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4 **Influence of viscosity on bone marrow-derived mesenchymal**
5 **stem cells trilineage differentiation during 3D culture**
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10 **Keywords:** viscosity, 3D culture, adipogenic differentiation, chondrogenic differentiation,
11 osteogenic differentiation, mesenchymal stem cells
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14 **Abstract**
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16 Stem cells can respond to mechanical stimuli such as stiffness, viscoelasticity, fluid shear
17 stress, micropatterned geometry and hydraulic pressure. However, viscosity as an
18 important cue is often overlooked. Thus, in this study, the influence of viscosity on
19 trilineage differentiation (adipogenesis, chondrogenesis and osteogenesis) of human bone
20 marrow-derived mesenchymal stem cells (hMSCs) was disclosed by three-dimensionally
21 culturing hMSCs in viscous media. The viscosity was modulated using bioinert
22 polyethylene glycol (PEG) at a range of 88.8 to 645.5 cP. A cuboid agarose hydrogel
23 container was used to encapsulate the cells and viscous media to prevent cell leakage and
24 PEG diffusion during cell culture. Viscosity showed inhibitory effects on trilineage
25 differentiation of hMSCs during 3D culture in viscous media containing PEG. The
26 inhibitory effect on adipogenic and chondrogenic differentiation was stronger than that on
27 osteogenic differentiation. Viscosity also affected cell proliferation. Viscosity strongly
28 promoted cell proliferation during chondrogenesis, and weakly promoted cell proliferation
29 during osteogenesis, while inhibited cell proliferation during adipogenesis. The influences
30 of viscosity on proliferation and trilineage differentiation of hMSCs were related to the
31 formation of cell aggregates and spheroids during 3D culture in the viscous media. The
32 results revealed the importance of viscosity on stem cell differentiation and could provide
33 some information for tissue engineering applications.
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39 **Introduction**
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41 Stem cells are highly valued in tissue engineering and regenerative medicine. Revealing
42 and understanding how environmental stimuli especially mechanical cues affect stem cell
43 differentiation is becoming increasingly important [1,2]. Many studies have explored the
44 influence of stiffness, viscoelasticity, ECM architecture and substrate nanopatterns on stem
45 cell differentiation potential [3–5]. The cells inhabiting physiological conditions
46 experience not only the stiffness stimulus generated by the surrounding matrix, but also the
47 dissipative property from the tissues. Therefore, many strategies have been envisaged to
48 enable mimicking of viscoelastic properties of the physiological tissues [6]. For example,
49 material designs have been used to tune viscoelastic properties by incorporating dynamic
50 covalent bonds such as hydrazone [7], oxime [8], boronate [9], disulfide and thioester
51 bonds [10,11]. Although these dynamic materials can provide tunable mechanical
52 parameters such as storage or loss moduli, they cannot fully recapitulate the pure viscous
53 environment existing in mucus [12], bone marrow [13,14] and synovial fluids [15–17].
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Thus, there is an impetus to culture cells in viscous conditions to elucidate how the viscosity affects stem cell differentiation.

To prepare a viscous culture condition, both natural polymers such as dextran [18,19], gelatin [20–22] and alginate [23], and synthetic polymers such as methylcellulose (MC), polyvinyl pyrrolidone (PVP) and polyethylene glycol (PEG) of different molecular weights have been adopted [18,24]. Some of these polymers are bioactive while some are bioinert. Bioactive polymers can provide not only a viscous microenvironment but also biological cues for matrix-cell interactions. However, bioinert polymers provide solely viscous microenvironment excluding specific interaction between the microenvironment and cells. The polymer type with or without bioactive moieties, which is used to adjust the viscosity of the culture medium, can affect the results. Previous studies employing bioactive gelatin to adjust the viscosity of the culture medium showed that high viscosity is detrimental to adipogenesis while encouraging both osteogenesis and chondrogenesis [21,22]. Intriguingly, another study using bioinert PEG showed that high viscosity induces an attenuated level of cartilaginous gene expression and matrix secretion [24]. Therefore, the bioactive moieties of polymers can alter stem cell fate commitment [25]. Furthermore, the content of bioactive moieties is dependent on the concentration of bioactive polymers, which may lead to undesired interference in the results [26,27]. To solely mimic the microenvironmental viscosity without interfering with other bioactive moieties, bioinert polymers such as PEG are preferable.

When cells are cultured in a viscous medium, both two-dimensional (2D) and three-dimensional (3D) culture systems can be used [28]. The 2D culture system has advantages of simplicity and convenience. However, it is difficult to suspend cells in the viscous medium because cells sink and adhere to the surfaces of culture plates. If non-adhesive culture plates are used to protect cell adhesion, medium change becomes difficult. The 3D culture system allows cells to suspend in a viscous medium and facilitates medium exchange. Some techniques such as layer-by-layer technique [23] and UV-assisted microfluidic system [29,30] have been developed to encapsulate cells in the viscous medium for 3D culture. However, these techniques require specialized equipment. In contrast to the aforementioned 3D culture systems, in this study, we used an agarose-based cuboid 3D culture system that could be simply fabricated. The cells suspended in a viscous medium could be encapsulated in cuboid agarose hydrogel containers. The system enabled the 3D culture of stem cells in media of different viscosities to disclose the influence of viscosity on adipogenic, chondrogenic and osteogenic differentiation of hMSCs. Viscosity showed an inhibitory effect on three differentiations (adipogenesis, chondrogenesis and osteogenesis) of hMSCs.

Materials and methods

Preparation of viscous media

The viscosities of different inductive culture media were regulated by PEG [24]. High molecular weight PEG (PEG 8 M, Mw ~8,000,000, Sigma-Aldrich), low molecular weight PEG (PEG 35 K, Mw ~35,000, Sigma-Aldrich) and their mixture at a weight ratio of 3:1

were used to prepare culture media with high, low and middle viscosity, respectively. Firstly, the polymers were weighed and dissolved in Milli-Q water to reach a concentration of 1.5 w/v%, followed by passing through a Millipore syringe filter (0.22 or 0.45 μm , Merck Millipore) for sterilization. Ten-fold concentrated Dulbecco's Modified Eagle's Medium-high glucose (H-DMEM, Sigma-Aldrich) medium and Dulbecco's Modified Eagle's Medium-low glucose (L-DMEM, Sigma-Aldrich) medium were prepared based on the manufacturer's instructions and sterilized by filtration. Thereafter, 1.5% of the stock polymer solution was mixed with the ten-fold concentrated DMEM medium and further supplemented with 74 g/L sodium bicarbonate, 0.4 mM L-proline, 50 mg/L ascorbic acid, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin to prepare the complete medium containing 1.0 w/v% PEG polymers. The induction media were prepared based on the previous report [22]. For the viscous adipogenic medium, the complete H-DMEM was further supplemented with 10% fetal bovine serum (FBS, Gibco), 1 μM dexamethasone (Dex, Sigma-Aldrich), 500 μM methyl-isobutylxanthine (IBMX, Sigma-Aldrich), 10 $\mu\text{g/ml}$ insulin (Sigma-Aldrich) and 100 μM indomethacin (Sigma-Aldrich). For the viscous chondrogenic medium, the complete H-DMEM was further supplemented with 0.1 μM dexamethasone, 10 ng/ml TGF- β 3 (Sigma-Aldrich), 1% ITS (Sigma-Aldrich) and 1X nonessential amino acids (Gibco). For the viscous osteogenic medium, the complete L-DMEM was further supplemented with 10% FBS, 10 nM dexamethasone and 10 mM β -glycerophosphate disodium salt hydrate (β -GP, Sigma-Aldrich). The induction medium without PEG polymers was prepared in the same way as the above-mentioned to serve as the normal viscosity group.

