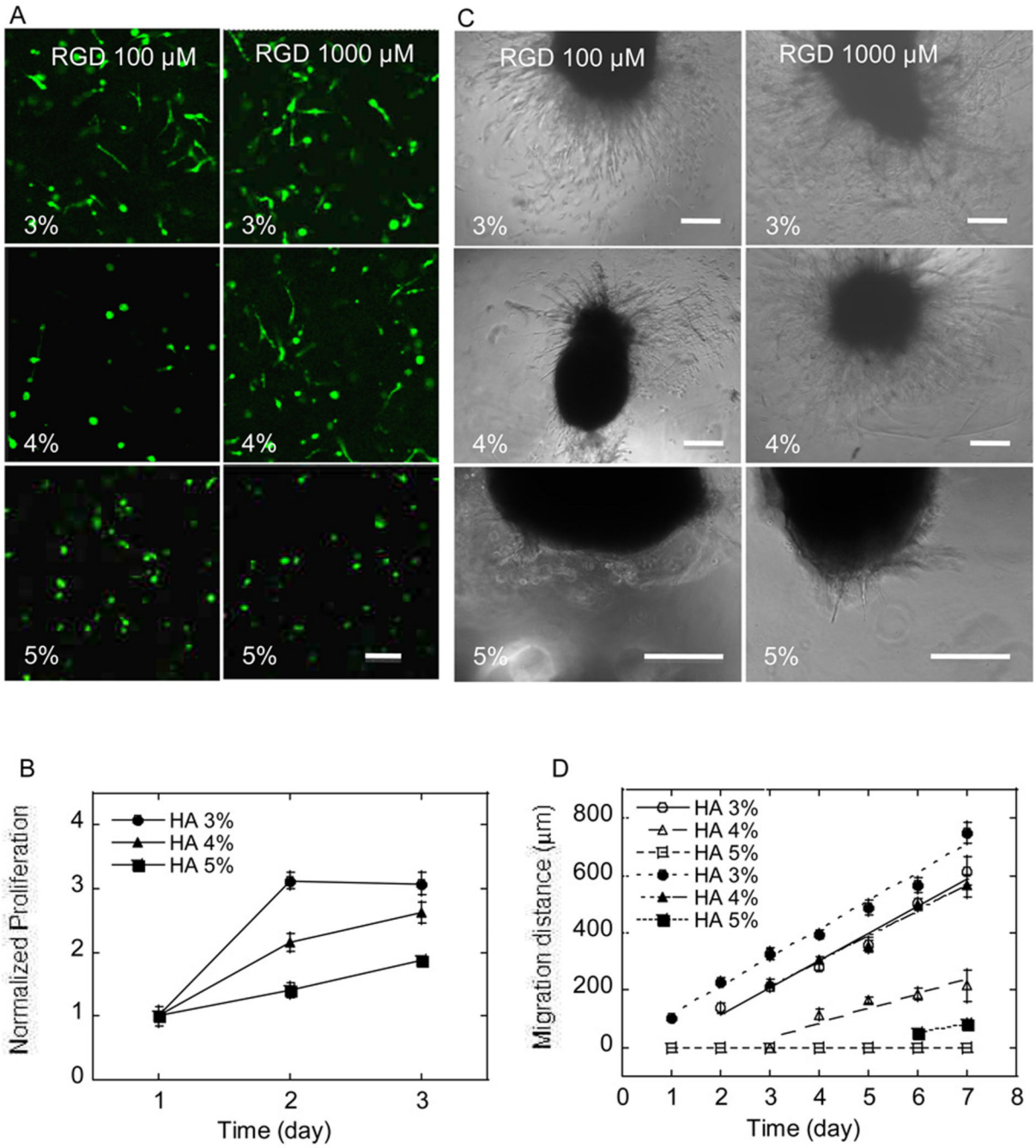


Figure 4.

Effect of HA concentration on D1 cells in HA-MMP hydrogels. (A) Cell viability and spreading at day 2 (green: live cell; red: dead cell). (B) Proliferation rates of cells in 3%, 4% and 5% HA gels with 100 μM RGD. (C) Cell migration. Snapshots were taken at day 6. The dash lines highlight the edges of the clots. “C” refers to the fibrin/cell clot (D) Migration rates were 95, 50, 0 μm/day in 3%, 4%, 5% hydrogels with 100 μM RGD (open symbols) and were 99, 88, 2 μm/day in 3%, 4%, 5% hydrogels with 1000 μM RGD (filled symbols), respectively. Scale bars for (A) and (C) are 200 and 100 μm, respectively. r = 0.25 for all gels.

