Influence of Solution Viscosity on Cell Functions in 3D Culture System Using Gelatin Biphasic Hydrogels

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Contents

List of abbreviations

ECM	Extracellular matrix
2D	Two-dimensional
3D	Three-dimensional
hMSCs	Human bone marrow-derived mesenchymal stem cells
BACs	Bovine articular chondrocytes
PNIPAAm	Poly(N-isopropylacrylamide)
GelMA	gelatin methacryloyl
PBS	Phosphate-buffered saline
^{1}H-NMR	^{1}H-nuclear magnetic resonance
DMEM	Dulbecco's modified Eagle's medium
sGAG	Sulfated glycosaminoglycan
EDTA-2Na	Ethylenediaminetetraacetic acid disodium salt dihydrate
RT-PCR	Real-time polymerase chain reaction
MSCGM^{TM}	Mesenchymal stem cell growth medium
PFA	Perfluoroalkoxy
FBS	Fetal bovine serum
cDNA	Complementary DNA
TGF-β3	Transforming growth factor-beta3
PEGDM	Polyethylene glycol dimethacrylate
PEG	Poly(ethylene glycol)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IBSP	Bone sialoprotein 2
SPP1	Secreted phosphoprotein 1
SP7	Osterix
RUNX2	Runt-related transcription factor-2
ALP	Alkaline phosphatase
LPL	Lipoprotein lipase
CEBPA	CCAAT/enhancer binding protein
FASN	Fatty acid synthase
FABP4	Fatty acid binding protein 4
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
pNPP	p-Nitrophenyl phosphate
IBMX	Methyl-isobutylxanthine
Indo	Indomethacin

Chapter 1

General introduction

Cells are surrounded by their respective extracellular matrix (ECM) microenvironment and their interaction can regulate cell functions through biochemical cues and biomechanical cues (Figure 1.1), such as cell adhesion, migration, self-renewal, proliferation and differentiation [1-7]. Although the effect of mechanical properties of ECM has long been overshadowed due to an emphasis on the contribution of biochemical cues, some recent studies have revealed the influence of mechanical cues on cell functions [8-11]. So far, most studies have used elastic hydrogels as synthetic ECM due to their hydrophilic polymer networks that are structurally similar to the native microenvironment with high water content [12-15]. Cell response to such elastic matrix is instrumental in understanding the process of mechanotransduction. However, these approaches are insufficient to fully understand the mechanical contribution of viscoelastic ECM to cells. In fact, native ECM exhibits viscous properties as well as elastic properties [16-19]. Nevertheless, most studies have focused on elastic properties and overlooked the viscous properties.

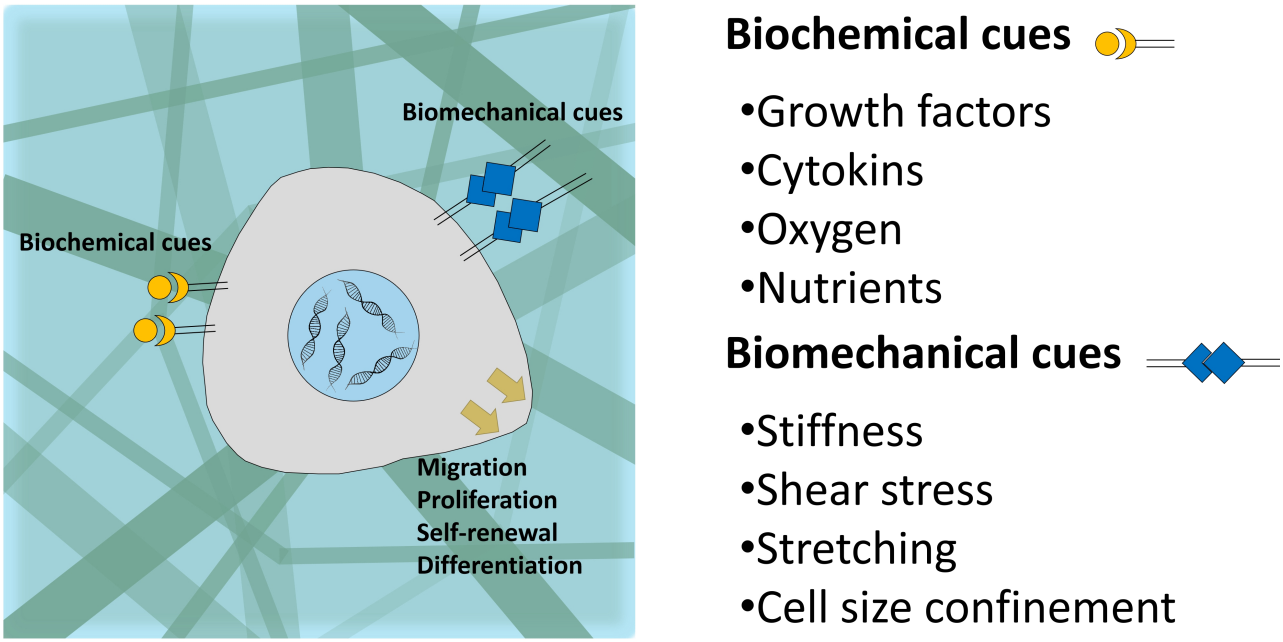

Figure 1.1 The interaction between cell and ECM through biochemical and biomechanical cues.

1.1 Material properties affecting cell functions

In our daily life, native tissues experience various mechanical stresses, such as stretching, pulling and loading [20-22]. The stresses can deform our tissues and also generate mechanical cues which can be determined by forces and material property (e.g. elasticity, viscosity and viscoelasticity). Our native tissues exhibit linear elastic or nonlinear elastic behaviors depending on the degree of forces [23-25]. For example, skin can be recovered without any damage after being stretched. However, it may be torn when they are overstretched. The properties of materials can be characterized by exerting mechanical stress (i.e. stress-strain test) [26]. Stress is a physical quantity that expresses the internal resistance generated by a material in response to its magnitude when an external force (load) such as compression, tension, bending, or twist is applied to a material. Strain refers to the geometrical deformation of a material caused by a mechanical stress.

1.1.1 Linear elasticity

Linear elastic material is a material whose stress or strain is proportional to the strain or stress (i.e. the relationship between the stress and strain is linear). When a force is applied to a linear elastic material material deformation will continue as long as the force persists and when the force is removed the linear elastic material will fully return to its original shape and size without any energy loss or irreversible deformation (Figure 1.2). The properties of elastic materials are characterized by Young's modulus (E), Poisson's ratio (v), bulk modulus (K) and shear modulus (G) (Figure 1.3) [27-30]. The stiffness of elastic material is defined as the measurement of the deformation resistance when a stress or strain is applied to an elastic material. In stress-stain test, stiffness of linear elastic materials can be measured by Young's modulus (E), bulk modulus (K) and shear modulus (G). Most of the existing studies have used linear elastic materials to investigate the influence of matrix elasticity or matrix stiffness on cell functions [31-34]. However, living tissues are not only limited to linear elasticity [35].

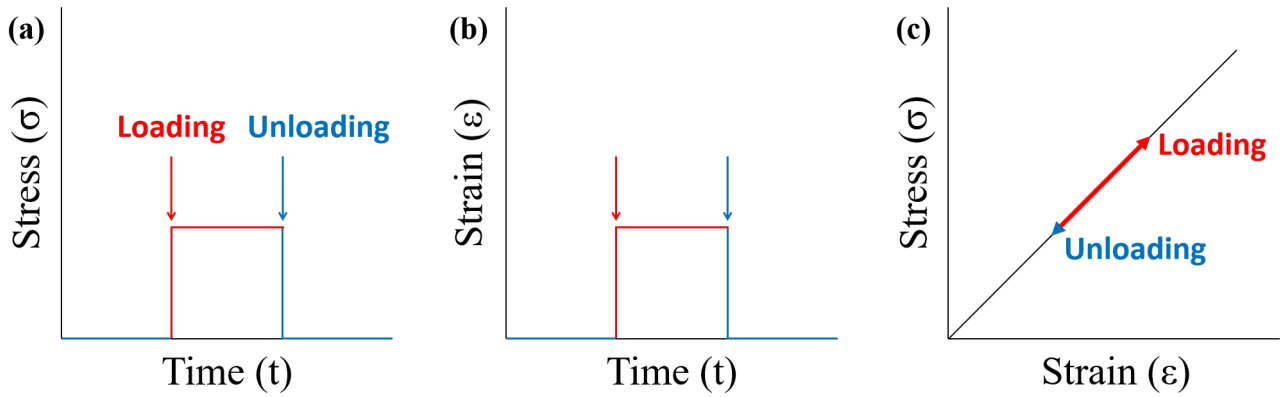

Figure 1.2 In a linear elastic material, (a) stress applied to the material can induce (b) strain response (the point at which the load is applied to the material is indicated by a red arrow, while the point at which the load is removed is indicated by a blue arrow). (c) The relationship between the stress and stain is linear without loss of mechanical energy and irreversible deformation (a red arrow represents the mechanical loading, while a blue arrow represents the removal of the mechanical loading).

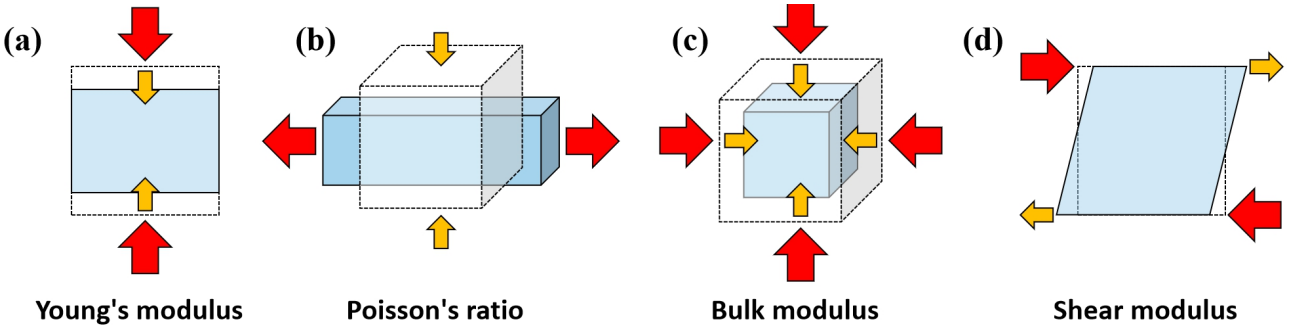

Figure 1.3 Elastic material has the following properties: (a) Young's modulus (E) which is a measurement of stiffness under an unidirectional mechanical loading (compression or tensile), (b) Poisson's ratio (v) which is the ratio of transverse strain to longitudinal strain under the axial mechanical loading, (c) bulk modulus (K) which is a measurement of volumetric stiffness under the uniform compression and (d) shear modulus (G) which is a measurement of stiffness under the opposite shear forces. Red arrow represents the direction of stress and orange arrow represents the direction of strain.

1.1.2 Nonlinear elasticity

Although *in vivo* tissues show linear elastic behaviors within a certain range of stress or strain, the tissues show nonlinear elastic behaviors when applied stress or strain exceeds yield point (Figure 1.4a). For example, the arterial vessel wall can be extended and return to its original shape and size under low blood pressure, whereas high blood pressure leads to eventual rupture [36]. The stretchable ligaments are helping to hold our skeleton in a normal alignment and prevent abnormal movements [37]. However, the ligaments can be torn and can't roil back to their original shape when high force is applied to a ligament. Therefore, biological tissues and ECM are not linear elastic materials (Figure 1.4b).

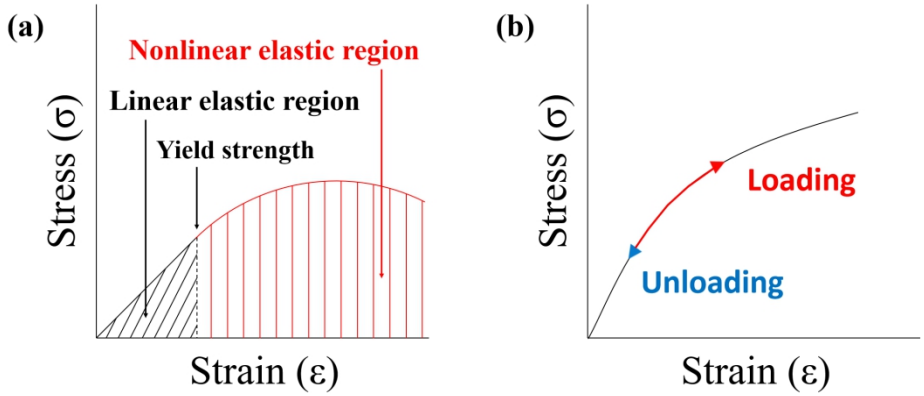

Figure 1.4 The stress-strain curves of elastic materials are measured in tensile or compression test. (a) A multilinear stress-strain curve of an elastic material where the nonlinear elastic region is observed when the stress or strain is over the yield strength. (b) The relationship between the stress and strain is nonlinear but no energy loss is observed (a red arrow represents the mechanical loading, while a blue arrow represents the removal of the mechanical loading).

1.1.3 Viscosity

Most exiting studies about cellular mechanotransduction have focused on static elastic materials to study cell behaviors. However, cells reside in native tissues with a dynamic and dissipative microenvironment [38-40]. Therefore, the property of viscous dissipation in a material is an important factor affecting cell behaviors [41-43]. Viscous material is a material whose strain increases linearly and irreversibly with time under a constant stress and the deformed structure will not be returned to its original shape after removing the stress (Figure 1.5).

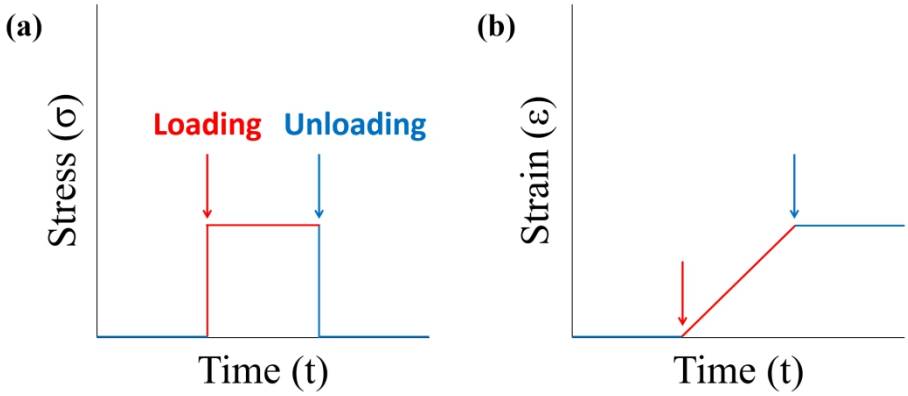

Figure 1.5 In viscous materials, (a, b) strain increases linearly but not instantaneously under a mechanical stress. (b) Deformation of the viscous material remains after removing the applied stress.

1.1.4 Viscoelasticity

Biological tissues and ECM exhibit both elastic and viscous properties because they are viscoelastic materials [44, 45]. The viscoelastic materials have the following properties: hysteresis, creep and stress relaxation (Figure 1.6) [46-50]. Dynamic mechanical analysis gives information about viscoelasticity where

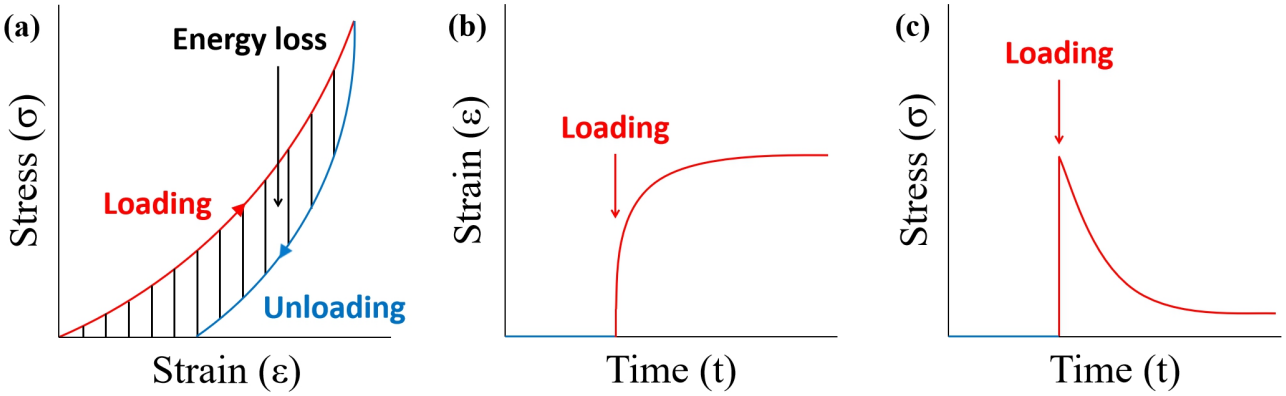

Figure 1.6 In viscoelastic materials, (a) hysteresis is observed because the material energy is dissipated during mechanical loading and unloading. (b) Creep and (c) stress relaxation are time-dependent increase or decrease in the response to strain or stress (a red arrow represents the mechanical loading, while a blue arrow represents the removal of the mechanical loading).

the storage modulus represents the storage of elastic energy (elastic resistance), while the loss modulus represents the loss of viscous energy (viscous dissipation) (Figure 1.7). The viscous dissipation is known as hysteresis in the strain-stress test where loading curve area is larger than the unloading curve area (Figure 1.6a). The area of irreversible deformation in a material is known as plasticity (Figure 1.7b). In addition, viscoelastic materials experience a molecular movement or rearrangement which is known as creep (Figure 1.6b). When a stress is applied to a viscous material, the strain increases linearly but irreversibly and polymer chain moves to another positions. On the other hand, viscoelastic materials have the properties to keep their structure in a strained conditions. When a strain is applied to a viscous material, the stress decreases linearly (Figure 1.6c).

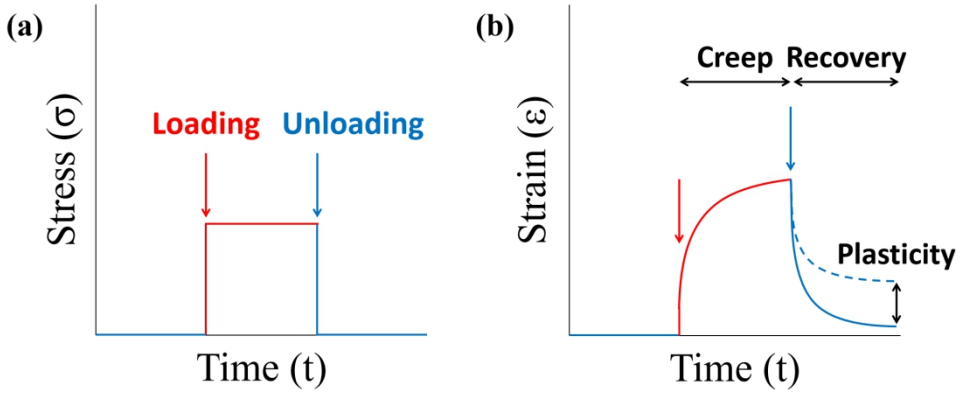

Figure 1.7 The nonlinearity of viscoelastic materials is described by (a, b) a combination of creep and stress relaxation under the constant mechanical loading and unloading. The materials exhibit (b) a permanent deformation (plasticity) when the stress is removed. (a red arrow represents the mechanical loading, while a blue arrow represents the removal of the mechanical loading).