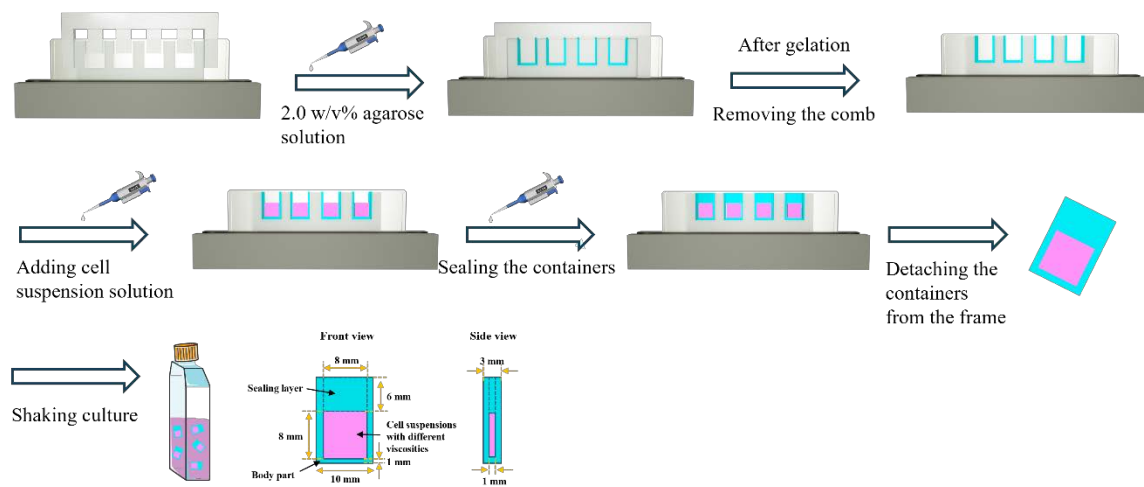
Viscosity measurement

The viscosity of the inductive culture media was measured by an MCR 302 rheometer (Anton Parr, Germany). PP-50 parallel plates (with a diameter of 50 mm) were used in the measurement at a testing gap of 1.0 mm. Around 2.0 ml of viscous media were pipetted into the gap and an oil trap was performed to avoid evaporation of water during the measurement. The tests were performed under shear rates ranging from 0.1 to 100 /s at a constant temperature of 37 $^{\circ}\text{C}$. The zero-shear viscosity was regarded as the viscosity at a shear rate of 0.1 /s.

Preparation of hMSC-encapsulated agarose hydrogel containers and cell culture

As shown in Scheme 1, cuboid agarose hydrogel containers with hMSCs/medium encapsulated were prepared. Firstly, hMSCs were subcultured (passage 4) and digested from the T175 flask by trypsin-EDTA solution (T4049-100 ml, Sigma-Aldrich). Thereafter, the digested cells were mixed with the above-mentioned viscous media to reach a cell concentration of 5×10^6 cells/ml. Then, agarose powder (A6013, Sigma-Aldrich) was sterilized by an autoclave and further dissolved in the appropriate amount of PBS at 110 $^{\circ}\text{C}$ to prepare 2 w/v% and 1 w/v% solutions. The 2% agarose solution was kept in a 65 $^{\circ}\text{C}$ water bath and used for the preparation of the agarose hydrogel container body with one side open. The 1% agarose solution was kept in a 37 $^{\circ}\text{C}$ water bath and used to seal the agarose hydrogel containers. To prepare hydrogel containers, the polytetrafluoroethylene (PTFE) holders, polycarbonate (PC) comb frames, glass slides and silicone frames were

sterilized and assembled in a clean bench (Scheme 1). Subsequently, 2% agarose solution was used to shape the container body and the inner cavity was formed by pressing the PC comb frame inside. After cooling at room temperature and removing the PC comb frame, cuboid agarose hydrogel containers containing the cavity were prepared. Then, 64 μ l of the cell suspension solution was pipetted into each cavity. Finally, another 48 μ l of 1% agarose solution was added on the top of cell suspension solution to seal the cavity. The agarose hydrogel containers had an outer dimension of $15 \times 10 \times 3$ mm and a cavity dimension of $8 \times 8 \times 1$ mm. The prepared containers were transferred into a T75 flask with 40 ml appropriate differentiation induction media and cultured under a shaking condition at 100 rpm in a CO₂ incubator at 37 °C. The culture medium was changed every 3 d.



Scheme 1. Schematic illustration of the preparation of hMSC-encapsulated agarose hydrogel containers. The cells were suspended in the medium of different viscosities to investigate the viscosity influence on stem cell differentiation.

Live/dead staining and cell proliferation of the encapsulated hMSCs

To check cell viability immediately after the preparation of hMSC-encapsulated hydrogel containers and after 21 d of different induction cultures under various viscosities, a double staining kit (CS01/341-07381, DOJINDO) was employed. Specifically, the hydrogel containers were washed with PBS thrice and followed by treatment with 2 μ M calcein-AM and 4 μ M propidium iodide at room temperature for 45 min. The stained cells inside the hydrogel containers immediately after preparation and after 21 d culture were observed by a fluorescent microscope (Olympus DP74, Japan) and a CLSM (confocal laser scan microscopy, ZEISS LSM 900, Germany), respectively.

The proliferation of hMSCs in hydrogel containers was evaluated on 7, 14 and 21 d by quantifying the DNA amount. At first, the hydrogel containers were washed with PBS thrice. Then, the containers were split with a steel spatula and washed again with PBS.

Thereafter, cells together with the split hydrogel containers were transferred to a 15 ml centrifugation tube for centrifugation. After centrifugation, the supernatant was removed and 500 μ l of Milli-Q water was added. Then, the samples were freeze-dried. To further digest cells and release DNA, 1 ml of 400 μ g/ml papain solution (P4762, Sigma-Aldrich) in 0.1 M phosphate buffer (pH 6.0) supplemented with 5 mM ethylenediaminetetraacetic acid disodium salt dihydrate (E5134-100G, Sigma-Aldrich) and 5 mM L-cysteine hydrochloride monohydrate (C7880-100G, Sigma-Aldrich) was applied and the samples were digested at 60 $^{\circ}$ C for 6 h under shaking. After digestion, an aliquot of the supernatant was mixed with bisbenzimidazole solution (Hoechst 33258) and the fluorescence was measured by an FP-8500 spectrofluorometer (JASCO, Japan) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. DNA amount was calculated based on a standard fluorescence curve. Triplicate samples were used for the measurements.