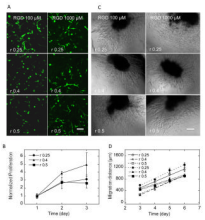
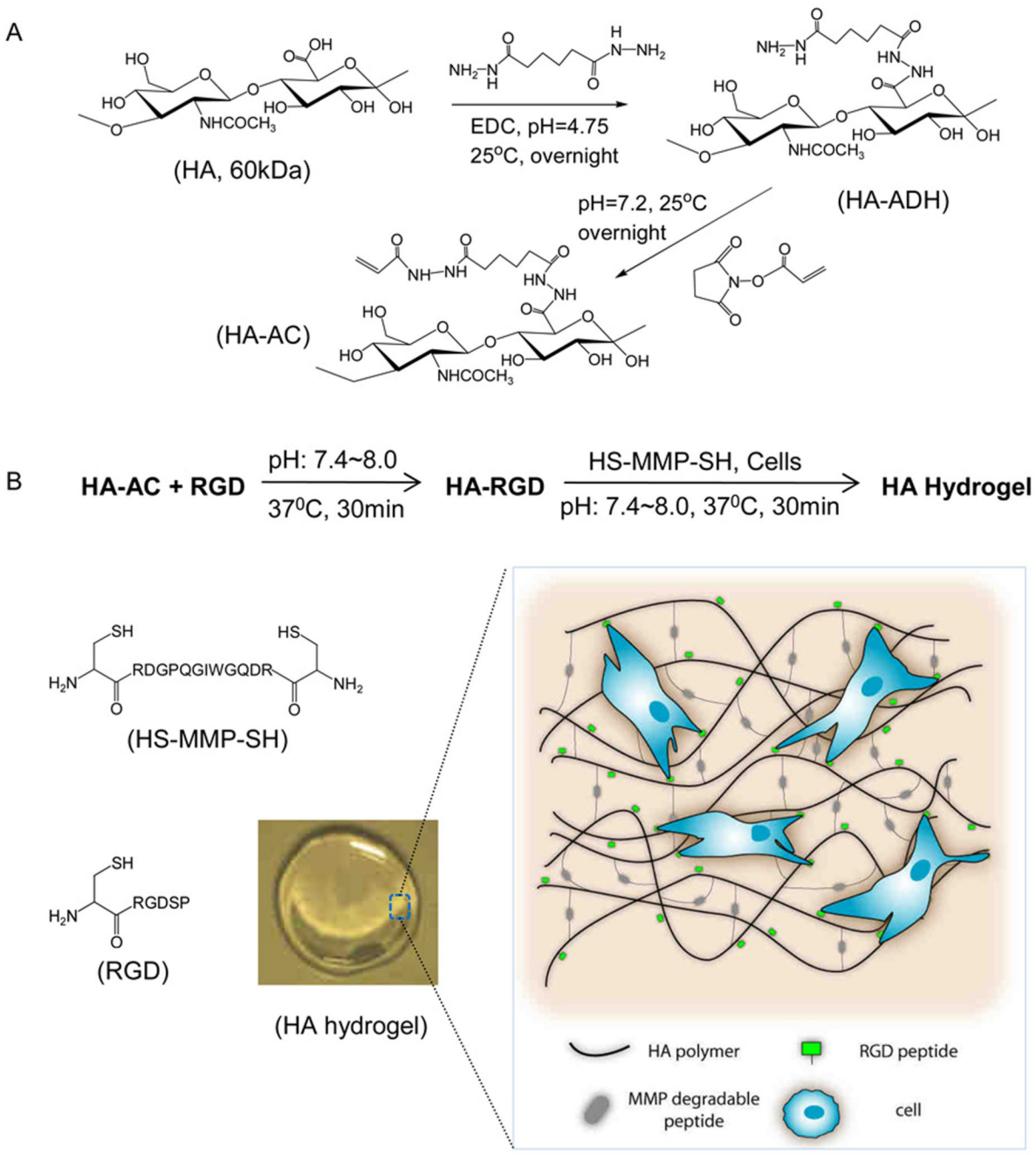


Figure 5.

Effect of crosslinking density on D1 cells inside HA-MMP hydrogels. (A) Cell viability and spreading at day 2 (green: live cell; red: dead cell). (B) Proliferation rates of cells in hydrogels with 100 μM RGD. (C) Cell migration. Snapshots were taken at day 3. The dash lines highlight the edges of the clots. “C” refers to the fibrin/cell clot (D) Migration rates were 220, 220, 210 μm/day in hydrogels at r = 0.25, 0.4, 0.5 at 100 μM RGD (open symbols) and were 240, 160, 160 μm/day hydrogels at r = 0.25, 0.4, 0.5 at 1000 μM RGD (filled symbols), respectively. Scale bars for (A) and (C) are 200 and 100 μm, respectively. 3% HA for all gels



Scheme 1.
(A) Synthesis of HA-AC and (B) Making HA hydrogels through Michael Addition