1.2 Influence of mechanical properties of hydrogels on cell functions

Since cells sense and respond to changes in mechanical properties of ECM, many studies have developed various kinds of biomaterials to understand cellular mechanical microenvironment *in vitro* [51-60]. Among them, hydrogels have been often used as synthetic ECM due to their hydrophilic polymer networks that are structurally similar to the native microenvironment and have high water content [61, 62]. A lot of studies have established fundamental cellular mechanotransduction based on cellular response to linear elasticity of hydrogels although they do not fully reflect viscoelastic ECM [63-65]. Hence, recent research has begun to pay attention to the role of nonlinear elasticity and viscoelasticity of hydrogels in regulating cell functions [66-68]. Furthermore, more recently, hydrogels have been used to investigate the influence of viscosity on cell functions by decoupling the influence of viscosity from other mechanical properties of hydrogels or embedding cell-laden viscous solution in the hydrogels [69-72]. Therefore, the tunable mechanical properties of hydrogels can enable researchers to investigate the influence of various mechanical cues on cell functions.

1.2.1 Influence of linear elasticity on cell functions

Although the influence of biochemical cues have been extensively studied over a long period of time, the influence of biophysical cues (especially mechanical cues) has only recently started to attract attention. Early approaches to manipulate the mechanical properties of hydrogels have utilized two-dimensional (2D) polyacrylamide (PAA) gel substrates with different elastic moduli which are linear elastic hydrogels [73-76]. The stiffness of linear elastic material is characterized by Young's modulus where material exhibits a constant strain or stress when a constant stress or strain applied. Stiffness of linear hydrogels can be controlled by changing polymer concentration, molecular weight and crosslinking density [77-80], and such gel substrates are found to affect various cell functions, including cell proliferation, migration and differentiation [81-90]. For example, cells response to PAA gel substrates with different stiffness where cell spreading area and growth increase with increasing substrate stiffness and the well-organized actin cytoskeleton is observed in cells cultured on a stiff substrate [91]. Furthermore, gradient elastic substrates cause cells to migrate from soft regions to stiff regions via a cell migration process known as durotaxis [92]. Besides, stem cell differentiation has been regulated by substrate stiffness. Human bone marrow-derived mesenchymal stem cells (hMSCs) are cultured on PAA gel substrates with different stiffness. hMSCs cultured on soft, intermediate and stiff PAA gel substrates that mimic the elasticity of brain (0.1-1 kPa), striated muscle (8-17 kPa) and premineralized bone (25-40 kPa) can differentiate to neurocytes, osteoblasts and skeletal muscle cells, respectively [93]. However, since 2D culture systems can not reflect a three-dimensional (3D) native microenvironment accurately, recent studies have been further extended from 2D to 3D elastic hydrogel models and have shown different results. For example, cells cultured on 2D PAA gel substrates show cell morphology-dependent cell differentiation, with a stiff substrate leading to cell spreading and stronger osteogenesis rather than adipogenesis [93]. On the other hand, hMSCs cultured in 3D alginate hydrogels show morphology-independent osteogenic differentiation, indicating that hMSCs use different integrin binding when they are cultured in 2D and 3D matrices [94-97]. Taken together, the results indicate that linear elasticity affects various cell functions but has limitations in completely mimicking complex tissue microenvironment. Materials used for hydrogel preparation, their stiffness and their influence on cell functions are summarized in Table 1.1.

Table 1.1 Influence of materials and their stiffness on cell functions

Hydrogel materials	Stiffness	Influences
GelMA hydrogels [98, 99]	3.8 - 29.9 kPa	Chondrocyte proliferation and phenotype
	5 - 180 kPa	
Recombinant elastin-like protein hydrogels [100]	715 - 3,919 Pa	Cardiomyocyte differentiation
PAA based hydrogels [101]	4 - 12 kPa	Osteogenic differentiation
	281 - 841 Pa	Neurogenesis
Gelatin-hydroxyphenylpropionic acid hydrogels [102-104]	1000 - 13,500 Pa	Osteogenic differentiation
	570 - 2,750 Pa	Chondrogenesis
N-isopropylacrylamide (PNIPAAm) based hydrogels [105]	5 - 63 Pa	Cardiac regeneration

Collagen based hydrogels [106]	3730 - 5,561 Pa	Chondrogenic differentiation
Aligned Poly (L-lactic acid) hydrogels [107]	22.76 MPa	Teno-lineage
Alginate hydrogels [108]	39 - 904 kPa	Fibroblast behaviors
Hyaluronic acid hydrogels [109, 110]	6.3 - 18 kPa 4 kPa	MSC morphology
Alginate/collagen-I hydrogels [111]	50 and 1200 Pa	Fibroblast morphology

1.2.2 Influence of nonlinear elasticity on cell functions

In our body, soft tissues and filamentous networks, including actin, collagen, fibrin, neurofilaments and vimentin, exhibit nonlinear elasticity which presents nonlinear stress-strain relationships even at small strains [112, 113]. The increase ratio between the stress and strain is nonlinear or not constant but increases with increasing strain after stress reaches a critical stress. Recent studies have demonstrated that 3D nonlinear elastic hydrogels which are close to the properties of native tissues can regulate cell functions, such as cell migration mode, mechanical homeostasis and cell differentiation [114-116]. For instance, human foreskin fibroblasts have been cultured in the linear cell-derived matrix and nonlinear collagen matrix. Results show that linear elasticity changes cells to lobopodia-based migration, whereas nonlinear elasticity supports lamellipodia-based migration. Another study reports that nonlinear polyisocyanopeptide (PIC)-based hydrogels are found to regulate cell lineage commitment where hMSC differentiation cultured in PIC is switched from adipogenic to osteogenic differentiation through stress stiffening [117].

1.2.3 Influence of viscoelasticity on cell functions

Despite a lot of studies on the influence of elastic materials on cell functions, native ECM microenvironment in tissues exhibits not only elastic properties but also viscous properties [118]. Contrary to elastic materials which exhibit a constant strain when a constant stress is applied, viscoelastic materials additionally exhibit viscous properties which show that strain increases when a constant stress is applied. Besides, viscoelastic materials are characterized by stress relaxation and creep which are phenomena observed in vicoelastic materials. Under applied forces, viscoelastic material undergoes a molecular rearrangement where the parts of long polymer chain change positions. For stress relaxation, the stress decreases when constant strain is applied, whereas for creep, the strain increases when constant stress is applied. Viscoelastic models prepared with various materials, methods and design have been found to regulate cell functions significantly. For example, stem cells cultured on high creep dissipative hydrogels are observed with increased cell spreading area but a decrease in the size of focal adhesion [119]. Another study reports that stem cells cultured on high-creep hydrogels proliferate and differentiate towards multiple lineages such as myogenesis, osteogenesis and adipogenesis [48]. In addition, viscoelastic hydrogels where stress is relaxed over time are prepared and used to study the role of stress relaxation in regulating cell volume [120]. In this report, chondrocytes have been cultured in 3D viscoelastic alginate hydrogels with different stress relaxation. The results indicate that stress relaxation can regulate cell volume. Faster relaxation allows cells to be expanded while slow relaxation confines cell volume. As a result, fast relaxation promotes cell proliferation and cartilage matrix formation while slow relaxation hinders cell proliferation

and restricts cell-cell communication and cartilage matrix formation. Another study demonstrates that stress-relaxation can regulate myoblast behaviors such as spreading and proliferation [121]. The influence of stress-relaxation has been compared with that of elasticity. The results show that myoblasts cultured on soft stress-relaxing substrates are promoted more than those cultured on purely elastic substrates with the same initial elastic modulus. Ionically crosslinked hydrogels enable the rearrangement of the polymer network, resulting in time-dependent stress relaxation which affects cell functions [122]. Taken together, these results suggest that viscoelastic hydrogels with stress relaxation play an important role in regulating cell functions.

1.2.4 Influence of viscosity on cell functions

Basically, viscoelastic materials have been characterized by a time-dependent viscous dissipation. However, there have been limitations in culturing cells in viscous environment due to lack of good culture system. Recent study has developed PAA gel substrates with independently tunable elastic properties and viscous dissipation to study the effect of viscous dissipation on cell functions [123]. In this report, PAA gel substrates have been prepared by sterically entrapping high molecular weight linear PAA molecules without covalent bonding in covalently crosslinked networks of PAA. Primary rat hepatic stellate cells are cultured on PAA gel substrates and the results show that PAA gel substrates reduce cell differentiation and maintain phenotypes with increasing viscous dissipation. Another approach has used some supported lipid bilayers with varying surface viscosity but same functionalized RGD ligands [124]. Murine myoblasts are cultured on such supported lipid bilayer substrates to study the mechanism of cell response to surface viscosity based on the molecular clutch model. Interestingly, the influence of surface viscosity is similar to that of matrix stiffness. Cells cultured on viscous surfaces exhibit substrate viscosity-dependent cell behaviors, with high viscous surfaces (low mobility) leading to a larger cell spreading area and well-organized cytoskeleton.

1.3 Motivation, objectives and outline

1.3.1 Motivation

In addition to the influence of biochemical cues on cell functions, recent studies have revealed the mechanical cues can regulate cell functions. A variety of materials and designs have been developed and used to study the cell responses to mechanical cues. However, previous approaches have the limitations in providing appropriate mechanical microenvironment for cells. Cells functions are affected by their surrounding microenvironments which are not static or elastic but dynamic and viscoelastic. Therefore, strategies to mimic the *in vivo* mechanical microenvironments are required to get more accurate information about cell behaviors.

Studies using elastic hydrogels have contributed to our understanding of the influence of mechanical cues on various cell functions including cell morphology, behaviors and differentiation. So far, the elastic hydrogel has been a suitable model for cellular mechanotransduction study. However, the elastic hydrogels with different stiffness have shown material dependent results. Furthermore, native tissues and ECM exhibit not only the properties of elastic solid but also the properties of viscous liquid. Therefore, an understanding of the contribution of viscous properties in ECM to cells is also required. Recent studies have decoupled the influence of viscous properties from that of other mechanical properties or used pure viscous surfaces to investigate the influence of viscosity on cell functions. However, these methods still have limitations in that they cannot provide a 3D viscous microenvironment to cells.

1.3.2 Objectives and outline

In this study, a biphasic solution/hydrogel model which was composed of an inner solution phase and an outer hydrogel phase was prepared. Solution viscosity was controlled by altering the concentration of gelatin solution. The biphasic solution/hydrogel culture system was used for 3D cell culture in viscous microenvironment to disclose the influence of solution viscosity on cell functions. The details are outlines as follows.

Chapter 2 describes the influence of solution viscosity on chondrocyte functions during 3D culture. The detailed preparation methods of the 3D biphasic solution/hydrogel culture system are described in this chapter. The cell functions including cell proliferation, sGAG production, cytoskeleton and cartilaginous gene expression were evaluated after cells were cultured in gelatin viscous microenvironment.

Chapter 3 describes the influence of solution viscosity on chondrogenic differentiation of hMSCs during 3D culture. In the presence or absence of dexamethasone and TGF-β3, hMSCs were cultured in gelatin solutions with different viscosities, and their viability, proliferation, sGAG production and chondrogenic differentiation were evaluated.

Chapter 4 describes the influence of solution viscosity on simultaneous osteogenic and adipogenic differentiation of hMSCs during 3D culture. The 3D biphasic gelatin solution/hydrogel system was used to culture hMSCs in gelatin solutions with different viscosities with a mixture of osteogenic and adipogenic induction media. Influence of solution viscosity on competing osteogenic and adipogenic differentiation of hMSCs was evaluated.

Chapter 5 provides the conclusions and future perspective.

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Chapter 2

Solution viscosity regulates chondrocyte proliferation and phenotype during 3D culture

2.1 Abstract

Cells are surrounded by an extracellular matrix (ECM), which controls cellular functions through biological or physicochemical cues. In particular, cartilage tissues have abundant ECM that is viscoelastic and provides the necessary signals for the maintenance of chondrocyte activity and metabolism. The influence of ECM stiffness on chondrocyte functions has been broadly investigated using elastic hydrogels. However, it is not clear how viscosity impacts chondrocyte functions. In this part, a biphasic gelatin solution/hydrogel system was established for the three-dimensional culture of bovine articular chondrocytes (BACs) to investigate the influence of gelatin solution viscosity on chondrocyte proliferation, ECM secretion and the maintenance of the chondrocyte phenotype. Gelatin solutions of different viscosities supported chondrocyte proliferation and ECM production. However, the cell morphology, proliferation rate, secreted ECM quantity and gene expression levels were different, and these were dependent on the viscosity of the gelatin solutions. Low-viscosity solutions were more beneficial for proliferation, while high-viscosity solutions were more beneficial for ECM production and the expression of collagen type II and aggrecan. Chondrocytes had a more spread morphology in a low-viscosity gelatin solution than in a high-viscosity gelatin solution. The results suggested that high-viscosity was more beneficial for the maintenance of the chondrocyte phenotype, while low viscosity was more beneficial for cell expansion. Viscosity was demonstrated as one of the key parameters affecting cell morphology, proliferation and phenotype.

2.2 Introduction

Cells in each tissue and organ are surrounded by their respective microenvironment, which have different biological and physicochemical properties [1-4]. Cells sense and respond to changes in their microenvironment to maintain their metastatic activities and phenotypes [5, 6]. In addition to biological factors, in recent years, the mechanical properties of the extracellular matrix (ECM) have attracted broad attention because they have significant modulatory effects on cell functions [7-12]. Many studies have reported the correlation of stiffness and viscoelastic properties with cell functions such as cell adhesion, morphology, mobility, self-renewal, proliferation and differentiation [13-22]. Various types of cells have

been cultured on two-dimensional (2D) surfaces or in three-dimensional (3D) hydrogel matrices to elucidate the interaction of cells with matrices of different stiffness and viscoelasticity [23-27].

Although matrix stiffness and viscoelastic properties have been widely studied to reveal their influences on cell functions, the effects of the viscous properties of matrices on cell functions have remained elusive due to the difficulty of culturing cells in viscous liquids in 3D. Hydrogels and elastomers used for cell cultures are viscoelastic, making it difficult to separate the impact of elasticity and viscous properties on cell functions. Recently, some efforts have been made to discriminate the impact of viscous properties from that of other mechanical properties of matrices [14, 28, 29]. Soft viscoelastic polyacrylamide (PAA) hydrogels with tunable elasticity and viscous dissipation have been reported to separate the viscosity influence from other factors [28]. In this report, high molecular weight linear PAA molecules were sterically entrapped without covalent bonding in covalently crosslinked networks of PAA to prepare soft viscoelastic hydrogels with the same permanent elastic modulus but with different viscous moduli. The viscous linear PAA reduced the probability of cell differentiation and facilitated the re-acquisition of an undifferentiated phenotype [28]. Another recent study reported the culture of human primary epidermal keratinocytes and human mesenchymal stem cells at the liquid-liquid interface [30]. Stem cell spreading, cytoskeletal organization and cell proliferation and fate were predominantly dependent on the mechanical properties of the liquid-liquid interface, rather than the bulk mechanical properties of the substrates [30]. Furthermore, the response of murine myoblasts to lipid bilayers of different viscosities has been reported [31]. The lipid bilayers were formed on glass surfaces by using different lipids with different diffusion coefficients but the same functionalized RGD ligands. The cell spreading area increased while the circularity decreased with increasing viscosity. The influence of the lipid viscosity on cell morphology was interpreted according to the mobility if RGD ligands were present in the lipid bilayers because surface mobility has a significant impact on cell functions [30]. Liver cancer cell lines have been cultured on solid-liquid interfaces of extracellular fluids of various viscosities [31]. High viscosity was shown to be beneficial for integrin-based cell adhesion, cell migration and the loss of cluster cohesiveness. Extracellular fluid viscosity was able to interfere with the cell response to ECM stiffness [31].

The above-mentioned studies on the influence of viscosity on cell functions have been conducted on the surfaces or interfaces with substrates. The influence of the bulk 3D matrix viscosity on cell functions remains unknown because there are no good 3D culture systems or materials that can be used to mimic the cellular microenvironment used for investigation. Furthermore, 2D conditions cannot reflect 3D conditions. In some cases, the mechanical properties of interfaces (2D) and bulk materials (3D) have been shown to influence cell behaviors differently [30]. Considering the viscoelastic 3D microenvironment surrounding cells *in vivo*, it is desirable to reveal the role of bulk matrix viscosity on cell functions.

Therefore, in this part, a biphasic hydrogel system consisting of an inner solution phase and an embedding hydrogel phase was prepared by embedding a gelatin viscous solution on gelatin hydrogels. The system was used for the 3D culture of chondrocytes to investigate the effect on chondrocytes of bulk matrix viscosity. The gelatin hydrogels were able to block the migration of cells and confine the cells within the viscous gelatin solutions, which could mimic the viscous 3D *in vivo* microenvironment of tissues and organs. The same matrix material, gelatin, was used to ensure that the two phases had the same chemical properties to avoid the effects of matrix differences on cell functions. Viscosity had an evident influence on the cell morphology and proliferation, ECM production and the phenotype of chondrocytes.

2.3 Materials and methods

2.3.1 Synthesis of gelatin methacryloyl (GelMA)

GelMA was synthesized by the reaction of gelatin with methacrylic anhydride [32]. Porcine skin gelatin (type A, 300 bloom, Sigma-Aldrich, USA) was dissolved at 10% (w/v) in phosphate-buffered saline (PBS) at 50 ºC. Methacrylic anhydride (Sigma-Aldrich, USA) was added dropwise to the gelatin solution at a rate of 0.5 mL $minute^{-1}$ under vigorous stirring at 50 ºC and allowed to react in the dark for 3 hours. After a five-fold dilution in warm (40 ºC) PBS to stop the reaction, the mixture was dialyzed against Milli-Q water using a dialysis tube (12-14 kDa molecular weight cut-off, Spectrum Laboratories Inc. USA) in the dark for 7 days, during which the water was changed 3 times every day at 40 ºC to remove the salts, free methacrylic anhydride and methacrylic acid. The dialyzed solution was lyophilized in the dark for 7 days to obtain the GelMA powder and stored at -30 ºC for subsequent experiments.

2.3.2 ^{1}H-nuclear magnetic resonance (^{1}H-NMR)

The chemical structures of pristine gelatin and GelMA were characterized by ^{1}H-NMR (AL300; JEOL, Tokyo, Japan) with a single axis gradient inverse probe at a frequency of 300 MHz according to a previously described method [32]. Twenty milligrams of pristine gelatin and GelMA were completely dissolved in 1 mL deuterium oxide containing 0.05% (w/v) 3-(trimethylsilyl)-propionic-2,2,3,3,-d_4 acid sodium salt (Sigma-Aldrich, USA).

2.3.3 Measurements of viscosity of gelatin solution and stiffness of gelatin hydrogels

The viscosity and gel-to-sol phase transition temperature of the gelatin aqueous solutions were measured using a MCR 301 rheometer (Anton Paar, Germany). The gelatin powder (porcine, type A, 300 bloom, Sigma-Aldrich, USA) was sterilized in an autoclave at 121 ºC for 15 min and the sterile gelatin powder was dissolved in high-glucose Dulbecco's modified Eagle medium (H-DMEM, D6546; Sigma-Aldrich, USA) at 50 ºC to prepare a gelatin solution (15% (w/v)). The diluted gelatin solutions (5 and 10% (w/v)) were prepared by diluting the 15% (w/v) gelatin solution with H-DMEM at 50 ºC. For the rheological measurements, the gelatin solutions were placed between two parallel PP-50 plates with a gap distance of 0.1 mm. The rotational shear experiment was performed at a fixed temperature (37 ºC) with a controlled shear rate from 0.1 S^{-1} to 1000 S^{-1}. To measure the sol-to-gel transition temperature, an oscillatory dynamic experiment was performed at a fixed amplitude (g = 5%) and frequency (1 Hz) under a controlled temperature sweep from 25 ºC to 37 ºC at a heating rate of 0.15 ºC per minute. The gel-to-sol phase transition temperatures were determined according to the intersection of the storage modulus (G′) and the loss modulus (G″).