Nile Red staining

To indicate the intracellular oil droplets accumulation during the adipogenic differentiation, Nile Red dye was used [31]. The Nile Red working solution was prepared by diluting the stock solution (1 mM in DMSO, 72485, Sigma-Aldrich) with PBS (1:1000 dilution). Firstly, the containers were taken out from the flask and transferred into a 6-well plate, followed by washing with PBS twice. Then, the samples were fixed with 4% paraformaldehyde phosphate buffer (163-20145, Wako) at 4 $^{\circ}$ C for 6 h and then washed with PBS twice. Thereafter, the samples were immersed into 3 ml Nile Red working solution at 37 $^{\circ}$ C for 30 min in the dark. After staining, the nuclei were stained with Hoechst (343-07961, Dojindo) at room temperature for 15 min. The stained cells inside the hydrogel containers were examined by a CLSM (ZEISS LSM 900, Germany).

Sulfated glycosaminoglycan (sGAG) production assay

Sulfated glycosaminoglycan (sGAG) was quantified after the cells were exposed to different viscous chondrogenic induction media for 21 d for the characterization of chondrogenesis. The sample preparation method was the same as that used for the DNA quantification. After papain digestion, the BlyscanTM Glycosaminoglycan Assay Kit (B1500, Funakoshi) was used to analyze the sGAG content in the papain lysates according to the manufacturer's instructions. The concentration of sGAG was measured by a microplate reader under the wavelength of 656 nm based on a standard curve obtained from the sGAG standard supplied with the kit. The contents of sGAG were further normalized by DNA amount. Quintuplicate samples were used for the analysis to calculate means and standard deviations.

Alkaline phosphatase (ALP) and calcium deposition quantification during osteogenic differentiation

ALP activity of each sample was evaluated with a SensoLyte[®] pNPP Alkaline Phosphatase Assay Kit (AS-72146, Anaspec) based on the manufacturer's instructions. Briefly, the samples cultured for 7 d were collected and rinsed with PBS twice. Thereafter, the samples were frozen in liquid nitrogen and crushed into powder using an electric crusher. The crushed sample powder was collected and transferred into a 1.5 ml centrifuge tube. 1 ml of

Triton-X 100 containing the assay buffer was added to each sample. The lysates were then mixed homogeneously and centrifuged at a speed of $10,000 \times g$ for 15 min at 4 °C. The supernatants were then incubated with p-nitrophenyl phosphate (pNPP) substrate solution at room temperature for 40 min and colorimetric detection was conducted at 405 nm. The amount was analyzed based on a standard calibration curve by an ALP standard solution. The results were further normalized with the DNA amount of each sample. Quintuplicate samples were used for the analysis to calculate means and standard deviations.

To characterize calcium deposition in different viscous environments during osteogenic induction. A calcium assay kit (DICA-500, BioAssay Systems) was employed according to the manufacturer's instructions. Briefly, the samples cultured for 21 d were collected and washed with PBS twice. Then, the hydrogel containers were split, freeze-dried and immersed in 500 μ l of 0.5 M HCl solution at 60 °C overnight for digestion. The lysates were mixed with the working reagent, incubated for 3 min at room temperature and analyzed by colorimetric detection at 612 nm. Calcium amount was calculated according to a standard curve and the data were normalized with the DNA content of each sample. Quintuplicate samples were used for the analysis to calculate means and standard deviations.

RNA isolation and gene expression analysis

After culture for 7, 14 and 21 d under different viscosities, the hydrogel containers were washed with PBS twice and then transferred to a 6-well plate. A spatula and a tweezer were used to cut the hydrogel into two pieces, the cell aggregations and spheroids were dispersed in PBS thoroughly. Thereafter, the hydrogel pieces were removed and cell-containing PBS was transferred to a 15 ml centrifugation tube. After washing with PBS thrice, 1 ml of Sepasol RNA I Super G (09379-97, Nacalai Tesque) was added into each tube. The samples were then frozen at -80 °C and further crushed into powder with an electric crusher. The total RNA of each sample was extracted based on the manufacturer's protocol. The extracted RNA was reversely transcribed into cDNA using a High-capacity cDNA Reverse Transcription Kit (4374966, Applied Biosystems). A 7500 Real-Time PCR system (Applied Biosystem, USA) was used to perform real-time PCR. Relative expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control and the expression level of interested targets was calculated with a $2^{-\Delta\Delta C_t}$ method. hMSCs at passage 4 (P4) was used as a reference. For adipogenic differentiation, below listed primer and probe sequences were used: *GAPDH* (Hs99999905_m1, Lot: 2104444, Applied Biosystems), *CEBPA* (Hs00269972_s1, Lot: 1993980, Applied Biosystems), *FASN* (Hs00188012_m1, Lot: 1875477, Applied Biosystems), *FABP4* (Hs00609791_m1, Lot: 1909263, Applied Biosystems), *LPL* (Hs00173425_m1, Lot: 1953315, Applied Biosystems); for chondrogenic differentiation, below listed primer and probe sequences were used: *type II collagen* (forward): 5'-GGCAATAGCAGGTTCACGTACA-3', *type II collagen* (reverse): 5'-CGATAACAGTCTTGCCCCACTT-3', *type II collagen* (probe): 5'-CCGGTATGTTTCGTGCAGCCATCCT-3'; *aggrecan* (forward): 5'-TCGAGGACAGCGAGGCC-3', *aggrecan* (reverse): 5'-TCGAGGGTGTAGCGTGTAGAGA-3', *aggrecan* (probe): 5'-ATGGAACACGATGCCTTTCACCACGA-3'. For osteogenic differentiation, below listed primer and probe sequences were used: *ALPL* (Hs01029144_m1, Lot: 2103375,

Applied Biosystems), *BMP2* (Hs00154192_m1, Lot: 2090101, Applied Biosystems), *RUNX2* (Hs00231692_m1, Lot: 2080647, Applied Biosystems) and *SPP1* (Hs00959010_m1, Lot: 2095770, Applied Biosystems). Triplicate samples were used for the analysis to calculate means and standard deviations.

Statistical analysis

The data were presented as means ± standard deviations (SD). For DNA quantification, relative gene expression assay and sGAG quantification, a two-way analysis of variance (ANOVA) with Tukey’s post hoc test for multiple comparisons was used. Instead, a one-way ANOVA was used for analyzing ALP activity and calcium deposition. Statistical analyses were processed by Prism GraphPad (version 10.1.2, USA). Significant levels were set at **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and *****p* < 0.0001.

Results

Viscosity of culture medium

The viscosity of induction media was adjusted by adding PEG of different molecular weights and ratios to the normal culture medium, as shown in Table 1. Normal viscosity represents normal induction medium without PEG supplementation (denoted as N-V). PEG 35 K and PEG 8 M were added into the induction medium solely or at a mixture ratio of 1:3 to prepare low viscosity (denoted as L-V), high viscosity (denoted as H-V) and middle viscosity medium (denoted as M-V), respectively. The final polymer concentration was kept the same for all groups at 1.0 w/v%. The viscosity changes upon increasing of the shear rate from 0.1 to 100 /s are shown in Figure S1. For adipogenic and osteogenic induction media, they showed a shear-thinning property in all viscosity groups because these media contained 10% FBS. Chondrogenic induction media did not show an obvious shear-thinning due to the absence of FBS. The viscosity at a shear rate of 0.1 /s was regarded as the zero-shear viscosity (Table 1 and Figure S1). The zero-shear viscosity of adipogenic and osteogenic media was kept almost the same within different viscosity groups, ranging from 74.2 ± 23.6 cP to 650.8 ± 60.6 cP. For the chondrogenic induction medium, the viscosity ranged from 1.6 ± 1.2 cP to 272.6 ± 8.2 cP. The prepared viscous media showed a stepwise increase in viscosity from L-V to H-V.

Table 1. Zero-shear viscosities of different viscous inductive media prepared by different ratios of PEG 35 K and PEG 8 M

Culture medium	PEG 35 K (w/v%)	PEG 8 M (w/v%)	Zero-shear viscosity of adipogenic medium (cP)	Zero-shear viscosity of chondrogenic medium (cP)	Zero-shear viscosity of osteogenic medium (cP)
Normal induction	0.0	0.0	74.2 ± 23.6	1.6 ± 1.2	86.4 ± 28.2

Culture medium	PEG 35 K (w/v%)	PEG 8 M (w/v%)	Zero-shear viscosity of adipogenic medium (cP)	Zero-shear viscosity of chondrogenic medium (cP)	Zero-shear viscosity of osteogenic medium (cP)
medium without PEG					
Low viscosity	1.00	0.00	88.8 ± 32.1	2.4 ± 0.5	105.9 ± 23.8
Middle viscosity	0.25	0.75	268.9 ± 46.6	62.7 ± 12.6	244.6 ± 47.8
High viscosity	0.00	1.00	645.5 ± 74.7	272.6 ± 8.2	650.8 ± 60.6

Cell morphology, viability and proliferation during the dynamic 3D culture

The cuboid agarose hydrogel container was used to encapsulate hMSCs to enable the 3D dynamic culture and sustain the viscous environment. After hMSCs were suspended in the normal induction medium without PEG supplement, low, middle and high viscosity media, they were encapsulated in agarose hydrogel containers. Cell morphology, viability and proliferation were evaluated. For cells immediately encapsulated in the cuboid containers, individual cells were observed (Figure S2-S4). Live/dead staining revealed that the cells in all groups showed high viability with a few dead cells at the beginning (Figure 1a-c). After 21 d culture in trilineage induction media within various viscosities, most of the cells remained alive and the cells showed different morphologies (loose aggregates or compact spheroids) depending on the induction medium type and viscosity of the media (Figure 1d-f).

During the ongoing culture, the cells aggregated and the cell aggregation behavior depended on the type of culture medium and viscosity. Phase-contrast microscopy observations showed that during adipogenic differentiation, irregular cell aggregates were formed after 1 d culture in N-V and L-V media (Figure S2). The size of cell aggregates decreased and the aggregates became more compact after 7 d culture. The circularity increased gradually with the culture time. Nonetheless, loose cell aggregates appeared first after 7 d culture in M-V and H-V media and the aggregates remained loose after 21 d culture, which reflected the inhibitory effect of high viscosity on cell-cell interactions. Meanwhile, cell proliferation during adipogenesis was also evaluated by DNA quantification after 7, 14 and 21 d of culture (Figure 2a). DNA amount showed a decreasing trend in all groups with the extension of culture. Furthermore, DNA amounts in high viscosity groups showed a greater decrease. The results indicated that high viscosity could inhibit cell proliferation more strongly than low viscosity during the 3D dynamic culture of adipogenic differentiation.

For chondrogenic differentiation, which differed from adipogenic differentiation, the cells formed several spherical spheroids immediately after 1 d culture in the N-V and L-V groups (Figure S3). These spheroids tended to merge during the subsequent culture. For hMSCs in the M-V and H-V groups, cells aggregated loosely after 1 d culture and formed numerous small compact spheroids during the continuing culture. These small spheroids were further merged during the dynamic culture. DNA amount quantification indicated that the cell number in the N-V and L-V groups remained unchanged while that in the M-V and H-V groups increased with culture time (Figure 2b). Cell number also increased with viscosity. The results indicated that viscosity promoted the proliferation of hMSCs during the 3D dynamic culture of chondrogenesis.

For osteogenic differentiation, cells in low viscosity media (N-V and L-V groups) formed cell aggregates after 1 d culture. The aggregates then became more compact and their size decreased with culture time. In the high viscosity groups, the cells formed loose aggregates after 7 d culture and some small spheroids were formed after culture for 14d and 21 d. DNA amount quantification showed that cell number in all groups decreased with culture time (Figure 2c). Cell number decrease in the low viscosity medium was more evident than that in the high viscosity medium. The results suggested that viscosity also promoted cell proliferation during the 3D dynamic culture of osteogenic differentiation, but the promotive effect was weaker than that during the 3D dynamic culture of chondrogenesis.

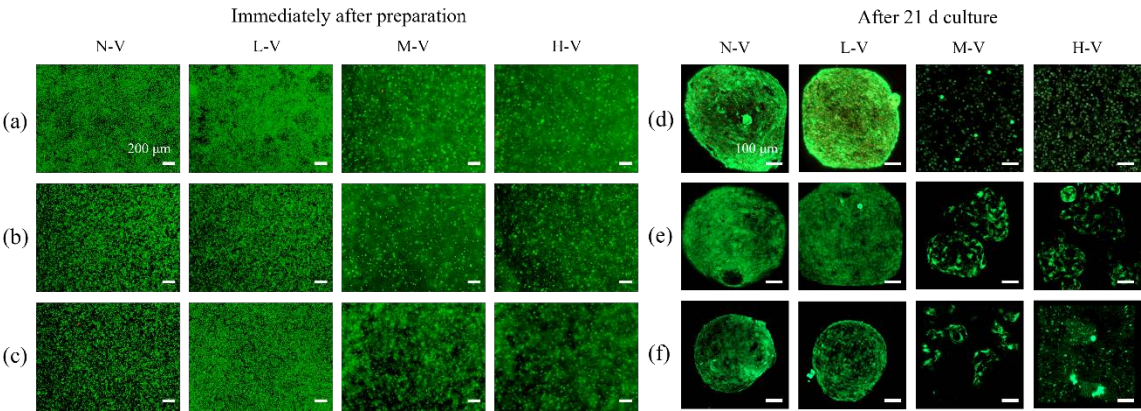


Figure 1. Live/dead staining of hMSCs during 3D culture in the media of different viscosities under trilineage differentiation. (a-c) Immediately after preparation of the hydrogel containers (0 d) and (d-f) after 21 d culture in (a, d) adipogenic induction medium, (b, e) chondrogenic induction medium and (c, f) osteogenic induction medium. Scale bar in the images immediately after preparation: 200 μm ; scale bar in the images after 21 d culture: 100 μm .