The stiffness of gelatin hydrogels was measured by atomic force microscopy (AFM, MFP-3D-Bio; Asylum Research, Santa Barbara, CA, USA). The GelMA hydrogels were prepared by the UV exposure of the sterile GelMA precursor solution. The hydrogels were incubated in PBS at 37 ºC overnight and then measured at room temperature. An optical microscope was used to observe hydrogels and control the position of the AFM tip. A silicon nitride cantilever (Bruker, Camarillo, CA, USA) with a 600 nm diameter glass ball was used as the probe. The exact spring constant was measured before each experiment using a

thermal tuning method. The force curves were obtained and fitted to the Hertz's contact model to calculate the Young's modulus [33]. Three different areas in each of three samples were measured for the calculation of the means and standard deviations (n = 3).

2.3.4 Isolation and subculture of chondrocytes

The isolation of bovine articular chondrocytes (BACs) from the knees of a 9 week-old calf was conducted according to a previously reported protocol [33]. The articular cartilage biopsy tissues were minced into small pieces using a sterile surgical scalpel and digested at 37 ºC for 24 hours in H-DMEM containing 0.2% (w/v) collagenase type II (Worthington Biochemical, Lakewood, NJ, USA) with shaking. After digestion, the solution was filtered through a 70 mm nylon filter to remove the undigested biopsy tissue. The isolated primary chondrocytes were harvested by centrifugation and cultured in 75 cm_2 tissue culture flasks in H-DMEM supplemented with 10% fetal bovine serum, 4500 mg L^{-1} glucose, 100 U mL^{-1} penicillin, 4 mM glutamine, 1 mM sodium pyruvate, 0.4 mM proline, 100 mg mL^{-1} streptomycin, 0.1 mM nonessential amino acids and 50 mg mL^{-1} ascorbic acid at 37 ºC in 5% CO_2. The cell culture medium was refreshed every 3 days. The chondrocytes were subcultured twice (P2 cells) and then used in the following experiments.

2.3.5 Preparation of chondrocyte-laden gelatin hydrogel microcubes

Chondrocytes were mixed with 5, 10 and 15% (w/v) gelatin solutions and the cell concentration was adjusted to 4×10^7 cells per mL. The cell suspensions were poured into a silicone frame with a 5 cm × 5 cm × 300 mm (height) space, which was placed on a perfluoroalkoxy (PFA) film-covered copper plate at 37 ºC and then a glass plate was placed on the silicone frame to flatten the solution. The construct was cooled at 4 ºC for 30 minutes to form cell-laden gelatin hydrogel sheets. The cell-laden gelatin hydrogel sheets were transferred onto a nylon mesh with a mesh opening size of 250 × 250 mm (AS ONE, Japan) and then gently pressed with a steel spatula to cut them into cube-shaped microgels (microcubes). The cell-laden gelatin hydrogel microcubes were used for the following experiments. All the materials were sterilized before use and the experiments were conducted in a clean bench.

2.3.6 Preparation of GelMA hydrogels loaded with cell-laden gelatin hydrogel microcubes

The GelMA precursor solution was prepared by dissolving the GelMA macromer (10% (w/v)) and the photoinitiator, 2-hydroxy- 1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (0.5% (w/v)) (I 2959, Sigma-Aldrich, USA), in PBS at 50 ºC in the dark. The GelMA/photo-initiator solution was sterilized using a 0.22 mm pore-size syringe filter and then cooled down to 34 ºC (GelMA was in a liquid state at this temperature) using a water bath in the dark for 1 hour. The GelMA/photo-initiator solution was mixed with the cell-laden gelatin hydrogel microcubes prepared earlier with microcubes and solution in the ratio of 1 : 2 (w/v). At 34 ºC, the cell-laden gelatin microcubes remained as hydrogels without dissolving. The mixture was poured into a silicone frame with a 5 cm × 5 cm × 2 mm (height) space, which was placed on a Teflon plate and then a quartz plate was placed on the silicone frame. The construct was irradiated with UV light at 365 nm using a CL-1000 UV crosslinker (Funakoshi Co., Ltd, Japan) at a distance of 20 cm for 5 minutes. After UV-initiated crosslinking, the hydrogel sheet was punched into discs using a 6 mm biopsy punch (Acu-Punch, Acuderm Inc., Fort Lauderdale, FL, USA). As a control, cell-laden GelMA hydrogels were prepared by UV exposure of a mixture of sterile GelMA precursor solution and chondrocytes by using the

same procedures. All the samples were cultured in H-DMEM in an atmosphere of 5% CO_2 at 37 ºC with shaking.

2.3.7 Evaluation of cell proliferation and sulfated glycosaminoglycan (sGAG) production

The proliferation of chondrocytes in each of the samples was evaluated by quantifying the amount of DNA. After being cultured for 3 hours, 7, 14 and 21 days, the samples were washed twice with PBS, freeze-dried and then digested by papain solution at 60 ºC for 6 hours with shaking. Papain powder (Sigma-Aldrich, USA) was dissolved at a concentration of 400 mg mL^{-1} in sterile 0.1 M phosphate buffer (pH 6.0) with 5 mM L-cysteine hydrochloride monohydrate (Sigma-Aldrich, USA) and 5 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-2Na, Sigma-Aldrich, USA). An aliquot of the papain-digested solution was mixed with bisbenzimide (Hoechst 33258) solution (Sigma-Aldrich, USA). Next, the fluorescence intensity was measured using an FP-8500 spectrofluorometer (JASCO, Tokyo, Japan) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The fluorescence values were used to calculate the DNA amount based on a standard curve. The sGAG content was measured by using a BlyscanTM Glycosaminoglycan Assay Kit (Biocolor, UK) according to a standard curve obtained from the sGAG standard supplied with the kit. All four samples were used for the analysis (n = 4).

2.3.8 Actin filament and cell nuclei staining

The samples were cultured for 1, 7, 14 and 21 days and were used for staining. The samples were washed with PBS twice and fixed with 4% paraformaldehyde-phosphate buffer solution (Wako, Japan) at 4 ºC for 24 hours. After being washed with PBS twice, the samples were immersed in PBS containing 0.2% Triton X-100 (Sigma-Aldrich, USA) for permeabilization of the cell membrane for 50 minutes at room temperature and then blocked with 1% bovine serum albumin (Wako, Japan) solution for 30 minutes at room temperature. Next, the samples were immersed in PBS containing 2.5% Alexa FluorVR 488 phalloidin (Thermo Fisher Scientific, USA) for 1 hour to stain the actin filaments and then placed in PBS containing 0.1% 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Dojindo Laboratories, Japan) to stain the cell nuclei for 10 minutes at room temperature. After washing with PBS, the stained samples were cut in a vertical direction, and the inner side of each sample was observed with a confocal laser scanning microscope.

2.3.9 Real-time polymerase chain reaction (RT-PCR) analysis

After being cultured for 21 days, each of the samples was washed twice with PBS. The samples were frozen in liquid nitrogen and crushed into powder using an electric crusher. The powder samples were dissolved in Sepasol RNA I Super G (1 mL per sample; Nacalai Tesque, Kyoto, Japan) to isolate the RNA. The RNA concentrations of each of the samples were measured and adjusted to almost the same concentration by adding nuclease-free water (Thermo Fisher Scientific, USA). Next, polymerase chain reaction (PCR) using a First-Strand cDNA Synthesis Kit (Applied Biosystems, USA) was conducted to transcribe the RNA extracted from each sample into cDNA. RT-PCR was used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Col1a2 (collagen type I), Col2a1 (collagen type II) and Acan (aggrecan) using a 7500 RT-PCR System (Applied Biosystems, USA). The expression level of GAPDH, a housekeeping gene, was used as an endogenous control, and the target gene expression relative to that of GAPDH was quantified with a $2^{-\Delta\Delta Ct}$ method. Chondrocytes cultured for 21 days in a GelMA hydrogel

loaded with cell-laden gelatin hydrogel microcubes of low-viscosity gelatin solution were used as a reference and the expression levels of each gene were normalized to 1. The primer and probe sequences were as follows [34]: GAPDH, forward primer, 5′-GCATCGTGGAGGGACTTATGA-3′, reverse primer, 5′-GGGCCATCCACAGTCTTCTG-3′, probe, 5′-CACTGTCCACGCCATCACTGCCA-3′; collagen type I, forward primer, 5′-TGCAGTAACTTCGTGCCTAGCA-3′, reverse primer, 5′-CGCGTGGTCCTCTATCTCCA-3′, probe, 5′-CATGCCAATCCTTACAAGAGGCAACTGC-3′; collagen type II, forward primer, 5′-AAGAAACACATCTGGTTTGGAGAAA-3′, reverse primer, 5′-TGGGAGCCAGGTTGTCATC-3′, probe, 5′-CAACGGTGGCTTCCACTTCAGCTATGG-3′; aggrecan, forward primer, 5′-CCAACGAAACCTATGACGTGTACT-3′, reverse primer, 5′-GCACTCGTTGGCTGCCTC-3′, probe, 5′-ATGTTGCATAGAAGACCTCGCCCTCCAT-3′. All three samples were used for the analysis (n = 3).

2.3.10 Statistical analysis

All statistical analyses were performed by a one-way analysis of variance (ANOVA) using KyPlot 5.0 software (KyensLab Inc., Tokyo, Japan). All quantitative data are expressed as the mean standard deviation (SD). Differences were considered to be statistically significant when $p < 0.05$. *, **, *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

2.4 Results

2.4.1 Rheological characteristics of gelatin solution and stiffness of gelatin hydrogels

Rheological measurements were conducted to evaluate the viscosity and gel-to-sol phase transition of gelatin solutions of different concentrations using a rotational shear rheometer and an oscillatory rheometer, respectively. The viscosity of the gelatin solution showed Newtonian behavior at 37 ºC regardless of the shear rate and gelatin concentration, which was in good agreement with the results of a previous report [36]. The viscosity increased with increasing gelatin concentration (Figure 2.1a). A cell culture medium (H-DMEM) was used as a control for comparison. These results indicated that the gelatin viscosity could be changed by altering the gelatin concentration. In this part, three concentrations of gelatin solutions (5, 10 and 15% (w/v)) were used to control the viscosity at three different levels (low, middle and high) to investigate the influence of viscosity on chondrocyte function.

The storage modulus (G′) and loss modulus (G″) of the three gelatin solutions were measured at a constant frequency of 1 Hz by changing the temperature from 25 ºC to 37 ºC (Figure 2.1b). As the temperature increased, the values of G′ and G″ of the samples gradually decreased and the gel-to-sol phase transition temperature was determined from the intersection point, where G″ exceeded G′. The gel-to-sol phase transition temperatures of the three gelatin solutions were almost the same and were below 37 ºC. The results indicated that the concentration of the pristine gelatin solution did not affect the gel-to-sol phase transition temperature. The gelatin solutions were still in a liquid state and could provide a viscous microenvironment for chondrocytes during cell culture at 37 ºC.

The Young's modulus of the GelMA hydrogels was measured by AFM nanoindentation and calculated from the obtained force curves according to the Hertz's model. The young's modulus of the GelMA hydrogels was 30.3 ± 3.3 kPa. The GelMA hydrogels had a high Young's modulus and were stiff.

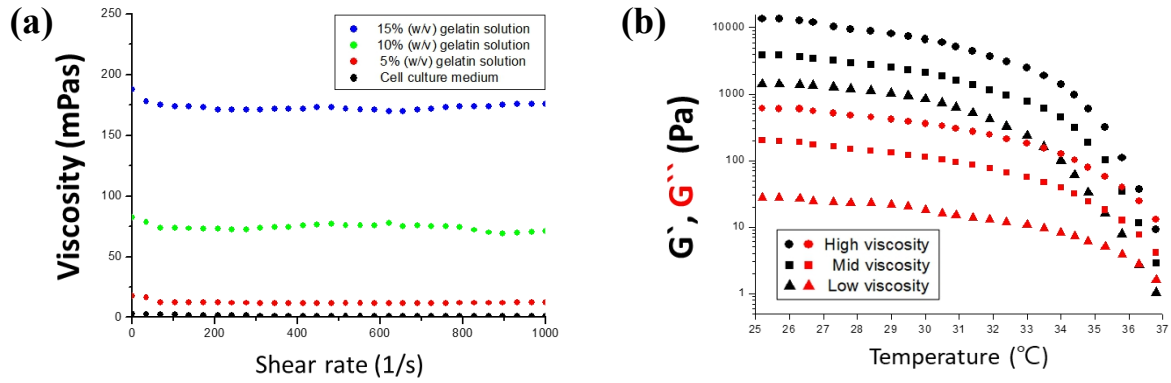

Figure 2.1 (a) Viscosity curves of the aqueous solutions of 5, 10 and 15 (w/v)% gelatin measured at 37 ºC. The cell culture medium used to prepare the gelatin solutions was used as a control. (b) Temperature-dependence curves of the G′ and G″ values of the 5, 10 and 15 (w/v)% gelatin aqueous solutions at a constant frequency of 1 Hz.

2.4.2 Preparation of cell-laden biphasic GelMA hydrogels

To investigate the effect of the viscosity of the gelatin solutions on cell functions, aqueous gelatin solutions with three different concentrations and viscosities were incorporated into the photocrosslinked GelMA hydrogels to construct the biphasic solution/hydrogel systems, in which microcubes of gelatin solution as the inner phase were encapsulated by the GelMA hydrogel as the outer phase (Figure 2.2a). The outer phase of the GelMA hydrogels was prepared by the photoinitiation of the crosslinking of the GelMA macromer, which was synthesized by the reaction of methacrylic anhydride with the amino groups in gelatin molecules. The synthesized GelMA macromer was analyzed by ^{1}H-NMR and the result indicated a high degree (91%) of methacrylation (Figure 2.3). Cell-laden GelMA hydrogels without microcubes of gelatin solution were prepared as a control.

First, the cell-laden gelatin microcubes were prepared by gelation of the mixture solution of chondrocytes and different concentrations of gelatin solution, followed by cutting with a nylon mesh (250 × 250 mm). The chondrocytes were homogeneously distributed in all the gelatin microcubes prepared with 5, 10 and 15% (w/v) gelatin solutions. The size of the microcubes was approximately 250 × 250 × 300 mm (Figure 2.2b). The preparation method enabled easy and efficient production of microcubes in a cell-friendly environment without the use of harmful reagents and solvents such as organic solvents and oils. Subsequently, the cell-laden gelatin microcubes were encapsulated within the photocrosslinkable GelMA hydrogel by UV irradiation. After culturing the constructs at 37 ºC, the cell-laden gelatin hydrogel microcubes were dissolved to yield a cell-laden gelatin solution. The cell-laden gelatin solution microcubes were encapsulated within the photocrosslinked GelMA hydrogel and were distributed within the GelMA hydrogel (Figure 2.2c). The cells and gelatin solution microcubes were constrained within the photocrosslinked GelMA hydrogel, allowing the 3D culture of chondrocytes in the viscous gelatin solution.

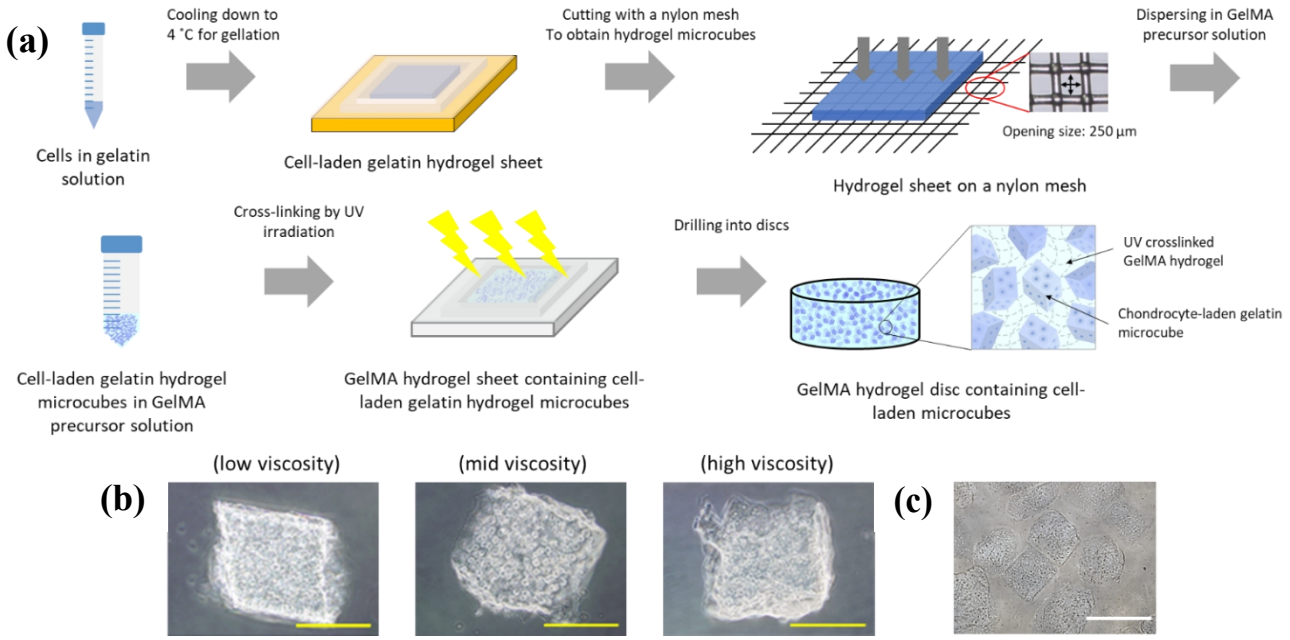

Figure 2.2 (a) Preparation scheme of the GelMA hydrogels containing cell-laden gelatin solution microcubes. (b) Representative photomicrographs of chondrocyte-laden gelatin hydrogel microcubes prepared with gelatin solutions of different viscosities at high magnification. (c) Representative photo of GelMA hydrogels containing cell-laden gelatin solution microcubes prepared with 10 (w/v)% gelatin solution at low magnification. The scale bars indicate (b) 200 µm and (c) 500 µm in the Figure legend.

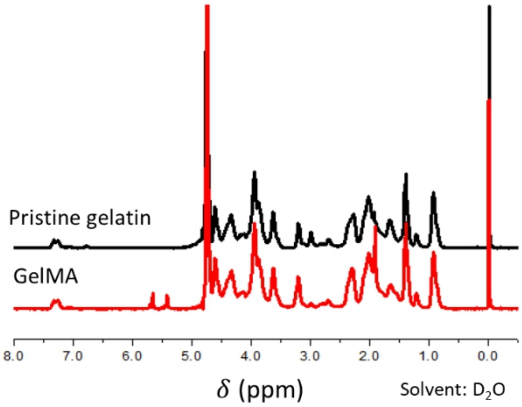

Figure 2.3 ^{1}H NMR spectra of pristine gelatin and GelMA macromer. The vinyl protons of the methacrylate groups were contributed to peaks at 5.4 ppm and 5.7 ppm. Peak at 2.9 ppm decreased due to reduction of the N-methylene protons in lysine groups after reaction with methacrylic anhydride.

2.4.3 Quantification of DNA and sGAG

The amount of DNA in the construct was quantitatively evaluated to investigate the effect of the viscosity of gelatin solutions on chondrocyte proliferation after 3 hours, 7, 14 and 21 days of culture (Figure 2.4a). The DNA amount after 3 hours of culture was the same for the three samples of different viscosities, indicating that the same number of chondrocytes was seeded in each sample. The amount of DNA in the GelMA hydrogels containing the chondrocyte-laden gelatin solution microcubes was lower than that in the chondrocyte-laden GelMA hydrogel without gelatin solution. This result should be due to the punching of the chondrocyte-laden biphasic GelMA hydrogels in discs during sample preparation, which could lead to

cutting of the cell-laden gelatin solution microcubes and therefore release the chondrocyte-laden gelatin solution located near the GelMA hydrogel surfaces.

The DNA amount showed a significant dependence on the viscosity of the gelatin solution (Figure 2.4a). The DNA amount was higher in the lower viscosity gelatin solution and was decreased with the increase in the gelatin solution viscosity. The results indicated that chondrocytes proliferated faster in the lower viscosity gelatin solution. Low viscosity was beneficial for chondrocyte proliferation. Compared to that in the viscous gelatin solution, chondrocyte proliferation was reduced in the stiff GelMA hydrogels (control).

The sGAG content, which is one of the main components of cartilage matrices, was quantitatively evaluated at the same time points as the DNA quantification (Figure 2.4b). The sGAG content was not detected after 3 hours of culture. After culturing for longer periods of 7, 14 and 21 days, the sGAG content was detected and was increased along with the culture time. The sGAG content was lower in the lower viscosity gelatin solution and was increased with increasing gelation solution viscosity. Chondrocytes cultured in the stiff GelMA hydrogels (control) showed the highest sGAG content. The sGAG/DNA ratio showed the same trend as that of the sGAG content, although it was more evident (Figure 2.4c). The ratio increased with increasing gelatin solution viscosity. The chondrocytes cultured in the stiff GelMA hydrogels showed the highest sGAG/DNA ratio.

The results indicated that chondrocytes cultured in gelatin solutions could proliferate and produce ECM, although the proliferation rate and ECM production capacity were dependent on the viscosity of the solutions. A low viscosity was more beneficial for chondrocyte proliferation, while a high viscosity was more beneficial for ECM production. However, chondrocytes cultured in the stiff GelM hydrogels had a high capacity for ECM production, but they almost did not proliferate. The stiff GelMA hydrogels showed the best effect on ECM production, while inhibiting the proliferation of chondrocytes.

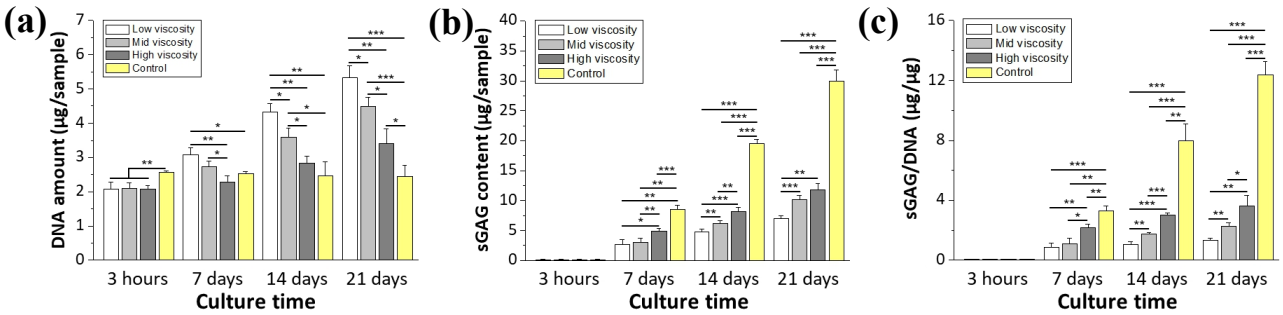

Figure 2.4 Quantification of (a) the DNA amount and (b) the sGAG content of GelMA hydrogels containing chondrocyte-laden gelatin solution microcubes prepared with gelatin solutions of different viscosities after culture for 3 hours and 7, 14 and 21 days. Chondrocyte-laden GelMA hydrogel without gelatin solution was used as a control. (c) The sGAG content was normalized to the DNA amount. Data are shown as the mean ± SD, n = 4. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

2.4.4 Cell morphology

It has been reported that cell morphology and cytoskeletal structures are correlated with cell proliferation and phenotypes [24, 34]. The nuclei and actin filaments of chondrocytes were stained after culturing for 1, 7, 14 and 21 days (Figure 2.5). After 1 day of culture in viscous gelatin solutions, the chondrocytes showed a rounded morphology. They spread and showed a spread cytoskeleton after culturing for 7, 14 and 21 days. Spreading was more evident in the chondrocytes cultured in the low-viscosity gelatin solution. Therefore, low viscosity could promote cell spreading more strongly than high viscosity. In the

high-viscosity gelatin solution, the chondrocytes were partially aggregated. Moreover, chondrocytes were observed only in the gelatin solution microcubes. No cells were observed in the surrounding GelMA hydrogels. The results indicated that no cells migrated from the viscous gelatin solution into the surrounding stiff GelMA hydrogel, which demonstrated that the biphasic system was effective for maintaining the cells in the viscous gelatin solution during the investigation of the influence of viscosity on cell functions.

In the case of the stiff GelMA hydrogel (control), the chondrocytes were homogenously distributed and maintained their spherical shape. Some aggregates were observed in the stiff GelMA hydrogel. Cell aggregates were more obvious after 21 days of culture.

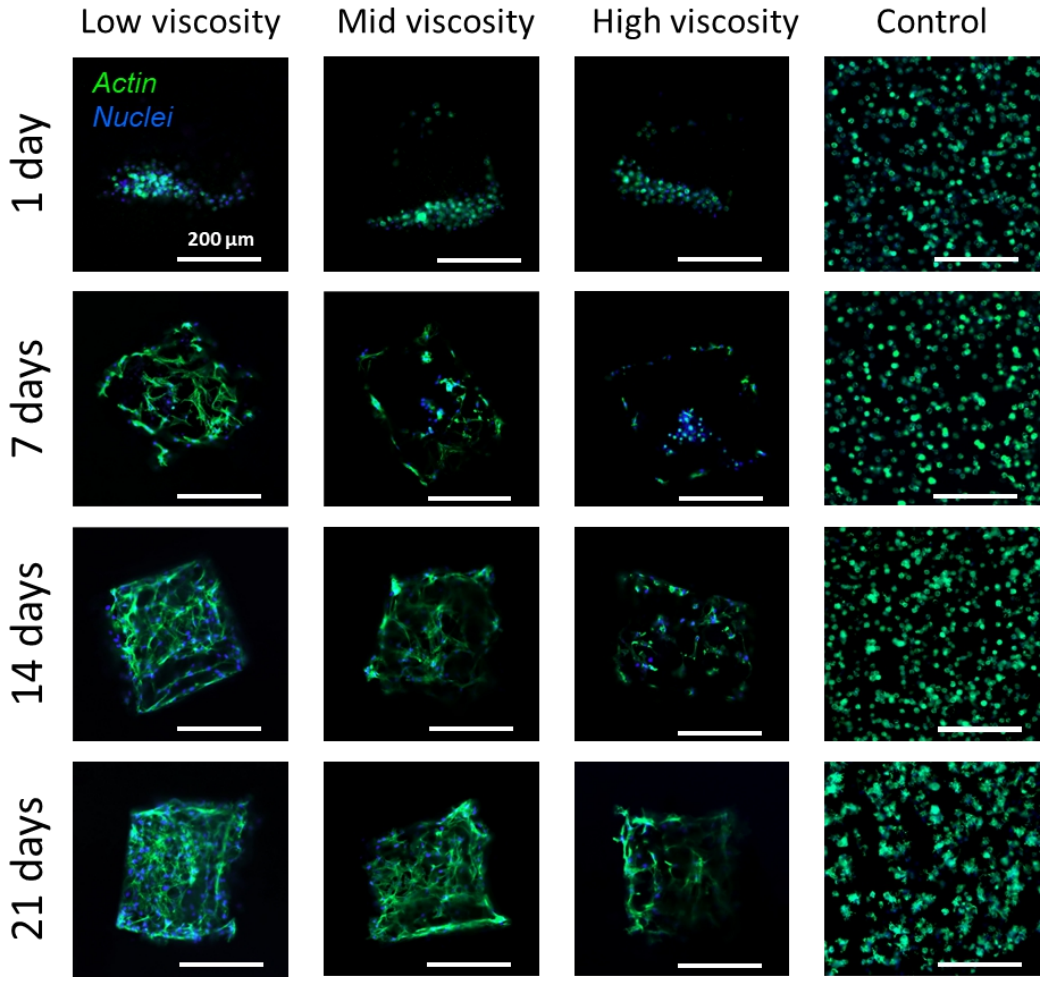

Figure 2.5 Representative confocal laser scanning microscopic images of chondrocytes cultured in gelatin solutions with different viscosities after 1, 7, 14 and 21 days. Actin filaments (green) and nuclei (blue) were fluorescently stained. Chondrocyte-laden GelMA hydrogel without gelatin solution was used as a control. Scale bar: 200 µm.

2.4.5 Quantification of cartilaginous gene expression

To further evaluate the chondrocyte responses to altered gelatin viscosity at the gene expression level, the gene expression levels of collagen type I, collagen type II and aggrecan were analyzed with RT-PCR after 21 days of culture (Figure 2.6). The expression of collagen type I, which is related to fibrocartilage formation, was high in the low-viscosity gelatin solution and its expression level decreased with increasing gelatin solution viscosity. Its expression was the lowest in the stiff GelMA hydrogel (control). On the other hand, the

two cartilaginous genes, collagen type II and aggrecan, were highly expressed in the high-viscosity gelatin solution. Their expression level decreased with decreasing viscosity. Their expression in the stiff GelMA

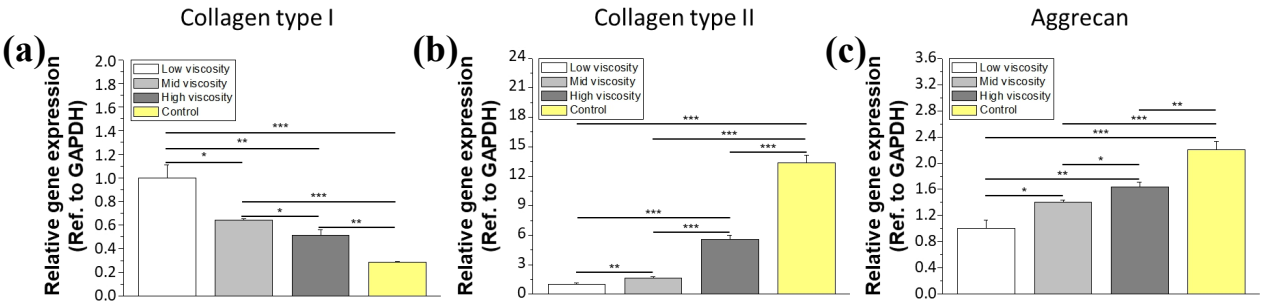

Figure 2.6 Quantification of the gene expression of (a) collagen type I, (b) collagen type II and (c) aggrecan in chondrocytes cultured in gelatin solutions with different viscosities after 21 days. Chondrocyte-laden GelMA hydrogel without gelatin solution was used as a control. Values were normalized according to the gene expression levels measured in chondrocytes cultured in a low-viscosity gelatin solution. Data are shown as the mean ± SD, n = 3. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

hydrogel (control) was the highest.

2.5 Discussion

In articular cartilage, the cartilaginous ECM surrounding chondrocytes provides numerous mechanical signals related to cellular functions, such as cell morphology, proliferation and differentiation [3]. Although viscoelastic properties have been shown to affect chondrocyte functions, the effect of ECM viscosity on chondrocyte functions, particularly in 3D culture, remains unclear. Therefore, in this part, a biphasic solution/hydrogel system composed of an inner gelatin solution phase and an embedding gelatin hydrogel phase was established to study the effects of fluid viscosity on chondrocyte functions.

The physically crosslinked gelatin hydrogel, which was a liquid solution at the cell culture temperature (37 ºC), was used as the inner phase. The gelatin was gelled at a low temperature (4 ºC) and dissolved at 37 ºC. Its viscosity could be adjusted by altering its concentration. Gelatin aqueous solutions at three different concentrations of 5, 10 and 15% (w/v) were used to produce low, medium and high viscosity for the 3D culture of chondrocytes. On the other hand, chemically crosslinked and stiff GelMA hydrogels were prepared by the photoinitiated crosslinking of photoreactive GelMA. The chemically crosslinked GelMA hydrogel was stable and retained its gelation structure during cell culture. The photoinitiated crosslinking was conducted after mixing the cell-laden physically crosslinked gelatin hydrogel microcubes and photoreactive GelMA, which led to the embedding of the physically crosslinked gelatin hydrogel microcubes in the chemically crosslinked gelatin hydrogel (GelMA hydrogel). During cell culture at 37 ºC, the physically crosslinked gelatin hydrogel microcubes were dissolved and the chondrocytes were suspended in the microcubic regions of gelatin solution surrounded by chemically crosslinked GelMA hydrogels. The solution and the embedding hydrogel matrix had the same composition of gelatin to avoid the influence of different compositions. During cell culture, no chondrocytes migrated from the gelatin solution microcubes into the embedding GelMA hydrogel. The cells were constrained in the microcubic regions of the gelatin solution. The system could be used for the 3D culture of cells in viscous liquids. To date, cells have been cultured at liquid-liquid or solid-liquid interfaces to investigate the effect of viscosity on cell functions. The biphasic hydrogel system used in the present study made it possible to culture cells in viscous 3D liquids.

When bovine primary articular chondrocytes were cultured in the biphasic hydrogel system, all three

gelatin solutions with different viscosities supported chondrocyte proliferation and ECM production. However, chondrocytes cultured in gelatin solutions of different viscosities showed different morphologies, proliferation rates, levels of ECM production and gene expression levels. Chondrocytes cultured in the low-viscosity gelatin solution proliferated more quickly than those cultured in the high-viscosity gelatin solution. High viscosity was beneficial for ECM production and the expression of articular cartilage-specific genes (collagen type II and aggrecan). The chondrocytes cultured in the low-viscosity gelatin solution had a more spread morphology than those cultured in the high-viscosity gelatin solution. The results indicated that high viscosity was more beneficial for the maintenance of the chondrocyte phenotype, while low viscosity was more beneficial for cell expansion.

The stiffness of cell culture substrates and scaffolds has been broadly investigated for its impact on chondrocyte functions. Some studies have reported that stiffer hydrogels or hydrogels with moderate stiffness are good for chondrogenic ECM deposition and the maintenance of the chondrocyte phenotype [35, 37, 38, 43]. For example, stiffer hydrogels show a higher capacity for inducing the expression of collagen type II and glycosaminoglycans than softer hydrogels when chondrocytes are cultured in chitosan-hyaluronic acid dialdehyde hydrogels with a stiffness range of 130.78-181.47 kPa [35]. Chondrocytes cultured in a poly(ethylene glycol) (PEG) hydrogel with a compressive modulus of 360 kPa showed enhanced secretion of cartilaginous matrices compared to those cultured in PEG hydrogels with higher (960 kPa) and lower (30 kPa) moduli [43] GelMA hydrogels of different stiffness were prepared from the same concentration of gelatin with different degrees of methacryloyl functionalization of the amino groups [32]. In comparison with GelMA hydrogels with three different stiffnesses within a relatively low range of stiffness (3.8, 17.1 and 29.9 kPa), GelMA hydrogels with a high stiffness (29.9 kPa) had a beneficial effect on the maintenance of the chondrogenic phenotype of bovine articular chondrocytes [32]. A further increase in the crosslinking degree and stiffness of GelMA hydrogels by utilizing their reaction with amino groups, hydroxyl and carboxyl groups (GelMAGMA hydrogels) can maintain the cartilaginous phenotypes of chondrocytes while inhibiting cell proliferation [37]. However, the capacity of GelMAGMA hydrogels to maintain a cartilaginous phenotype is inferior to that of GelMA hydrogels [37]. When chondrocytes were cultured in enzymatically crosslinked gelatin-hydroxyphenylpropionic acid hydrogels with a storage modulus from 570 to 2750 Pa, the hydrogels with a medium stiffness (1000 Pa) showed the best effect on the chondrocyte phenotype, cartilage regeneration and integration with the adjacent tissue [38].

The influence of stiffness on cartilaginous matrix deposition and neocartilage formation is also dependent on the culture time and culture mode (*in vitro* culture or *in vivo* implantation). Poly(ethylene glycol) dimethacrylate hydrogels functionalized with chondroitin sulfate, hyaluronic acid and heparin sulfate with a tunable stiffness range of ~1-33 kPa have been used for *in vitro* and *in vivo* cartilage regeneration, and hydrogels with a stiffness range of ~7-33 kPa have been shown to be preferable scaffolds for neocartilage formation [39]. Gelatin hydrogels with a three-dimensional architecture with high and low elastic properties have been used to investigate the influence of these properties on *in vitro* and *in vivo* chondrogenesis. Soft, fast-degrading hydrogels better promote *in vitro* chondrogenic differentiation, while stiffer, slow-degrading hydrogels are superior for neocartilage formation [40].

On the other hand, some studies have shown that softer hydrogels are more beneficial for the deposition of cartilaginous ECM and maintenance of the chondrocyte phenotype [41, 42]. For example, when chondrocytes were cultured in a polyethylene glycol dimethacrylate (PEGDM) hydrogel system with a Young's modulus gradient range of ~27-3.8 kPa, hydrogels with a lower Young's modulus induced an increased ECM content [43]. Softer collagen hydrogels promoted the expression of cartilaginous genes in comparison with stiffer collagen hydrogels [44]. Low stiffness (4 kPa) is good for maintaining the chondrocyte phenotype by up-regulating the expression of collagen type II and aggrecan while suppressing

actin organization and cell proliferation when chondrocytes are cultured on the surfaces of PAA hydrogels with a Young's modulus between 4 and 100 kPa [45]. The culture of chondrocytes in PEG hydrogels with a compressive modulus range between 60 and 670 kPa showed that highly crosslinked hydrogels with a high compression modulus have a greater impact on the inhibition of cell proliferation and proteoglycan synthesis [46]. PEG hydrogels with three different crosslinking densities (compressive moduli of 60, 320 and 590 kPa) had different impacts on ECM production [42]. PEG hydrogels with the lowest degree of crosslinking most significantly enhanced glycosaminoglycan production [42]. High stiffness is always accompanied by high polymer concentration and high crosslinking density, which are considered to impede secretion and re-distribution of ECM [37, 46].

Furthermore, viscoelastic hydrogels and substrates with stress relaxation have been used for cell culture to separate the influence of viscosity from that of elastic properties. Viscous linear PAA physically entrapped in covalently crosslinked PAA networks reduced the differentiation probability and facilitated the re-acquisition of an un-differentiated phenotype in rat hepatic stellate cells [28]. Cell culture at liquid-liquid interfaces or liquid-substrate interfaces has been adopted to investigate the influence of viscosity on cell functions [30, 31] Viscosity has been shown to play an important role in controlling cell spreading, cytoskeletal organization, cell proliferation and fate. However, all these studies were based on interfaces that were 2D.