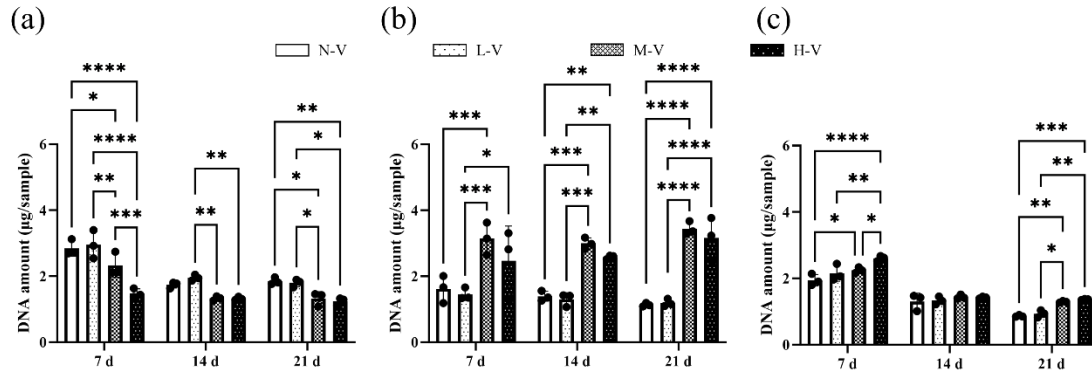


Figure 2. Quantification of DNA amount during 21 d culture in different viscosity media during (a) adipogenic induction, (b) chondrogenic induction and (c) osteogenic induction. Data are shown as mean $\pm$ SD. Significant difference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Influence of viscosity on adipogenic differentiation of hMSCs

The influence of viscosity on adipogenic differentiation of hMSCs was investigated by Nile Red staining and expression analysis of adipogenic differentiation-related genes (*CEBPA*, *FASN*, *FABP4* and *LPL*). Nile Red staining showed that the fluorescence intensity of the stained cells decreased with the increase of viscosity (Figure 3a). The cells cultured in the high viscosity medium (H-V) showed the lowest fluorescence. The results suggested that the cells in the low viscosity medium generated more oil droplets than those in the high viscosity medium. Real-time PCR showed that the expression of *CEBPA*, *FASN*, *FABP4* and *LPL* decreased with the increase of viscosity (Figure 3b-e). Meanwhile, expression of these genes in N-V and L-V steadily increased with the culture time. However, expression of the genes remained very low during the 21 d culture in M-V and H-V groups. The results indicated viscosity inhibited adipogenic differentiation of hMSCs during the 3D dynamic culture of adipogenic differentiation.

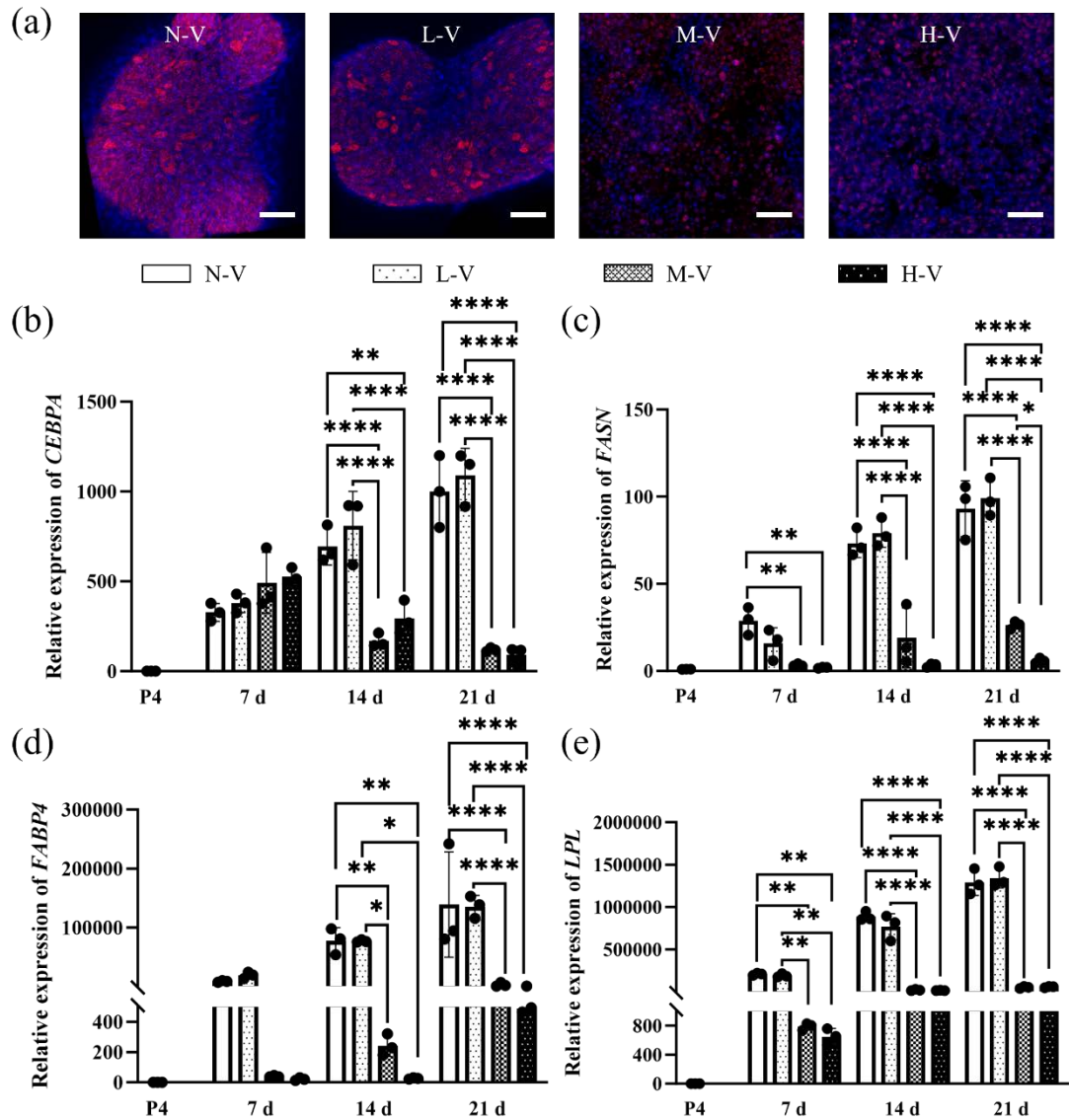


Figure 3. Influence of viscosity on adipogenic differentiation of hMSCs during 3D induction culture. (a) Fluorescent photomicrographs of Nile Red staining. Oil droplets were stained red and nuclei were stained blue. Scale bar: 100 μm. Quantitative analysis of genes encoding (b) CEBPA, (c) FASN, (d) FABP4 and (e) LPL. Data were normalized by expression level of the respective genes in P4 cells. Data are shown as mean ± SD. Significant difference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Influence of viscosity on chondrogenic differentiation of hMSCs

To investigate the influence of viscosity on chondrogenic differentiation of hMSCs, the production of sGAG and expression of chondrogenesis-related genes were evaluated. As

shown in Figure 4a and 4b, the sGAG amount was almost the same when the cells were cultured in N-V and L-V media. The sGAG amount decreased with the increase of viscosity and the sGAG/DNA ratio also showed the same trend. The sGAG amount did not increase with culture time and even showed a tapering off trend. Gene expression results indicated that gene encoding type II collagen and aggrecan showed a decreasing trend with the increase of viscosity (Figure 4c-d). Expression of these genes increased with culture time. The results indicated that viscosity had an inhibitory effect on the chondrogenic differentiation of hMSCs during the 3D dynamic culture of chondrogenic differentiation.