The biphasic solution/hydrogel culture system used in this part allowed the 3D culture of chondrocytes. The influence of viscosity on chondrocyte functions was different from that of stiffness. Viscosity supported chondrocyte proliferation and showed different effects on ECM production and chondrocyte phenotypes, which were dependent on viscosity. The different results observed in the present study and previous studies are mainly due to the different properties of viscous liquids and elastic hydrogels. Previous studies have used elastic or viscoelastic hydrogels, which could not completely separate the influence of viscosity from that of other parameters. The influence of the viscosity of the gelatin solution could be completely separated from other factors and individually investigated. Chondrocytes cultured in a high-viscosity gelatin solution were less spread and partially aggregated. Chondrocytes cultured in the stiff GelMA hydrogel (control) showed an obviously rounded morphology and aggregation. The rounded morphology and cell aggregation may have contributed to the enhancement of the maintenance of the chondrocyte phenotype. The formation of cell aggregation can enhance cell-cell interactions, change cell morphology from spread to round and stimulate the secretion of cartilaginous matrices [47, 48]. Culture in viscous gelatin solutions not only increased the secretion of cartilaginous matrices and the maintenance of the chondrocyte phenotype but also supported chondrocyte proliferation.

2.6 Conclusions

A biphasic solution/hydrogel 3D culture system was established for the 3D culture of bovine primary articular chondrocytes to investigate the influence of viscosity on chondrocyte functions. Highly viscous gelatin solutions promoted cartilaginous gene expression and ECM secretion but also resulted in slow cell proliferation. On the other hand, chondrocytes cultured in low-viscosity gelatin solution showed fast proliferation and a more elongated cell morphology but less ECM production and cartilaginous gene expression. The results suggested that viscosity was a very important factor in the regulation of chondrocyte function. Viscosity could affect cartilaginous ECM production and the chondrocyte phenotype and supported chondrocyte proliferation.

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Chapter 3

Influence of viscosity on chondrogenic differentiation of mesenchymal stem cells during 3D culture in viscous gelatin solution-embedded hydrogels

3.1 Abstract

Differentiation of human bone marrow-derived mesenchymal stem cells (hMSCs) is regulated by a variety of cues of their surrounding microenvironment. In particular, mechanical properties of cell culture matrices have been recently disclosed to play a pivotal role in stem cell differentiation. However, it remains elusive how viscosity affects the chondrogenic differentiation of hMSCs during three-dimensional (3D) culture. In this part, a 3D culture system that was established by embedding viscous gelatin solution in chemically crosslinked gelatin hydrogels was used for 3D culture of hMSCs in gelatin solutions with different viscosities. The influence of solution viscosity on chondrogenic differentiation of hMSCs was investigated. Viscous gelatin solutions promoted cell proliferation in the order of low, middle and high viscosity while elastic hydrogels restricted cell proliferation. High viscosity gelatin solution led to increased production of the cartilaginous matrix. Under the synergistic stimulation of chondrogenic induction factors, high viscosity was beneficial for the chondrogenic differentiation of hMSCs. The results suggested the role of viscosity should be considered as one of the dominant mechanical cues affecting stem cell differentiation.

3.2 Introduction

Extracellular matrix (ECM) microenvironment is a three-dimensional (3D) network that surrounds and interacts with cells. Cell functions are regulated by various biological and physicochemical cues from ECM [1-6]. Although influence of biological and chemical cues on cell functions, such as stem cell differentiation, has been intensively studied so far, accumulating evidences indicate that physical cues, such as elasticity and viscoelasticity, also play an important role in regulating cell functions [7, 8]. Many approaches have been developed to explore the effect of such a physical cue, stiffness, on stem cell differentiation [9-14]. Initial studies of stiffness influence on cell differentiation have been performed on two-dimensional (2D) substrates [15-19]. Human bone marrow-derived mesenchymal stem cells (hMSCs) were seeded and grown on

polyacrylamide (PAA) gels with different stiffness and the results showed stiffness-dependent cell differentiation. hMSCs cultured on soft, intermediate and stiff PAA gels that respectively mimic the elasticity of brain (0.1-1 kPa), striated muscle (8-17 kPa) and premineralized bone (25-40 kPa) could differentiate to neurocytes, osteoblasts and skeletal muscle cells, respectively [20]. It has been well recognized that on 2D hydrogel substrates, stiff matrix is beneficial for osteogenic differentiation, while soft matrix is beneficial for adipogenic differentiation [21]. Studies have been further extended from 2D to 3D culture systems because 2D culture systems do not accurately reflect the complexity of 3D native tissue microenvironment. hMSCs encapsulated in alginate hydrogels with different elastic moduli differentiate to osteogenic and adipogenic lineages, which is dependent on elastic modulus [22]. Adipogenic differentiation primarily occurred in softer matrix with an elastic modulus of 2.5-5 kPa, while osteogenic differentiation occurred in stiffer matrix with an elastic modulus of 11-30 kPa, indicating that cell lineages were largely affected by matrices with similar stiffness to the microenvironment where cells reside. Softer elastomer scaffolds enhance chondrogenic differentiation of hMSCs, while stiffer ones promote their osteogenic differentiation [23, 24]. Thixotropic polyethylene glycol-silica gels have been used for 3D culture of hMSCs [25]. The thixotropic gels of different liquefaction stress, which is the minimum shear stress required for liquefaction of the gel, show different effect on differentiation of hMSCs. The gels with low (7 Pa), middle (25 Pa) and high (75 Pa) liquefaction stress showed the highest expression of neural, myogenic and osteogenic transcription factors, respectively. Enhanced stiffness of hydrogel scaffolds promotes osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells and accelerates bone regeneration [25]. On the other hand, soft gels prepared from self-assembling peptide facilitate adipogenic differentiation of MSCs [26]. ECM microenvironment in tissues exhibit elastic properties as well as viscous properties. In spite of extensive studies about the influence of matrix elastic modulus on cell functions have been reported, influence of matrix viscosity on cell functions has been less investigated due to lack of good culture systems. A recent approach has been reported by using viscoelastic PAA hydrogels with independently tunable elasticity and viscous dissipation to disclose their influence on rat primary hepatic stellate cells [27]. Cell phenotype is much closer to non-differentiated stellate cells on highly dissipative gels. In another study, RGD-functionalized lipid bilayers with controlled ligand density and different viscosity were designed to investigate the influence of pure viscous surfaces on murine myoblast functions [28]. A lot of focal adhesions as well as cell spreading were observed on high viscous surfaces (low mobility). These studies performed on viscoelastic or viscous surfaces reveal the viscosity influence on cell functions during 2D culture. However, influence of matrix viscosity on cell functions during 3D culture remains elusive. Recently, a 3D culture system has been established by embedding gelatin solutions of different viscosities in a chemically crosslinked hydrogel [29]. The system is applicable for 3D culture of hMSCs in viscous microenvironment. Meanwhile, osteogenic and adipogenic differentiation of hMSCs has been frequently conducted in either osteogenic or adipogenic induction medium. It is unclear how osteogenesis and adipogenesis of hMSCs competes each other at the same culture condition in 3D viscous microenvironment. Mixture medium of osteogenic and adipogenic media is useful for investigation of competing osteogenic and adipogenic differentiation of stem cells [30]. Therefore, in this part, hMSCs were cultured in 3D gelatin solutions of different viscosities with a mixture medium of osteogenic and adipogenic induction media to investigate how solution viscosity balances the osteogenic and adipogenic differentiation of hMSCs.

3.3 Materials and methods

3.3.1 Synthesis of gelatin methacryloyl macromer

Gelatin methacryloyl (GelMA) macromer was synthesized by introducing methacrylate groups in gelatin molecules [16]. 5 g of gelatin powder from porcine skin (type A, 300 bloom, Sigma-Aldrich, St Louis, Missouri, USA) was dissolved at 10 (w/v)% in phosphate buffered saline (PBS, pH 7.4, Nacalai Tesque, USA) at 50 ºC under stirring until gelatin was dissolved. 5 mL of methacrylic anhydride (Sigma-Aldrich, St Louis, Missouri, USA) was added dropwise to the gelatin solution at a rate of 0.5 mL min^{-1} under vigorous stirring and allowed to react in the dark at 50 ºC for 3 h. A five-fold warm (40 ºC) PBS was added into the mixture to stop the reaction and then the product was dialyzed against Milli-Q water using a dialysis tube (12-14 kDa molecular weight cut-off, Spectrum Laboratories, USA) in the dark for 7 days. During dialysis, water was changed 3 times every day at 40 ºC to remove salts, free methacrylic anhydride and methacrylic acid. The dialyzed solution was lyophilized to obtain the GelMA macromer. The degree of methacryloyl functionalization was 91.7 % [16].

3.3.2 Culture and osteogenic differentiation of hMSCs

Gelatin powder was sterilized in an autoclave at 121 ºC for 15 min and then dissolved in serum-free Dulbecco's Modified Eagle's Medium with high glucose (4500 mg L^{-1} glucose, H-DMEM, Sigma-Aldrich, St Louis, Missouri, USA). H-DMEM was supplemented with 4 mM glu- tamine, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 0.1 mM nonessential amino acids, 0.4 mM proline, 1 mM sodium pyruvate and 50 µg mL^{-1} ascorbic acid. Gelatin solutions with a concentration of 5, 10 and 15 (w/v)% were prepared. An MCR Rheometer (Anton Paar, Germany) was used to measure the viscosity of the gelatin solutions. The samples were placed between two parallel plates (PP-50) with a gap distance of 0.1 mm. The viscosity was measured by using a rotational shear mode at 37 ºC under a controlled shear rate from 0.1 S^{-1} to 1000 S^{-1}. The viscosity of the serum-free H-DMEM was also measured as a control. Gelatin solutions supplemented with or without chondrogenic induction factors were used for following cell culture experiments. To prepare gelatin solutions containing chondrogenic induction factors, the gelatin solutions were prepared with 10-7 M dexamethasone (Sigma-Aldrich, St Louis, Missouri, USA) and 10 ng mL^{-1} transforming growth factor-beta3 (TGF-β3; Sigma-Aldrich, St Louis, Missouri, USA).

3.3.3 Preparation of hMSC-laden gelatin microcubes

The preparation of hMSC-laden gelatin microcubes is shown in Figure 3.1. hMSCs were purchased from Lonza (Verviers, Belgium) at passage 2 and sub-cultured using MSCGM™ (Lonza, Verviers, Belgium) under an atmosphere of 5% CO_2 at 37 ºC. The subcultured hMSCs (P5) were detached by trypsin/EDTA (Sigma-Aldrich, St Louis, Missouri, USA) treatment, counted and re-suspended in the above prepared gelatin solutions (5, 10 and 15 (w/v)%) with or without dexamethasone and TGF-β3 at a concentration of 5 × 10^6 cells mL^{-1}. The cell/gelatin suspension solution was poured into a 300-µm-thick silicone frame (AS ONE, Japan) which was placed on a perfluoroalkoxy (PFA) film-covered copper plate at 37 ºC, and then a glass plate was placed on the silicone frame to flatten the solution. The hMSC-laden hydrogel sheets were formed by cooling down at 4 ºC, transferred onto a nylon mesh with a mesh opening size of 250 µm × 250 µm (AS

ONE, Japan) and pressed with a steel spatula through the mesh to obtain hMSC-laden gelatin hydrogel microcubes.

3.3.4 Preparation of GelMA hydrogels containing hMSC-laden gelatin microcubes

As shown in Figure 3.1, the as-prepared hMSC-laden gelatin hydrogel microcubes with or without dexamethasone and TGF-β3 were embedded in GelMA hydrogels by the initiation of chemical crosslinking of GelMA macromer under the presence of hMSC-laden gelatin hydrogel microcubes. GelMA macromer solution was prepared by dissolving GelMA macromer (10 (w/v)%) and photoinitiator (0.5 (w/v)%), 2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (Irgacure 2959, Sigma-Aldrich, St Louis, Missouri, USA), in PBS at 50 ºC in dark and then sterilized by syringe filter having a pore size of 0.22 μm. For the samples containing the chondrogenic induction factor, 10^{-7} M dexamethasone and 10 ng mL^{-1} TGF-β3 were added in the GelMA macromer solution. The GelMA macromer solution and the hMSC-laden gelatin hydrogel microcubes containing dexamethasone and TGF-β3 were mixed at a ratio of gelatin hydrogel microcubes and GelMA macromer solution of 1:2 (w/v) homogeneously. The mixture solution was added in a 2-mm-thick silicone frame placed between a Teflon plate and a quartz plate, followed with UV light irradiation at 365 nm using a CL-1000 UV crosslinker (Funakoshi, Japan) at a distance of 20 cm for 5 min. The UV-polymerized GelMA hydrogel sheets were punched into the discs by using a 6-mm biopsy punch (Acu-Punch, USA). After these procedures, GelMA hydrogel discs embedded with hMSC-laden gelatin hydrogel microcubes containing chondrogenic induction factors were prepared. The hMSC-laden gelatin hydrogel microcubes without chondrogenic induction factors were embedded in the GelMA hydrogels without chondrogenic induction factors by the same procedures. GelMA hydrogels loaded with hMSCs without gelatin hydrogel microcubes were prepared as controls. GelMA macromer solution was mixed with hMSCs at a final concentration of 5×10^6 cells mL^{-1}. In the case of samples containing chondrogenic induction factor, 10^{-7} M dexamethasone and 10 ng mL^{-1} TGF-β3 were added into the GelMA macromer solution before mixing with cells. hMSC-laden GelMA hydrogels were obtained after UV irradiation by the same procedures. hMSC-laden GelMA hydrogels were also punched into 6-mm discs. All these hydrogel discs were cultured in serum-free H-DMEM under shaking in an incubator with an atmosphere of 5% CO_2 at 37 ºC. For the culture of samples with chondrogenic induction factors, the culture

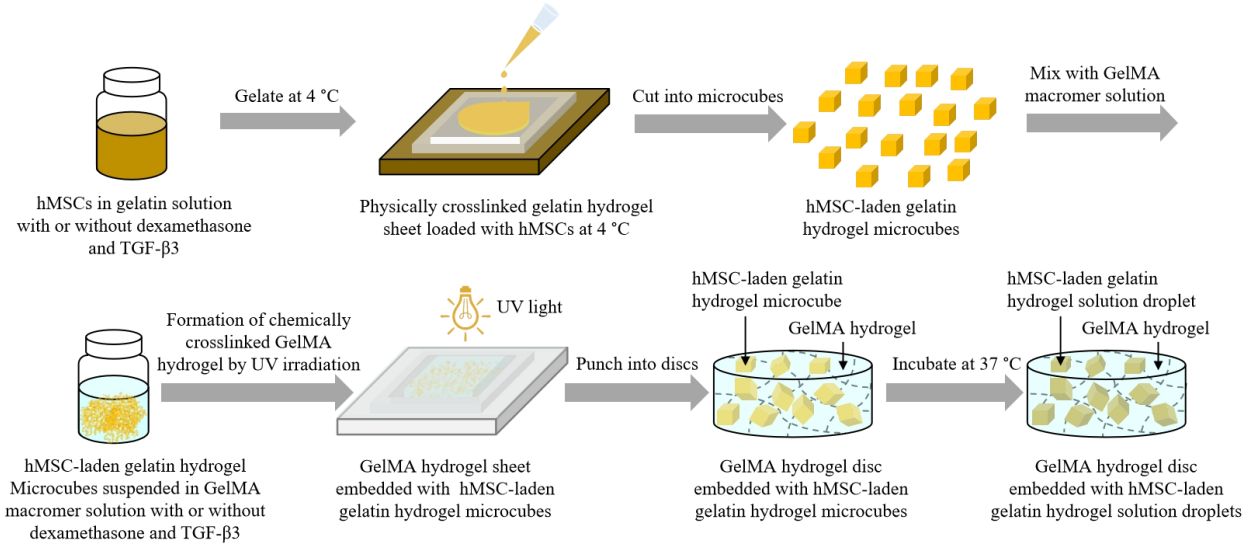

Figure 3.1 Schematic illustration of preparation of cell-laden gelatin solution-embedded GelMA hydrogel.

medium was added with 10^{-7} M dexamethasone. The samples were cultured for 21 days and the medium was changed every 3 days.

3.3.5 Cell viability assay

Cell viability was assessed by live/dead staining by using a Cell stain Double Staining Kit (Dojindo Laboratories, Japan) immediately after preparation of the samples and after 21 days of culture. All hydrogel discs were washed with PBS twice and incubated in serum-free medium containing calcein-AM (2 μM) and propidium iodide (4 μM) at 37 ºC for 15 min. The stained samples were cut in a vertical direction and observed with a confocal laser microscope (Zeiss LSM 510 Meta, Germany).

3.3.6 Cell proliferation and sGAG production assay

The hydrogel discs were harvested from their culture medium and then washed with PBS two times. After being frozen and lyophilized, they were digested in 500 μL papain solution at 60 ºC for 6 h under shaking. Papain solution (400 μg mL^{-1}) was prepared by dissolving papain powder (Sigma-Aldrich, St Louis, Missouri, USA) in 0.1 M phosphate buffer (pH 6.0) with 5 mM L-Cysteine hydrochloride monohydrate (Sigma-Aldrich, St Louis, Missouri, USA) and 5 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-2Na, Sigma-Aldrich, St Louis, Missouri, USA). The proliferation of hMSCs cultured in each condition was evaluated by quantification of DNA amount. A DNA quantification Kit (Sigma-Aldrich, St Louis, Missouri, USA) was utilized to measure the DNA amount in each sample. 5 μL of papain lysates aliquot was mixed with 2 mL bisbenzimide (Hoechst 33258) solution (Sigma-Aldrich, St Louis, Missouri, USA) in a cuvette. An FP-8500 spectrofluorometer (JASCO, Japan) was used to measure the fluorescence intensity at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The DNA amount was determined by calculating the recorded fluorescence values according to a standard calibration curve. The ECMs secretion was evaluated by quantifying sGAG content. The samples for measurement of sGAG content in the above-mentioned papain lysates were prepared with Blyscan™ Glycosaminogly can Assay Kit (Biocolor, UK) according to the manufacturer's in structions. The concentration of sGAG was measured by a microplate reader at 656 nm according to a standard curve. The sGAG content of each sample was calculated from its concentration and dilution ratio. Triplicate samples were used for the analysis to calculate means and standard deviations (n = 3).

3.3.7 RNA isolation and RT-PCR analysis

The hydrogel discs were collected, washed twice with PBS and frozen in liquid nitrogen. The frozen samples were crushed into powder using an electric crusher. The powder from each sample was dissolved in Sepasol RNA I Super G (1 mL per sample; Nacalai Tesque, Japan). Chloroform was added to the lysates to extract RNA through phase separation. The concentration of the extracted RNA was measured using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, USA). RNA concentration was between 101.2 ng μL^{-1} and 198.2 ng μL^{-1} and then adjusted to the same level by adding Nuclease-free Water (Thermo Fisher Scientific, USA). Next, the RNA extracted from each sample (2024 ng/sample) was converted to cDNA by using the first-strand cDNA synthesis kit (Applied Biosystems, USA). RT-PCR was performed to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH), type I collagen, type II collagen and aggrecan by using a 7500 RT-PCR System (Applied Biosystems, USA). The expression level of GAPDH, a

housekeeping gene, was used as endogenous control and the target gene expression relative to GAPDH was quantified with a $2^{-\Delta\Delta Ct}$ method. The P5 hMSCs used for cell seeding were used as a reference. The data were analyzed using a QuantStudio Design & analysis software. The following primer and probe sequences were used [17]: GAPDH (forward): 5′-ATGGGGAAGGTGAAGGTCG-3′, GAPDH (reverse): 5′-TAAAAGCAGCCCTGGTGACC-3′, GAPDH (probe): 5′-CGCCCAATACGACCAAATCCGTTGAC-3′; Type I collagen (forward): 5′-CAGCCGCTTCACCTACAGC-3′, Type I collagen (reverse): 5′-TTTTGTATTCAATCACTGTCTTGCC-3′, Type I collagen (probe): 5′−CCGGTGTGACTCGTGCAGCCATC-3′; 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′. Triplicate samples were used for the analysis to calculate means and standard deviations (n = 3).