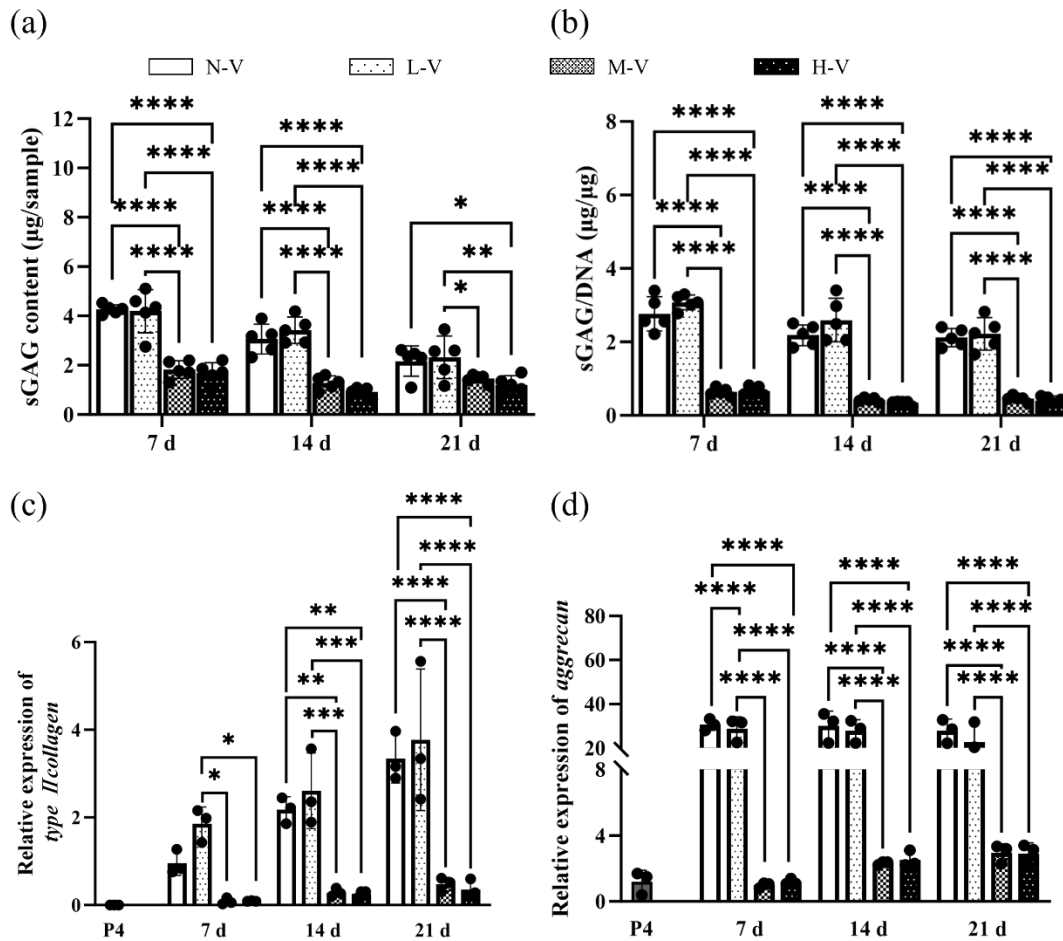


Figure 4. Influence of viscosity on chondrogenic differentiation of hMSCs during 3D induction culture. (a) Quantification of sGAG amount and (b) normalized sGAG/DNA ratio. Quantitative analysis of expression of genes encoding (c) type II collagen and (d) aggrecan. Data were normalized by expression level of the respective genes in P4 cells. Data are shown as mean $\pm$ SD. Significant difference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Influence of viscosity on osteogenic differentiation of hMSCs

ALP activity, calcium deposition and expression of osteogenesis-related genes were investigated to disclose the influence of viscosity on osteogenic differentiation of hMSCs. As an early-stage marker of osteogenesis, ALP activity was analyzed after 7 d culture. The results indicated that ALP activity slightly increased with the increase of viscosity (Figure 5a). After normalizing to DNA amount, the high viscosity groups still showed a higher tendency of ALP activity (Figure 5b). The calcium deposition, as a late-stage marker of osteogenesis, was investigated after 21 d culture. The results indicated that calcium deposition slightly increased with the increase of viscosity (Figure 5c). However, the normalized calcium deposition to DNA amount decreased with viscosity (Figure 5 d). The expression of osteogenesis-related genes (*ALPL*, *BMP2*, *RUNX2* and *SPPA*) showed that the expression of these genes decreased with the increase of viscosity (Figure 5e-h). Their expression increased with the culture time. These results suggested that high viscosity slightly inhibited osteogenic differentiation during the 3D dynamic culture of osteogenic differentiation.

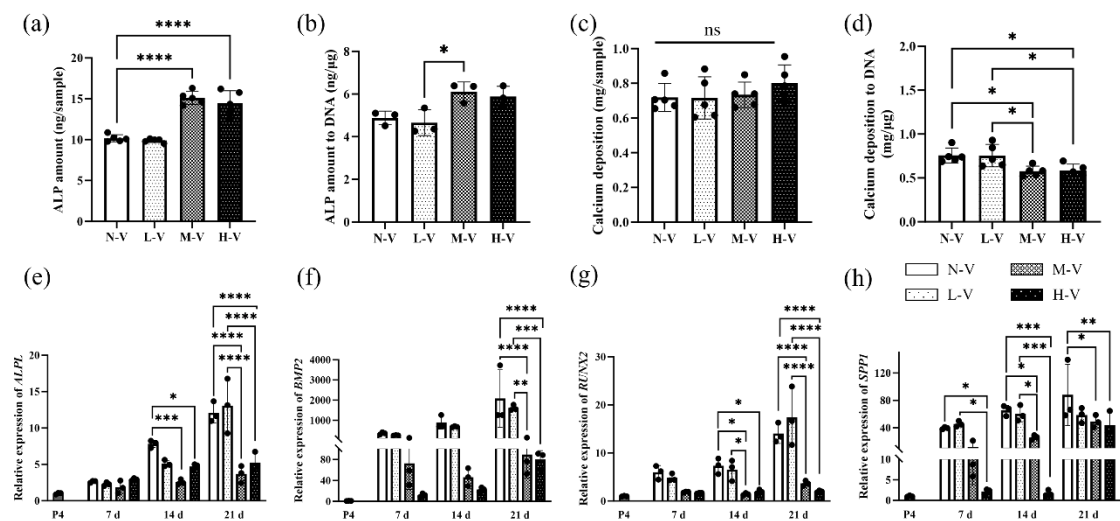


Figure 5. Influence of viscosity on osteogenic differentiation of hMSCs during 3D induction culture. (a) Quantification of ALP activity and (b) normalized ALP/DNA ratio of hMSCs after 7 d culture. (c) Quantification of calcium deposition and (d) normalized calcium deposition/DNA ratio of hMSCs after 21 d culture. Quantitative analysis of expression of genes encoding (e) *ALPL*, (f) *BMP2*, (g) *RUNX2* and (h) *SPP1*. Data were normalized by expression level of the respective genes in P4 cells. Data are shown as mean $\pm$ SD. Significant difference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; ns: not significant.

Discussion

Stem cells can respond to external mechanical cues, which are reflected in cell behaviors such as proliferation and differentiation through mechanotransduction [5,32–35]. Currently, tremendous attention has been paid to unraveling how viscoelasticity affects stem cell differentiation. However, the influence of viscosity on cell behavior is still in its infancy. Furthermore, current studies have focused more on 2D cell culture [36–41]. However, 3D cell culture exploration is lacking due to device limitations and difficulties in cell behavior characterization. Recently, several 3D cell culture methods have been developed and the effect of viscosity on stem cell differentiation has been explored [20–23]. However, these studies using gelatin to modulate viscosity or introducing plasma-treated nanoparticles into the alginate viscous solution cannot separate the influence of viscosity from the influence of cell-matrix interactions [42]. The usage of bioinert PEG can not only provide viscous conditions but also avoid specific cell interactions. Therefore, in this study, we employed bioinert PEG to modulate the viscosity of culture media. By dispersing cells in a viscous culture medium and further encapsulating them in the cuboid agarose hydrogel container, a dynamic 3D cell culture system was established. Therefore, the influence of viscosity on hMSCs trilineage differentiation was elucidated.

During 3D culture, the cells formed aggregates and spheroids spontaneously (Figure S2-4). The speed to form aggregates and spheroids was dependent on the differentiation conditions and viscosity of the culture medium. The cells formed aggregates and spheroids more quickly in the low viscosity medium than in the high viscosity medium (Figure S2-S4). The cells formed more compact spheroids in chondrogenic and osteogenic induction media than in the adipogenic induction medium. After the formation of compact spheroids, the exchanges of gases, nutrients and metabolites are decreased. Meanwhile, the cell cycle can be arrested in the G0/G1 phase resulting in reduced cell proliferation [43,44]. Therefore, Cell proliferation was also dependent on differentiation conditions and viscosity (Figure 2). Viscosity inhibited the proliferation of hMSCs during adipogenesis, while promoted cell proliferation during chondrogenesis, and slightly promoted cell proliferation during osteogenesis.

The different effects of viscosity on cell proliferation should be due to the different degrees of cell-cell interactions and limited nutrient diffusion in spheroids. Cell-cell interactions are beneficial for cell proliferation, whereas spheroid can inhibit cell proliferation. When the cells formed spheroids, cell-cell interactions became strong. However, large spheroids could pose much hypoxic stress for the inner cells within spheroids, which led to the formation of an avital, necrotic core in the spheroids [45–47]. Therefore, the balance between cell-cell interactions and nutrient diffusion is important for cell proliferation. The formation of small spheroids could provide sufficient cell-cell interactions without excessive inhibition on nutrient diffusion in the high viscosity medium of chondrogenesis, which might explain the promotive effect of viscosity on cell proliferation during the 3D dynamic culture of chondrogenesis. The hypoxic condition in spheroids has also been reported to promote proliferation of stem cells during chondrogenic differentiation [48]. The cell aggregates formed in the high viscosity medium of osteogenesis were looser than the spheroids in the chondrogenesis condition, which might induce less cell-cell interactions for a strong promotive effect on cell proliferation. The cell aggregates in the

high viscosity medium of adipogenesis were further loose, thus resulting in an inhibitory effect of viscosity on cell proliferation.

Viscosity of the culture medium during 3D dynamic culture showed inhibitory effects on hMSCs trilineage differentiation. The inhibitory effect on adipogenic and chondrogenic differentiation of hMSCs during 3D dynamic culture was stronger than that on the osteogenic differentiation of hMSCs. This can be explained by the cell-cell interactions and hypoxic conditions in spheroids. The low viscosity facilitated the formation of cell aggregates and spheroids during 3D dynamic culture. The formation of cell aggregates and spheroids can induce hypoxic conditions that are beneficial for adipogenic and chondrogenic differentiation [49]. High viscosity showed an inhibitory effect on the formation of cell aggregates and spheroids, therefore inhibiting adipogenic and chondrogenic differentiation of hMSCs. On the other hand, the hypoxic condition has been reported to inhibit osteogenesis of hMSCs [50]. The cells in both the low and high viscosity media formed spheroids. The spheroids in the high viscosity medium were smaller, while cell-cell interactions were also weaker than those in the low viscosity medium. The balance of the influences of hypoxia and cell-cell interactions might explain the weak inhibitory effect of viscosity on the osteogenic differentiation of hMSCs.

The results differed from the effects of viscous media prepared with bioactive molecules, such as gelatin. It has been reported that high viscosity of gelation solution facilitates both osteogenic and chondrogenic differentiation while inhibiting adipogenic differentiation [20–22]. In contrast to previous studies, we used PEG in this study to modulate viscosity and the cell suspensions were confined within the agarose hydrogels. Both PEG and agarose are bioinert and cannot provide extra cell-matrix interactions at the initial stage of differentiation, whereas the balance between cell-cell interactions and cell-matrix interactions is important in steering stem cell differentiation potential [51]. The results of this study reflected cell-cell interactions, other than cell-matrix interactions, which might explain the different results of this study compared to the previous report.

The results could provide some useful insights into the influence of viscosity on adipogenic, chondrogenic and osteogenic differentiation for tissue engineering applications.

Conclusion

The effect of viscosity on hMSCs trilineage differentiation in the 3D culture condition was disclosed by designing a novel agarose-based cuboid hydrogel container. Equal concentrations of PEG with different molecular weights were used to control the viscosity of the medium. Cells in low viscosity were prone to aggregate and form spheroids, whereas high viscosity delayed cell aggregation depending on different differentiation types. High viscosity had detrimental effects on adipogenic, chondrogenic and osteogenic differentiations. High viscosity decreased cell proliferation during adipogenic differentiation while promoting cell proliferation in chondrogenic and osteogenic differentiations. The results revealed how viscosity influences stem cell differentiation in the 3D condition under the bioinert environment. The study gained knowledge to elucidate how viscosity affects stem cell differentiations in a 3D environment.

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Supplementary information

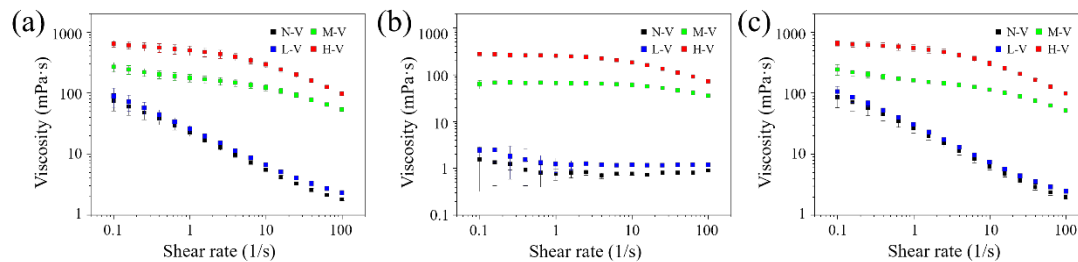


Figure S1. Rheological properties of inductive media with varied viscosities. (a) Adipogenic induction media; (b) Chondrogenic induction media; (c) Osteogenic induction media. N-V, L-V, M-V and H-V represented normal inductive culture medium without PEG supplementation, low viscosity, middle viscosity and high viscosity inductive culture medium, respectively.