3.3.8 Statistical analysis

All experiments were measured with at least three replicate samples. One-way analysis of variance (ANOVA) statistical analysis was performed using a KyPlot 5.0 software (Kyenslab, Tokyo, Japan). All quantitative data were indicated as the mean ± standard deviation (SD). The significance level was considered when $p < 0.05$. The data were marked as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

3.4 Results

3.4.1 Viscosity of gelatin solution of different concentration and stiffness of GelMA hydrogel

To obtain gelatin solutions of different concentrations and viscosities, different amount of gelatin powder was dissolved in serum-free H-DMEM. The three gelatin solutions showed a clear difference in viscosity. Their viscosities increased with an increase in gelatin concentration (Figure 3.2). Therefore, the 5, 10 and 15 (w/v) % gelatin solutions were used as cell culture matrix with low, medium and high viscosity, respectively.

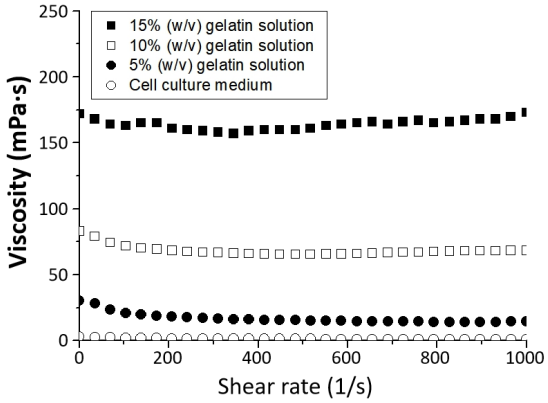

Figure 3.2 Viscosity curves of gelatin aqueous solutions having a concentration of 5, 10 and 15 (w/v)% and serum-free cell culture medium at 37 ºC.

3.4.2 Preparation of biphasic hydrogels loaded with cell-laden gelatin solutions

To investigate the influence of gelatin solution viscosity on chondrogenic differentiation of hMSCs, the biphasic hydrogels loaded with hMSC-laden gelatin solution were fabricated (Figure 3.1). The biphasic hydrogels consist of two phases. One phase was an inner phase that was composed of the hMSC-laden gelatin solution of different viscosity that was formed after the hMSC-laden gelatin hydrogel microcubes were dissolved at a solution temperature of gelatin (37 ºC). The other phase was chemically crosslinked GelMA hydrogels as an outer phase. The outer phase of GelMA hydrogels was prepared by UV-initiated crosslinking of the GelMA macromer. hMSCs were homogeneously distributed in all the gelatin microcubes regardless of the concentration of gelatin solution (Figure 3.3a and c). As a control, hMSCs were encapsulated in GelMA hydrogels without a gelatin solution. hMSCs were homogeneously distributed in the control GelMA hydrogels (Figure 3.3b and d). To investigate the synergistic effect of both chondrogenic induction factors and viscosity, chondrogenic induction factors (dexamethasone and TGF-β3) were added in both gelatin solutions and GelMA hydrogels (Figure 3.3c). GelMA hydrogels containing dexamethasone and TGF-β3 without gelatin solution were used as a control (Figure 3.3d). Cell distribution was almost the same

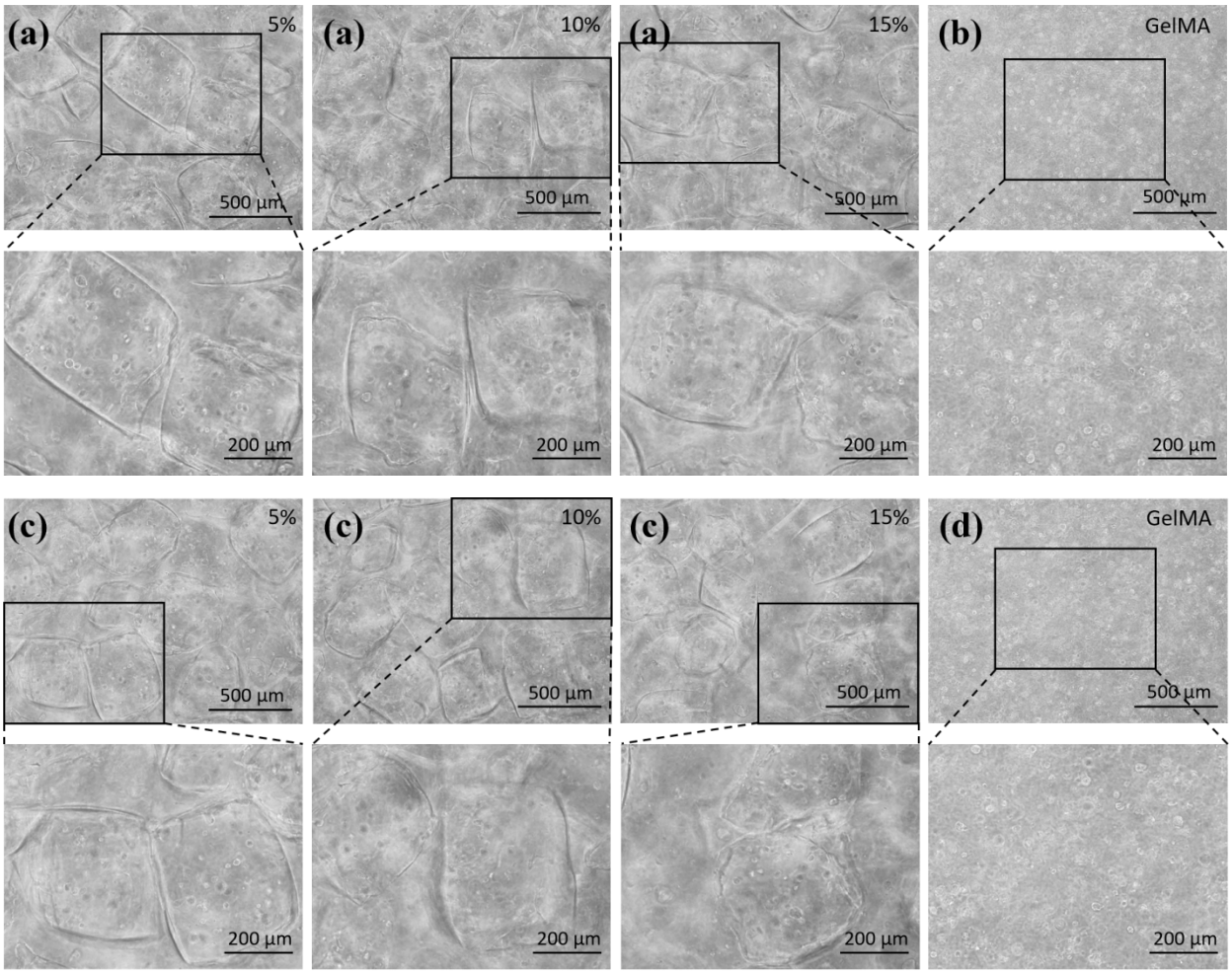

Figure 3.3 Phase-contrast micrographs of (a, c) biphasic hydrogels embedded with cell-laden gelatin microcubes and (b, d) cell-laden GelMA hydrogels without gelatin microcubes immediately after preparation. Dexamethasone and TGF-β3 were added in the hydrogels shown in (c) and (d), but not added in hydrogels shown in (a) and (b). 5, 10 and 15 (w/v)% indicate the concentrations of gelatin solutions. GelMA indicates cell-laden GelMA hydrogels.

in samples with or without dexamethasone and TGF-β. Gelatin microcubes in the biphasic hydrogels became gelatins solution droplets of different viscosities after being cultured at 37 ºC.

3.4.3 Cell viability

The cells were stained with calcein acetoxymethyl (AM) for live cells and propidium iodide for dead cells immediately after embedding and after 21 days of culture. The samples immediately observed after preparation of the biphasic hydrogels showed high cell viability and homogeneous cell distribution in gelatin solution regardless of different concentrations of gelatin solution and control GelMA hydrogel (Figure 3.4a and c). After 21 days of culture, most of cells were alive. Cell migration was restricted in the GelMA hydrogels (control) due to the stiff environment of the hydrogels. The cells in GelMA hydrogels had round

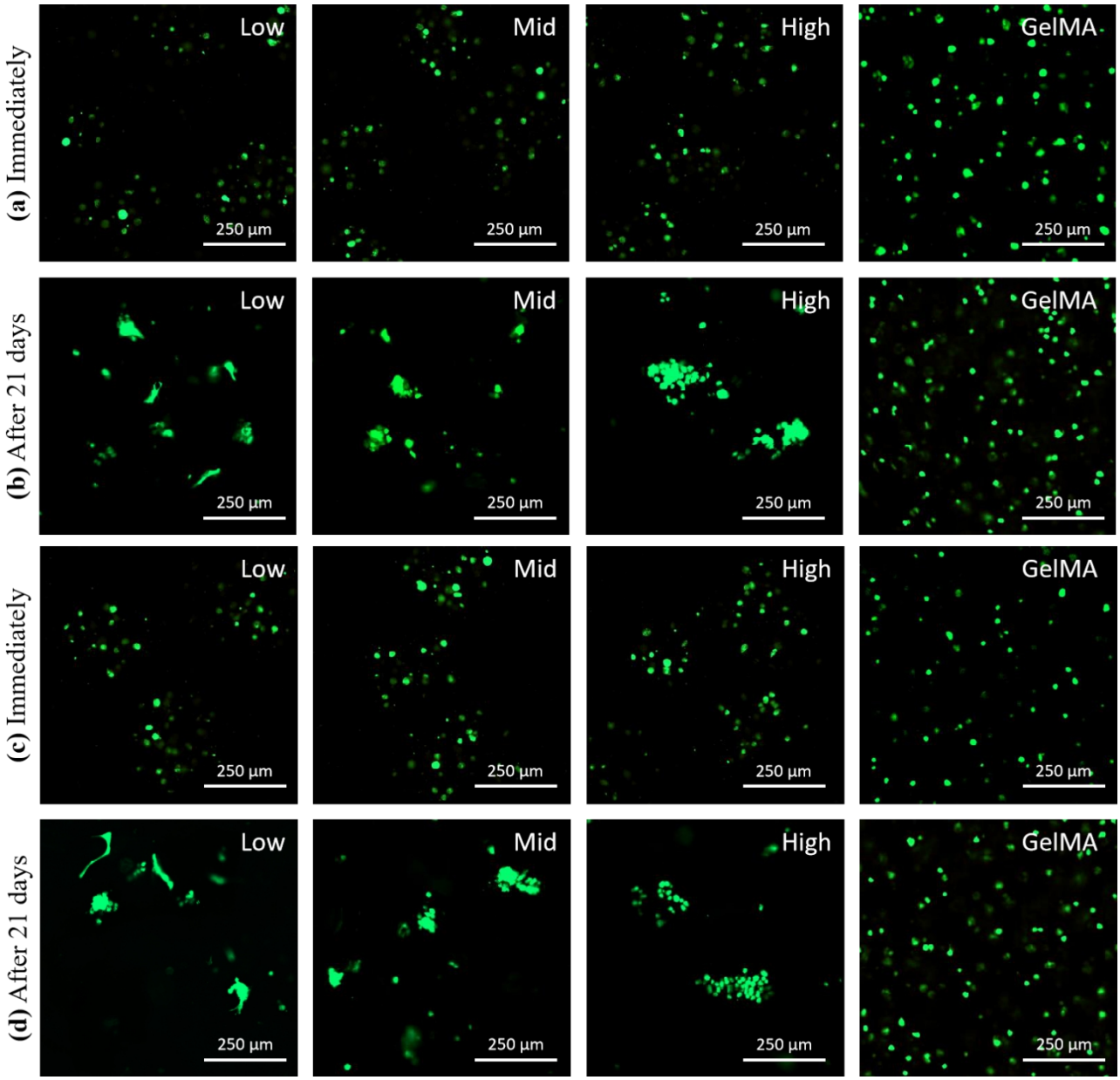

Figure 3.4 Live/dead staining of hMSCs in biphasic hydrogels embedded with cell-laden gelatin microcubes and cell-laden GelMA hydrogels (a, c) immediately after preparation and (b, d) after 21 days of culture. (c, d) Dexamethasone and TGF-β3 were added in the hydrogels, (a, b) but not added in the hydrogels. Live cells were stained green, while dead cells stained red.

morphology and were homogeneously distributed throughout the GelMA hydrogels during cell culture of 21 days. On the other hand, the cells in the gelatin solution embedded in the GelMA hydrogels spread and aggregated after culture at 37 ºC for 21 days (Figure 3.4b and d). Interestingly, cells cultured in a gelatin solution showed different cell morphologies according to the extent of the gelatin viscosity. Larger aggregates and less spreading of cells were observed in higher viscous solutions, whereas smaller aggregates and more spreading of cells were observed in lower viscous gelatin solution. When hMSCs were cultured under the presence of dexamethasone and TGF-β3 (Figure 3.4c and d), the cells had the same viability and morphology as those cultured without dexamethasone and TGF-β3 (Figure 3.4a and b). The results indicated that the addition of dexamethasone and TGF-β3 didn't affect cell viability and morphology.

3.4.4 Influence of gelatin solution viscosity on proliferation and sGAG production of hMSCs

To investigate the influence of gelatin solution viscosity on hMSCs proliferation, DNA amount in each sample was quantitatively evaluated after culture for 3 h, 7 days and 21 days (Figure 3.5a and d). After culturing for 3 h, the DNA amount in the biphasic hydrogels was lower than that in the GelMA hydrogels (control). DNA amount at this time point reflected the seeded cell number in each sample. Gelatin solution droplets that were located at the peripheral regions of the biphasic hydrogel discs could come out of the biphasic hydrogel discs because of the punching process, which might result in partial loss of seeded cells. The partial loss of seeded cells should explain why the initial DNA amount in the biphasic hydrogel discs was a little lower than that in the GelMA hydrogels without gelatin solution droplets. During cell culture without dexamethasone and TGF-β3, the DNA amount of hMSCs cultured in the biphasic hydrogels increased with culture time, while the DNA amount in the GelMA hydrogels remained almost unchanged (Figure 3.5a). The results indicated that hMSCs could proliferate in biphasic hydrogels. After culture for 21 days, the DNA amount of hMSCs cultured in low viscous gelatin solution was significantly higher than that of hMSCs cultured in high viscous gelatin solution. The DNA amount of hMSCs cultured in the biphasic

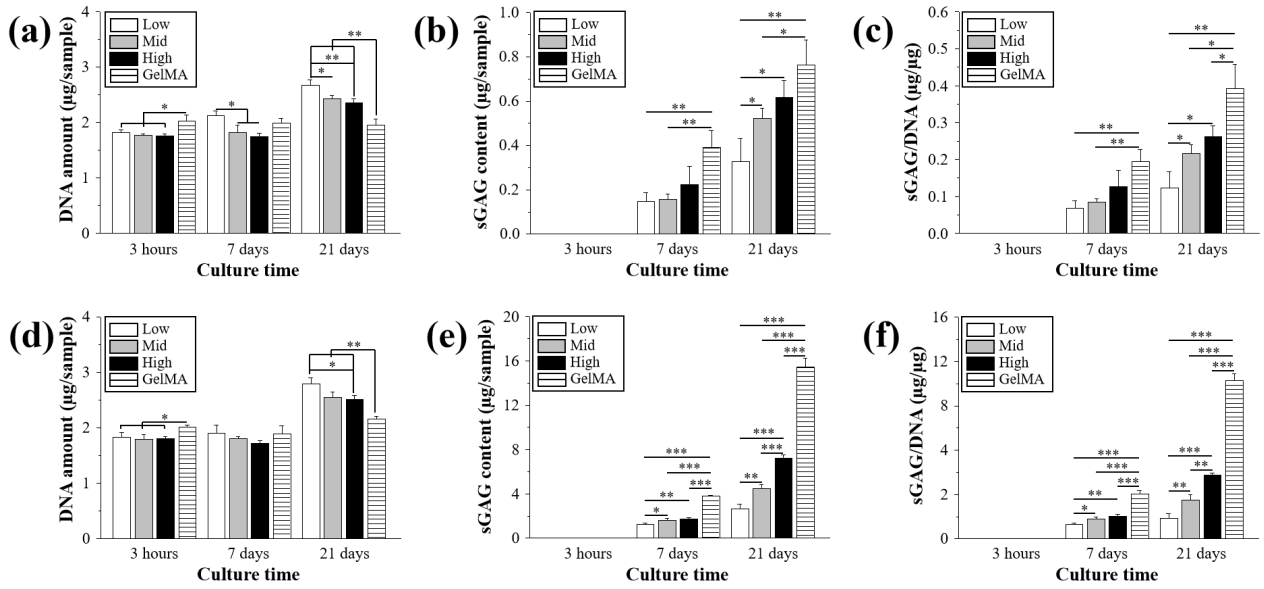

Figure 3.5 Quantification of (a, d) DNA amount, (b, e) sGAG content (c, f) normalized sGAG/DNA ratio in biphasic hydrogels embedded with cell-laden gelatin microcubes and cell-laden GelMA hydrogels (a, b, c) without and (d, e, f) with dexamethasone and TGF-β3. Lw, Mid and High indicate the viscosity of gelatin solution at a concentration of 5, 10 and 15(w/v)%. GelMA indicates cell-laden GelMA hydrogels. Data are shown as mean ± SD, n = 3. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$.

hydrogels and GelMA hydrogels under the presence of dexamethasone and TGF-β3 (Figure 3.5d) showed the same trend as that cultured without dexamethasone and TGF-β3 (Figure 3.5a). Production of sGAG content which is one of the main components of cartilage matrix was quantitatively evaluated to investigate chondrogenic differentiation of hMSCs. No sGAG was detected in the samples cultured for 3 h regardless of the presence or absence of dexamethasone and TGF-β3. After culture for 7 and 21 days, sGAG content increased with culture time. The cells cultured in the GelMA hydrogels showed the highest level of sGAG production. The cells cultured in the biphasic hydrogels showed increasing sGAG content with the increase of gelatin solution viscosity (Figure 3.5b and e). Normalization of sGAG content to DNA amount also revealed that sGAG/DNA value increased with culture time and increase of viscosity (Figure 3.5c and f). When hMSCs were cultured under the presence of the dexamethasone and TGF-β3, sGAG content and sGAG/DNA value were much higher than those cultured without dexamethasone and TGF-β3 (Figure 3.5e and f). However, the increasing trend was the same as that of hMSCs cultured without dexamethasone and TGF-β3. These results indicated that both cell proliferation and sGAG production were affected by gelatin solution viscosity. Supplement of dexamethasone and TGF-β3 showed a synergistic effect with the that of high viscosity.

3.4.5 Influence of ECM scaffolds on osteogenesis of hMSCs

Expression of genes encoding type I collagen, type II collagen and aggrecan of hMSCs cultured in the biphasic hydrogels and GelMA hydrogels was measured by RT-PCR (Figure 3.6). Without stimulation with dexamethasone and TGF-β3, expression of type I collagen increased, while expression of aggrecan decreased with culture time when hMSCs were cultured the biphasic hydrogels (Figure 3.6a and b). Expression of both type I collagen and aggrecan decreased with culture time when hMSCs were cultured in the GelMA hydrogels. Under the presence of dexamethasone and TGF-β3, the expression of type II collagen was

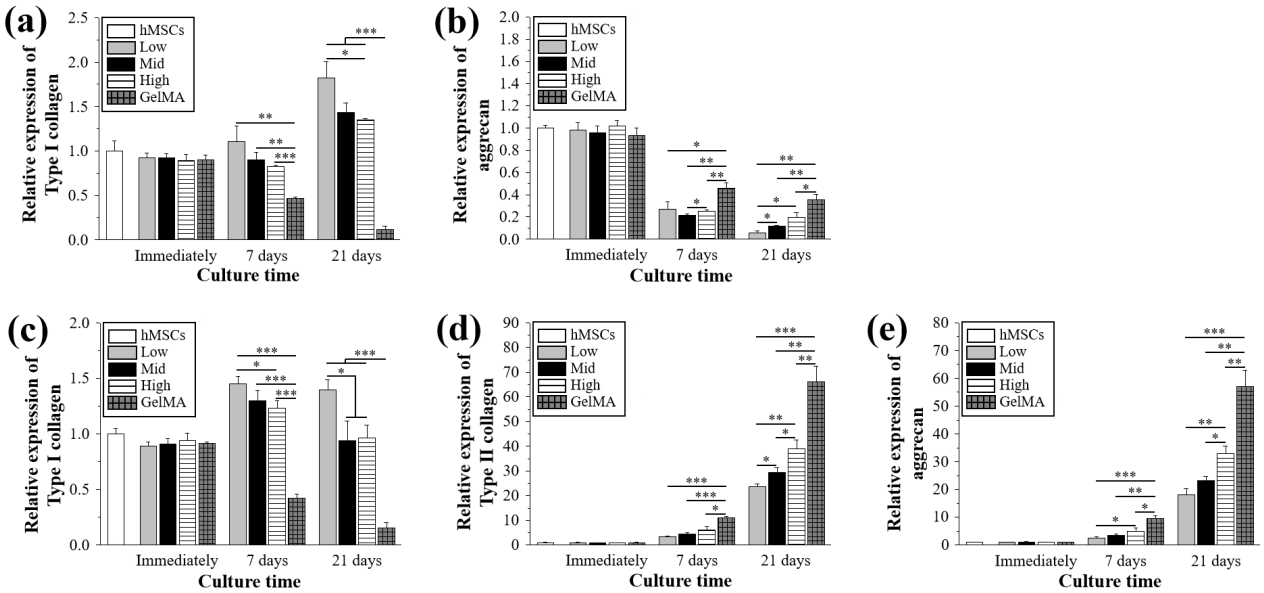

Figure 3.6 Quantification of gene expression of (a, c) type I collagen, (d) type II collagen and (b, e) aggrecan of hMSCs in biphasic hydrogels embedded with cell-laden gelatin microcubes and cell-laden GelMA hydrogels (a, b) without and (c, d, e) with dexamethasone and TGF-β3. Low, Mid and High indicate the viscosity of gelatin solution at a concentration of 5, 10 and 15(w/v)%. GelMA indicates cell-laden GelMA hydrogels. Data are normalized by gene expression levels measured in P5 hMSCs. Data are shown as mean ± SD, n = 3. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$.

detected (Figure 3.6d). The cells expressed a much higher level of aggrecan than did the cells cultured without dexamethasone and TGF-β3 (Figure 3.6e). Expression of type I collagen of hMSCs cultured in the biphasic hydrogels for 7 and 21 days was higher that of the starting point, whereas expression of type I collagen of hMSCs cultured in the GelMA hydrogels for 7 and 21 days was lower than that of the starting point (Figure 3.6c). Expression of type II collagen and aggrecan increased with culture time and increase of viscosity. Expression of type II collagen and aggrecan was the highest in hMSCs cultured in the GelMA hydrogels. These results indicated that gelatin solution viscosity could not promote the expression of chondrogenic genes under the absence of chondrogenic stimulation factors. However, high viscosity of gelatin solution showed a promotive effect on the expression of chondrogenic genes under the presence of chondrogenic stimulation factors.

3.5 Discussion

A variety of materials such as mucin, dextran and glycol (PEG) have been used to create viscous microenvironment [18, 19]. Unlike these materials, gelatin derived from thermal denaturation of the collagen which is a major component of ECMs can provide more closely mimic- king ECM microenvironment [20]. In addition, hMSCs can be encapsulated in gelatin hydrogels because gelatin solution can form hydrogels by physical crosslinking at a low temperature such as 4 ºC. Gelatin hydrogels can change to aqueous gelatin solution during culture at 37 ºC. Such phase change property of gelatin was used to embed gelatin solution in chemically crosslinked hydrogels for 3D cell culture. The influence of gelatin solution viscosity of stem cell proliferation and differentiation was disclosed. Live/dead staining showed that hMSCs cultured in the biphasic hydrogels and GelMA hydrogels had high viability (Figure 3.4). The cells in the gelatin solution of the biphasic hydrogels spread and aggregated during cell culture. The viscous gelatin solution allowed cell spreading. The cells could migrate in the viscous matrix to form aggregates. However, hMSCs in the GelMA hydrogels had round morphology and did not form aggregates. The GelMA hydrogels had Young's modulus of 30.3 kPa [21]. The stiff GelMA hydrogels inhibited cell spreading and migration. DNA quantification revealed that gelatin viscous solutions provided a beneficial environment for cell proliferation compared to elastic hydrogels (Figure 3.5a and d)). When gelatin solutions with low, middle and high viscosity were compared, low viscosity was beneficial for cell proliferation. Previous studies have reported that cell proliferation and aggregation are limited in stiff hydrogel matrices due to their high elastic stresses [22-24]. Viscosity also affected sGAG production (Figure 3.5b and e). hMSCs cultured in higher viscous gelatin solution produced higher content of sGAG. Interestingly, in the absence of chondrogenic induction factors (dexamethasone and TGF-β3), hMSCs deposited cartilaginous sGAG in decreasing order of elastic hydrogels, high-, middle and low-viscosity solution (Figure 3.5b). The results suggested that viscosity could affect cell proliferation and ECMs production. High viscosity was more beneficial for sGAG production, while low viscosity was more beneficial for cell proliferation. Synergistic effects of viscosity and chondrogenic induction factors were observed when dexamethasone and TGF-β3 were added in the biphasic hydrogels and GelMA hydrogels. Dexamethasone and TGF-β3 have been widely used for inductions of chondrogenic differentiation of hMSCs [25-27]. Cells cultured in the biphasic hydrogel and GelMA hydrogels with dexamethasone and TGF-β3 showed much higher production of cartilaginous sGAG and expression level of genes encoding type II collagen and aggrecan than did the cells cultured without dexamethasone and TGF-β3 (Figure 3.5 and 3.6). Under the presence of dexamethasone and TGF-β3, the stiff GelMA hydrogels showed the highest sGAG production and chondrogenic gene expression. The biphasic hydrogels showed different influences depending on the viscosity of the gelatin solution. The sGAG

production and chondrogenic gene expression increased with the increase of gelatin solution viscosity. However, hMSCs cultured without dexamethasone and TGF-β3 neither expressed type II collagen nor led to the up-regulation of aggrecan. The results suggested that viscosity could affect the chondrogenic differentiation of hMSCs and the influence could be magnified by the supplement of chondrogenic induction factors. Previous studies have focused on the influence of elastic and viscoelastic properties on stem cell differentiation [28, 29]. The 3D biphasic hydrogel culture system in this part could separate the influence of viscosity from elastic or viscoelastic parameters to investigate the viscosity influence on chondrogenic differentiation during the 3D culture of stem cells. The culture system was also used for the culture of other cell types, such as chondrocytes, to investigate viscosity impact on cell functions [21]. Hydrogels and biodegradable scaffolds have been broadly used in chondrocyte culture and autologous chondrocyte transplantation [30-32]. The 3D biphasic solution/hydrogel culture system may be useful to combine viscous solutions and hydrogels of a variety of biological molecules for autologous chondrocyte transplantation. The results should provide an insight into the influence of matrix viscosity on chondrogenic differentiation, moreover, a new strategy to design a viscous solution-based 3D hydrogel model for chondrogenic differentiation of hMSCs and tissue engineering applications.

3.6 Conclusions

This study disclosed the influence of gelatin solution viscosity on proliferation and chondrogenic differentiation of hMSCs in a 3D biphasic hydrogel culture system. The cells showed high viability during the 3D culture. High viscosity promoted cartilaginous sGAG production while inhibited cell proliferation. On the other hand, low viscosity promoted cell proliferation while suppressed cartilaginous sGAG production. Under the presence of chondrogenic induction factors, high viscosity gelatin solution had a higher promotive effect on chondrogenic differentiation of hMSCs than did low viscosity gelatin solution. The promotive effect of viscosity on the chondrogenic differentiation of hMSCs needed the synergistic effect of chondrogenic induction factors.

3.7 References

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Chapter 4

Osteogenic and adipogenic differentiation of mesenchymal stem cells in gelatin solutions of different viscosities

4.1 Abstract

Accumulating evidence indicates that stem cell fate can be regulated by mechanical properties of the extracellular matrix. Most studies have focused on the influence of matrix elasticity and viscoelasticity on stem cell differentiation. However, how matrix viscosity affects stem cell differentiation has been overlooked. In this part, a biphasic gelatin solution/hydrogel system was used for three-dimensional (3D) culture of human bone marrow-derived mesenchymal stem cells (hMSCs) to investigate the influence of gelatin solution viscosity on simultaneous osteogenic and adipogenic differentiation at the same culture condition. Gelatin solution promoted cell proliferation, while its promotive effect decreased with the increase of viscosity. The influence of viscosity on osteogenic and adipogenic differentiation of hMSCs showed opposite trends. A high-viscosity gelatin solution resulted in an increase of alkaline phosphatase (ALP) activity, calcium deposition and expression of osteogenesis-related genes. On the other hand, in a low-viscosity gelatin solution, a lot of lipid vacuoles were formed and adipogenesis-related genes were highly expressed. The results indicated high viscosity was beneficial for osteogenic differentiation, while low viscosity was beneficial for adipogenic differentiation. These findings suggested the importance of matrix viscosity on stem cell differentiation in 3D microenvironment.

4.2 Introduction

Extracellular matrix (ECM) microenvironment is a three-dimensional (3D) network that surrounds and interacts with cells. Cell functions are regulated by various biological and physicochemical cues from ECM [1-6]. Although influence of biological and chemical cues on cell functions, such as stem cell differentiation, has been intensively studied so far, accumulating evidences indicate that physical cues, such as elasticity and viscoelasticity, also play an important role in regulating cell functions [7, 8]. Many approaches have been developed to explore the effect of such a physical cue, stiffness, on stem cell differentiation [9-13]. Initial studies of stiffness influence on cell differentiation have been performed on two-dimensional (2D) substrates [14-18]. Human bone marrow-derived mesenchymal stem cells (hMSCs) were seeded and grown on polyacrylamide (PAA) gels with different stiffness and the results showed stiffness-dependent cell

differentiation. hMSCs cultured on soft, intermediate and stiff PAA gels that respectively mimic the elasticity of brain (0.1-1 kPa), striated muscle (8-17 kPa) and premineralized bone (25-40 kPa) could differentiate to neurocytes, osteoblasts and skeletal muscle cells, respectively [19]. It has been well recognized that on 2D hydrogel substrates, stiff matrix is beneficial for osteogenic differentiation, while soft matrix is beneficial for adipogenic differentiation [20].

Studies have been further extended from 2D to 3D culture systems because 2D culture systems do not accurately reflect the complexity of 3D native tissue microenvironment. hMSCs encapsulated in alginate hydrogels with different elastic moduli differentiate to osteogenic and adipogenic lineages, which is dependent on elastic modulus [21]. Adipogenic differentiation primarily occurred in softer matrix with an elastic modulus of 2.5-5 kPa, while osteogenic differentiation occurred in stiffer matrix with an elastic modulus of 11-30 kPa, indicating that cell lineages were largely affected by matrices with similar stiffness to the microenvironment where cells reside. Softer elastomer scaffolds enhance chondrogenic differentiation of hMSCs, while stiffer ones promote their osteogenic differentiation [22, 23]. Thixotropic polyethylene glycol-silica gels have been used for 3D culture of hMSCs [24]. The thixotropic gels of different liquefaction stress, which is the minimum shear stress required for liquefaction of the gel, show different effect on differentiation of hMSCs. The gels with low (7 Pa), middle (25 Pa) and high (75 Pa) liquefaction stress showed the highest expression of neural, myogenic and osteogenic transcription factors, respectively. Enhanced stiffness of hydrogel scaffolds promotes osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells and accelerates bone regeneration [24]. On the other hand, soft gels prepared from self-assembling peptide facilitate adipogenic differentiation of hMSCs [25].

ECM microenvironment in tissues exhibits elastic properties as well as viscous properties. In spite of extensive studies about the influence of matrix elastic modulus on cell functions have been reported, influence of matrix viscosity on cell functions has been less investigated due to lack of good culture systems. A recent approach has been reported by using viscoelastic PAA hydrogels with independently tunable elasticity and viscous dissipation to disclose their influence on rat primary hepatic stellate cells [26]. Cell phenotype is much closer to non-differentiated stellate cells on highly dissipative gels. In another study, RGD-functionalized lipid bilayers with controlled ligand density and different viscosity were designed to investigate the influence of pure viscous surfaces on murine myoblast functions [27]. A lot of focal adhesions as well as cell spreading were observed on high viscous surfaces (low mobility). These studies performed on viscoelastic or viscous surfaces reveal the viscosity influence on cell functions during 2D culture. However, influence of matrix viscosity on cell functions during 3D culture remains elusive.

Recently, a 3D culture system has been established by embedding gelatin solutions of different viscosities in a chemically crosslinked hydrogel [28]. The system is applicable for 3D culture of hMSCs in viscous microenvironment. Meanwhile, osteogenic and adipogenic differentiation of hMSCs has been frequently conducted in either osteogenic or adipogenic induction medium. It is unclear how osteogenesis and adipogenesis of hMSCs competes each other at the same culture condition in 3D viscous microenvironment. Mixture medium of osteogenic and adipogenic media is useful for investigation of competing osteogenic and adipogenic differentiation of stem cells [29]. Therefore, in this part, hMSCs were cultured in 3D gelatin solutions of different viscosities with a mixture medium of osteogenic and adipogenic induction media to investigate how solution viscosity balances the osteogenic and adipogenic differentiation of hMSCs.

4.3 Materials and methods

4.3.1 Preparation of gelatin solutions and their viscosity measurement

Gelatin powder (type A, 300 bloom, Sigma-Aldrich, St Louis, Missouri, USA) was sterilized in a glass bottle by using an autoclave at 121 ºC for 15 min. The sterile gelatin powder was dissolved in Dulbecco's modified Eagle's medium with low glucose (1000 mg L^{-1}) (L-DMEM, D6046, Sigma-Aldrich, St Louis, Missouri, USA), Dulbecco's modified Eagle's medium with high glucose (4500 mg L^{-1}) (H-DMEM, D6546, Sigma-Aldrich, St Louis, Missouri, USA), or a mixture medium of L-DMEM and H-DMEM at a ratio of 7:3 v/v to prepare high-concentration gelatin solution (15%, w/v) using a water bath at 50 ºC with slow stirring. L-DMEM and H-DMEM contained 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), 0.1 × 10^{-3} m MEM NEAA, 1 × 10^{-3} m MEM sodium pyruvate, 100 U-100 µg mL^{-1} penicillin-streptomycin, 50 mg mL^{-1} L-Ascorbic acid, 584 mg mL−1 (4 × 10^{-3} m) L-Glutamine and 0.4 × 10^{-3} m proline. Low- and middle-concentration gelatin solutions (5 and 10%, w/v) were prepared by diluting the high-concentration gelatin solution (15%, w/v). Totally 9 types of gelatin solutions were prepared as follows: gelatin solutions (5, 10 and 15%, w/v) in L-DMEM, H-DMEM and L-DMEM/H-DMEM mixture medium, respectively. The viscosity of gelatin solutions of different concentrations (5, 10 and 15%, w/v) prepared in L-DMEM, H-DMEM or mixture medium was measured with an MCR Rheometer (Anton Parr, Germany) using a rotational shear mode. The sample solutions were placed between two parallel plates (PP-50) with a gap size of 0.1 mm and then measured at shear rates ranging from 0.1 to 1000 S^{-1} at a constant temperature of 37 ºC. The viscosity of L-DMEM, H-DMEM and mixture medium was measured as controls.

4.3.2 Preparation of cell-laden gelatin hydrogel microcubes

hMSCs at passage 2 were purchased from Lonza (Verviers, Belgium) and sub-cultured in Mesenchymal Stem Cell Growth Medium (MSCGM™, Lonza, Verviers, Belgium) under a humidified atmosphere of 5% CO_2 at 37 ºC. The sub-cultured hMSCs at passage 5 were used for the following experiments. hMSCs were embedded in physically crosslinked gelatin hydrogel by suspending them in gelatin solution at 37 ºC and gelation at 4 ºC [28]. The sub-cultured hMSCs were detached by treatment with trypsin/EDTA solution (Sigma-Aldrich, St Louis, Missouri, USA) and re-suspended in the as-prepared 9 types of gelatin solutions (5, 10 and 15%, w/v) at a final density of 5 × 10^6 cells mL^{-1}. Each of the cell-laden gelatin solution was poured into a 300 µm thick silicone frame (AS ONE, Japan) which was placed on a perfluoroalkoxy (PFA) film-wrapped copper plate at 37 ºC. Next, a glass plate was placed on the silicone frame to flatten the solution. The constructs were placed at 4 ºC to allow gelation of the cell-laden gelatin solutions (physically crosslinked gelatin hydrogels). The cell-laden gelatin hydrogel sheets were transferred onto a nylon mesh having a mesh opening size of 250 × 250 µm (AS ONE, Japan). The cell-laden gelatin hydrogel microcubes were obtained by gently pressing the gelatin hydrogel sheets through the nylon mesh with a steel spatula and used for following experiments (Figure 2).

4.3.3 Preparation of gelatin methacryloyl hydrogels embedded with cell-laden gelatin hydrogel microcubes

To prepare chemically crosslinked gelatin hydrogels, gelatin methacryloyl macromers were synthesized by introducing methacryloyl groups in gelatin molecules [28]. Porcine skin gelatin (type A, 300 bloom, Sigma-Aldrich, St Louis, Missouri, USA) was dissolved at 10% w/v in phosphate buffered saline (PBS) at 50 ºC and stirred until fully dissolved. Methacrylic anhydride (Sigma-Aldrich, St Louis, Missouri, USA) was added to the gelatin solution at a rate of 0.5 mL min^{-1} under stirring condition at 50 ºC and allowed to react in the dark for 3 h. Following a fivefold dilution with additional warm (40 ºC) PBS to stop the reaction, the mixture was dialyzed against Milli-Q water using a dialysis tube (12-14 kDa molecular weight cut-off, Spectrum Laboratories Inc. USA) in the dark for 7 days at 40 ºC to remove the salts and free methacrylic acid. The solution was lyophilized for 7 days to obtain GelMA macromer and stored at -80 ºC until further use. GelMA macromer (10%, w/v) was dissolved in L-DMEM, H-DMEM or mixture medium, respectively and then mixed with photo-initiator, 2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (Irgacure 2959, Sigma-Aldrich, St Louis, Missouri, USA), at a concentration of 0.5% w/v to prepare GelMA precursor solutions. These solutions were sterilized by filtration through syringe filters with 0.22 μm pore size. The sterilized solutions were homogeneously mixed with the cell-laden gelatin hydrogel microcubes at a ratio of gelatin hydrogel microcubes/GelMA precursor solution of 1:2 w/v. The same medium was used for both the cell-laden hydrogel microcubes and the GelMA precursor solutions of each system. The mixtures were placed between a Teflon plate and a quartz plate separated by 2 mm thick silicone frame and then irradiated with UV light at 365 nm using a CL-1000 UV crosslinker (Funakoshi Co., Ltd., Japan) at a distance of 20 cm for 5 min. After UV irradiation, the GelMA macromers were chemically crosslinked to form GelMA hydrogel sheets embedded with the cell-laden gelatin hydrogel microcubes. The chemically crosslinked hydrogel sheets were punched with punches having an inner diameter of 6 mm to obtain 6 mm diameter discs.