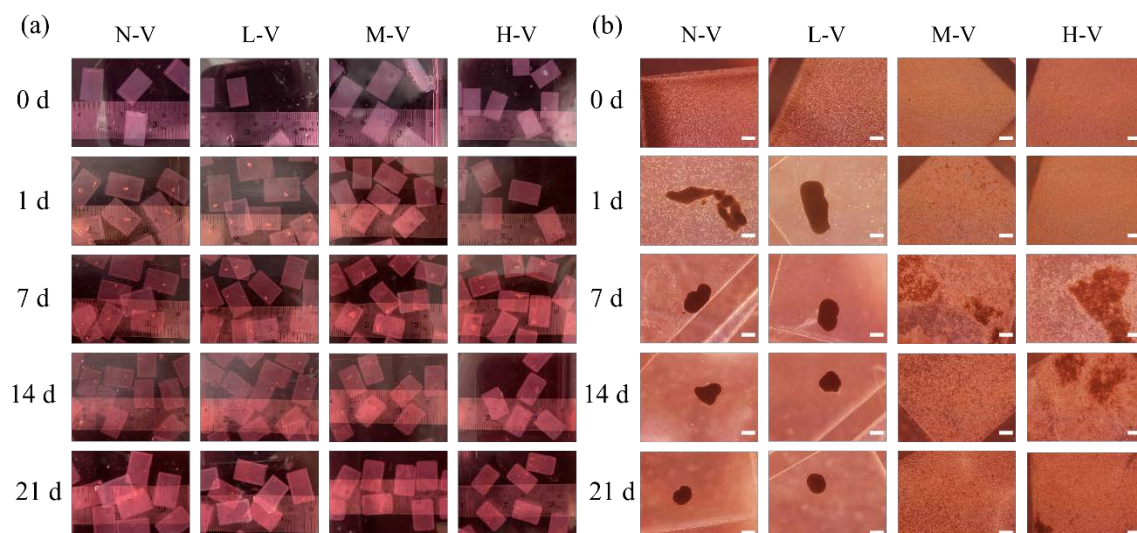


Figure S2. (a) Gross appearance of hydrogel containers as well as (b) phase-contrast photomicrographs of hMSCs during 3D culture in different viscous media under

adipogenic differentiation. Scale bar: 500 μ m. N-V, L-V, M-V and H-V represented normal inductive culture medium without PEG supplementation, low viscosity, middle viscosity and high viscosity inductive culture medium, respectively.

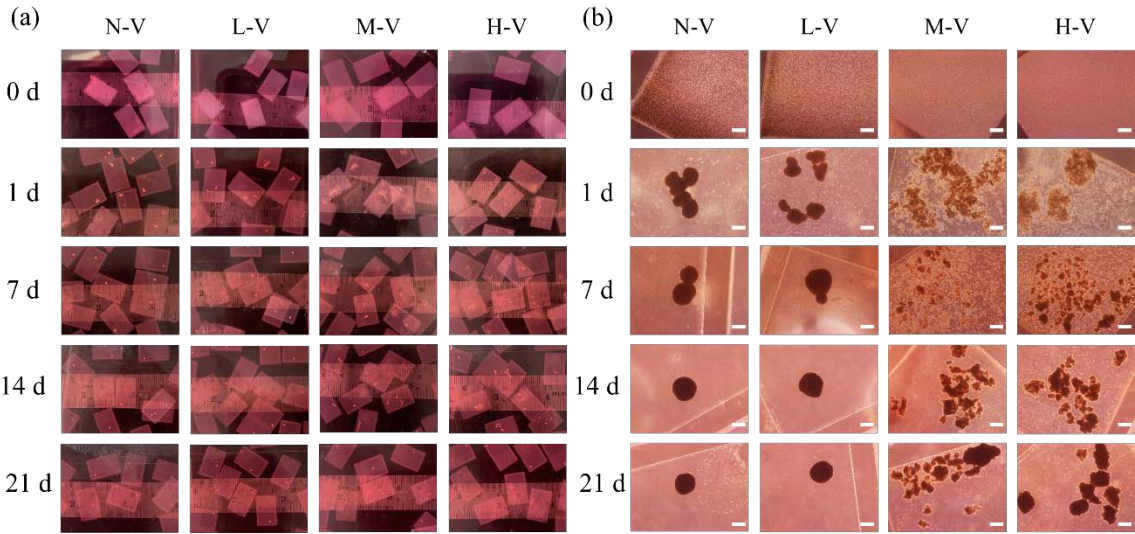


Figure S3. (a) Gross appearance of hydrogel containers as well as (b) phase-contrast photomicrographs of hMSCs during 3D culture in different viscous media under chondrogenic differentiation. Scale bar: 500 μ m. N-V, L-V, M-V and H-V represented normal inductive culture medium without PEG supplementation, low viscosity, middle viscosity and high viscosity inductive culture medium, respectively.

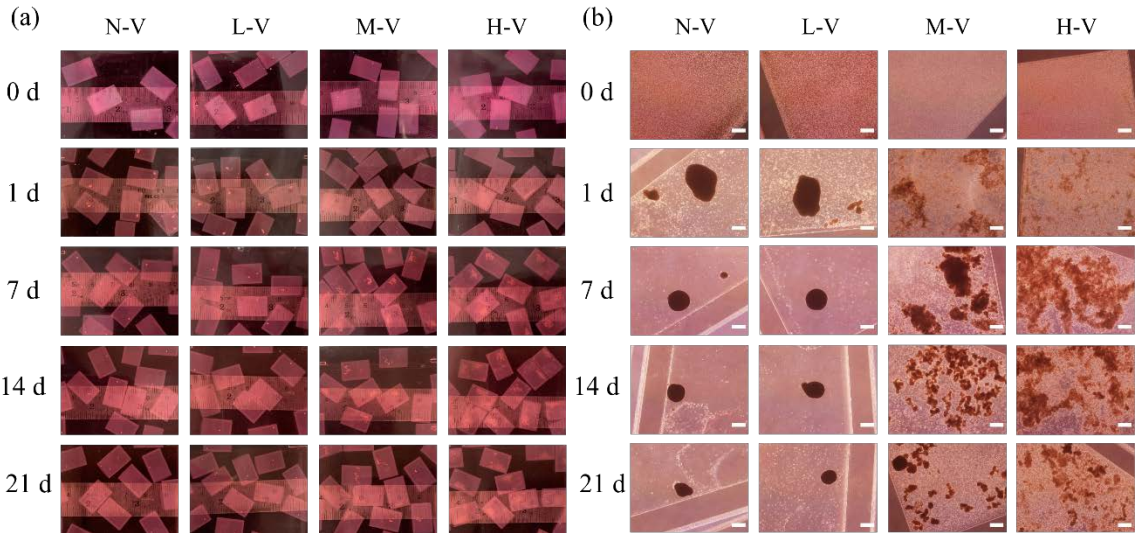


Figure S4. (a) Gross appearance of hydrogel containers as well as (b) phase-contrast photomicrographs of hMSCs during 3D culture in different viscous media under osteogenic differentiation. Scale bar: 500 μm . N-V, L-V, M-V and H-V represented normal inductive culture medium without PEG supplementation, low viscosity, middle viscosity and high viscosity inductive culture medium, respectively.

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