4.3.4 3D culture and competing osteogenic and adipogenic differentiation of hMSCs in gelatin solutions of different viscosities

The above-prepared GelMA hydrogel discs embedded with cell-laden gelatin hydrogel microcubes were cultured in osteogenic induction medium, adipogenic induction medium or mixture medium of osteogenic and adipogenic induction media under an atmosphere of 5% CO_2 at 37 ºC with shaking. During culture at 37 ºC, the cell-laden gelatin hydrogel microcubes were melted to cell-laden gelatin microdroplets where hMSCs were suspended. The medium was changed every 3 days. The osteogenic induction medium was the above-mentioned L-DMEM supplemented with 10×10^{-9} m dexamethasone (Sigma-Aldrich, St Louis, Missouri, USA) and 10×10^{-3} m glycerol 2-phosphate disodium salt hydrate (β-GP, Sigma-Aldrich, St Louis, Missouri, USA). The adipogenic induction medium was the above-mentioned H-DMEM supplemented with 1×10^{-6} m Dex, 0.5×10^{-3} m methyl-isobutylxanthine (IBMX), 10 μg mL^{-1} insulin and 100×10^{-6} m indomethacin (Indo). The mixture medium was prepared by mixing osteogenic induction medium and adipogenic induction medium at a ratio of 7:3 v/v.

4.3.5 Cell proliferation

DNA amount in each sample was measured immediately after preparation of the GelMA hydrogel discs embedded with cell-laden gelatin hydrogel microcubes and after culture for 7, 14 and 21 days. A DNA quantification Kit (Sigma-Aldrich, St Louis, Missouri, USA) was used to measure the DNA amount. At first, the samples were collected, washed with PBS twice and freeze-dried. Next, they were digested in papain solution (400 μg mL^{-1}) which was prepared by dissolving papain powder (Sigma-Aldrich, St Louis, Missouri, USA) in 0.1 m phosphate buffer (pH 6.0) with 5×10^{-3} m L-cysteine hydrochloride monohydrate (Sigma-Aldrich, St Louis, Missouri, USA) and 5×10^{-3} m ethylenediaminetetraacetic acid disodium salt dihydrate (Sigma-Aldrich, St Louis, Missouri, USA). Finally, an aliquot of the papain digestion solution was mixed with bisbenzimide Hoechst 33258 solution (Sigma-Aldrich, St Louis, Missouri, USA) and measured with an FP-8500 spectrofluorometer (JASCO, Tokyo, Japan) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The fluorescence intensity values were calculated to determine DNA amount according to a standard calibration curve. Triplicate samples were used for the measurements ($n = 3$).

4.3.6 Cell viability

Live/dead staining was conducted with a Cellstain double staining kit (Dojindo Laboratories, Japan) to evaluate cell viability in each sample immediately after sample preparation and after culture for 21 days. The samples were washed with PBS twice and incubated in serum-free medium containing calcein-AM (2×10^{-6} m) and propidium iodide (4×10^{-6} m) at 37 ºC for 15 min. The stained cells were observed with a confocal laser microscope (Zeiss LSM 510 Meta).

4.3.7 Alkaline phosphatase activity assay

ALP activity of each sample was analyzed with a sensolyte pNPP alkaline phosphatase assay kit (Anaspec, USA) according to the manufacturer's instructions. Briefly, the samples cultured for 7 days were collected, washed with PBS twice, frozen in liquid nitrogen and crushed into powder by an electric crusher. The powder samples were dissolved in 0.2% Triton X-100 solution in ice/water mixture. The lysates were mixed homogeneously and centrifuged at a speed of 10,000 g at 4 ºC to collect supernatants. Next, the supernatants were incubated with a p-nitrophenyl phosphate (pNPP) substrate solution and colorimetric detection was conducted at 405 nm. An ALP standard solution was used to prepare a standard calibration curve. The ALP amount was normalized with DNA content of each sample. Triplicate samples were used for the measurements.

4.3.8 Calcium deposit assay

Calcium assay kit (DICA-500, BioAssay Systems, USA) was used to measure calcium deposition of the samples according to the manufacturer's instructions. Briefly, the samples cultured for 21 days were collected, rinsed with PBS twice, frozen and immersed in a 0.5 m HCl solution to digest the samples. The lysates were mixed with working reagent, incubated for 3 min at room temperature and determined by colorimetric detection at 612 nm. Calcium amount was calculated according to a standard curve and the data were was normalized with DNA content of each sample. Triplicate samples were used for the measurements.

4.3.9 Oil Red O staining and quantification

The samples were rinsed with PBS twice, fixed with 4% paraformaldehyde for 6 h at 4 ºC and rinsed with water. After being treated with a 60% 2-propanol solution for 2 h, the samples were immersed in 60% Oil Red O working solution for 10 min at room temperature. The 60% Oil Red O working solution was prepared by diluting Oil Red O stock solution (0.3 mg mL^{-1} Oil Red O powder in 99% 2-propanol) with water. After the cells were stained, the samples were washed with water and observed with an optical microscope. Next, these samples were dried in the air and the Oil Red O dye was extracted with 2-propanol at room temperature for 2 h. The absorbance of Oil Red O dye extracted from the stained cells was measured with a microplate reader at 540 nm. The absorbance results were subtracted with background values of the hydrogels containing undifferentiated cells. Triplicate samples were used for the measurements.

4.3.10 RNA isolation and Real-Time PCR analysis

After culture for 7, 14 and 21 days, the samples were collected, washed with PBS twice, frozen in liquid nitrogen and crushed into powder using an electric crusher. The powder samples were dissolved in Sepasol RNA I Super G (1 mL per sample; Nacalai Tesque, Japan). Chloroform was added to extract RNA through phase separation. Concentration of the extracted RNA was measured with a NanoDropTM Lite Spectrophotometer (Thermo Fisher Scientific, USA) and unified with nuclease-free water (Thermo Fisher Scientific, USA). A first-strand cDNA synthesis kit (Applied Biosystems, USA) was used to convert RNA to cDNA. A 7500 Real-Time PCR system (Applied Biosystems, USA) was used to perform real-time PCR. Expression of GAPDH (a housekeeping gene) was used as an endogenous control and relative gene expression was calculated with a $2^{-\Delta\Delta Ct}$ method. Data were normalized with gene expression levels of P5

Table 4.1 Primers and probes for real-time PCR analysis.

mRNA	Description	Oligonucleotide
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
ALP	Alkaline phosphatase	Forward 5’-GACCCTTGACCCCCACAAT-3’ Reverse 5’-GCTCGTACTGCATGTCCCCT-3’ Probe 5’-TGGACTACCTATTGGGTCTCTTCGAGCCA-3’
IBSP	Bone sialoprotein 2	Forward 5’-TGCCTTGAGCCTGCTTCC-3’ Reverse 5’-GCAAAATTAAAGCAGTCTTCATTTTG-3’ Probe 5’-CTCCAGGACTGCCAGAGGAAGCAATCA-3’
SPP1	Secreted phosphoprotein 1	Forward 5’-CTCAGGCCAGTTGCAGCC-3’ Reverse 5’-CAAAAGCAAATCACTGCAATTCTC -3’ Probe 5’-AAACGCCGACCAAGGAAAACTCACTACC-3’
SP7	Osterix	Hs00541729_m1
RUNX2	Runt-related transcription factor-2	Hs00231692_m1
LPL	Lipoprotein lipase	Hs00173425_m1
FABP4	Fatty acid binding protein 4	Hs00609791_m1
FASN	Fatty acid synthase	Hs00188012_m1
CEBPA	CCAAT/enhancer binding protein	Hs00269972_s1

hMSCs used for cell seeding. Expression of ALP, Sp7 transcription factor (SP7), secreted phosphoprotein 1, runt-related transcription factor-2 and bone sialoprotein 2 was analyzed to check osteogenic differentiation. Expression of fatty acid binding protein 4 (FABP4), fatty acid synthase (FASN), CCAAT/enhancer binding protein (CEBPA) and lipoprotein lipase (LPL) was analyzed to check adipogenic differentiation. The primer and probe sequences of target genes used in this part are listed in Table 4.1.

4.3.11 Statistical analysis

Each quantitative analysis experiment was carried out in triplicate. One-way analysis of variance (ANOVA) statistical analysis was performed to evaluate the significance of the experimental data using KyPlot 5.0 software (KyensLab Inc., Tokyo, Japan). All quantitative data were reported as the mean ± standard deviation (SD). When $p < 0.05$, the difference was considered significant. The data are shown as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

4.4 Results

4.4.1 Gelatin solutions of different viscosities

The gelatin solutions with different concentrations (5, 10 and 15%, w/v) were prepared by dissolving gelatin powder in three types of culture media. There are Dulbecco's modified Eagle's medium with low (1000 mg L^{-1}) or high (4500 mg L^{-1}) glucose or their mixture medium. The viscosity of gelatin solutions increased with their concentration (Figure 4.1). Gelatin solutions of the same concentration had almost the same viscosity even different medium was used to prepare the solutions (Figure 4.1d). The results indicated that the viscosity of gelatin solution could be controlled by gelatin concentration, whereas the three types of media used to dissolve the gelatin powders had no effect on viscosity. Therefore, 5, 10 and 15% w/v gelatin solutions were used for following experiments as low-, middle- and high-viscosity gelatin solutions, respectively.

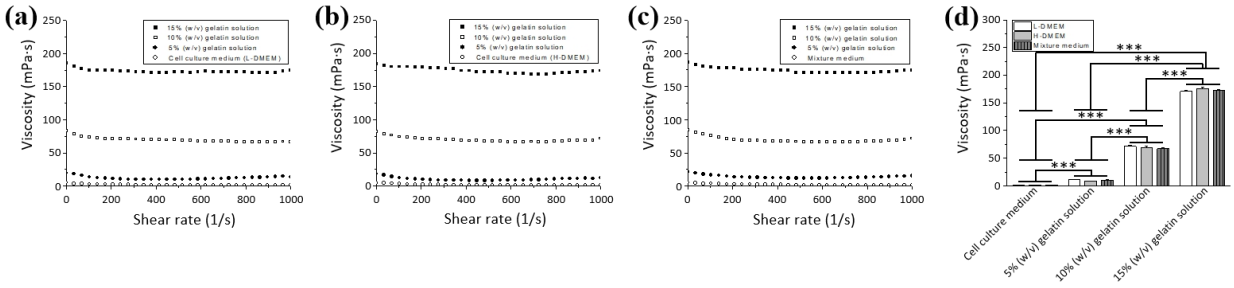

Figure 4.1 Representative curves of viscosity measurements of gelatin aqueous solutions with a concentration of 5, 10, and 15% w/v in (a) L-DMEM, (b) H-DMEM and (c) mixture medium of L-DMEM and H-DMEM at a ratio of 7:3 v/v. (d) The viscosity of gelatin aqueous solutions with differentconcentrations in different media was compared by calculating the averages of viscosity values measured at a shear rate from 0.1 to 1000 S^{-1}. The viscosity was measured at 37 ºC. Each medium without gelatin was used as controls. Data are shown as mean ± SD, n = 3. ***, $p < 0.001$.

4.4.2 GelMA hydrogels embedded with hMSC-laden gelatin solutions and cell viability

hMSCs were suspended in gelatin solutions of different viscosities and droplets of the hMSC-laden gelatin solutions were embedded in a chemically crosslinked gelatin methacryloyl (GelMA) hydrogel (Figure 4.2). The culture system allowed 3D cell culture in viscous gelatin solution for a long term. At first, hMSC-laden gelatin hydrogel sheets were prepared through physical gelation of hMSC/gelatin suspension solutions at 4 ºC after mixing hMSCs in the gelatin solutions of three different concentrations (i.e., low, middle and high viscosity). The hMSC-laden gelatin hydrogel sheets were cut into discs by passing through a nylon mesh to obtain hMSC-laden gelatin hydrogel microcubes. Next, the hMSC-laden gelatin hydrogel microcubes were dispersed in GelMA macromer solution and the mixture solution was irradiated with UV

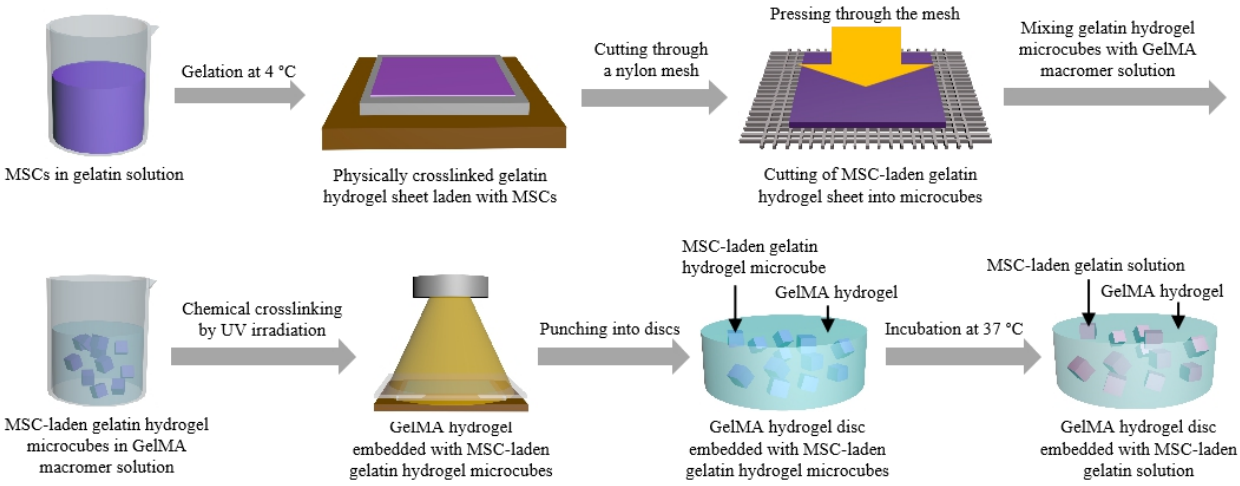

Figure 4.2 Preparation scheme of GelMA hydrogel embedded with hMSC-laden gelatin solution.

light to form chemically crosslinked GelMA hydrogels. The hMSC-laden gelatin hydrogel microcubes were embedded in the GelMA hydrogels as shown in Figure 4.3. Finally, the as-prepared hydrogels were cultured at 37 ºC and the hMSC-laden gelatin hydrogel microcubes were melted to form hMSC-laden gelatin solution

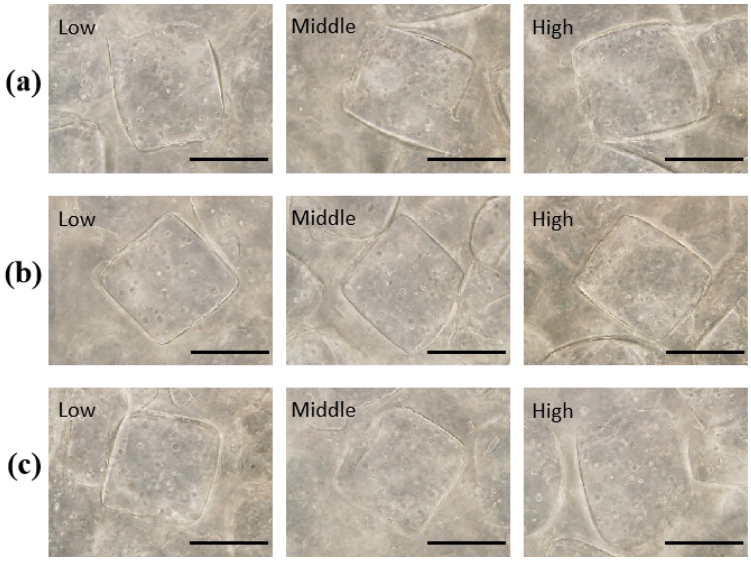

Figure 4.3 Representative photomicrographs of GelMA hydrogels embedded with hMSC-laden gelatin hydrogel microcubes in (a) osteogenic induction medium, (b) adipogenic induction medium and (c) mixture medium immediately after preparation. Low, Middle and High indicate low-, middle- and high-viscosity gelatin solutions at a concentration of 5, 10 and 15% w/v, respectively. Scale bar: 200 μm.

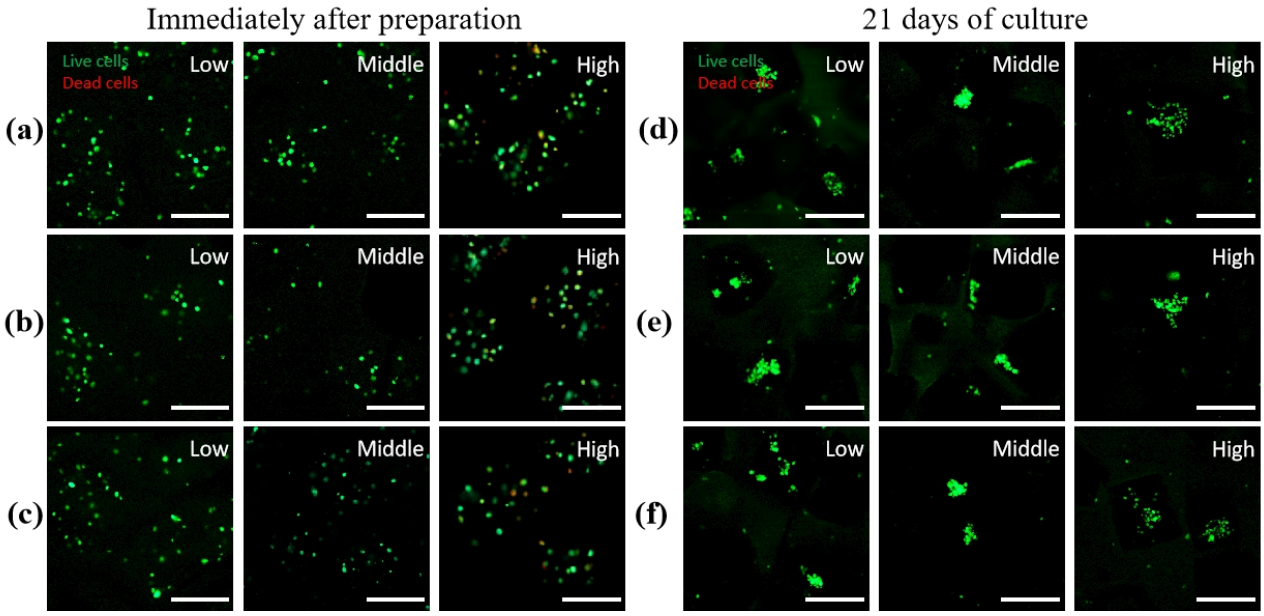

Figure 4.4 Live/dead staining of hMSCs encapsulated in the biphasic hydrogels (a, b, c) immediately after preparation and (d, e, f) after 21 days of culture in (a, d) osteogenic induction medium, (b, e) adipogenic induction medium and (c, f) mixture medium. Scale bar: 250 μm.

droplets that were embedded in GelMA hydrogels, forming hMSC-laden biphasic hydrogels. The hMSC-laden biphasic hydrogels were cultured in osteogenic induction medium, adipogenic induction medium and their mixture medium. Live/dead staining of the samples immediately after preparation and after 21 days of culture showed high viability of hMSCs in the biphasic hydrogels (Figure 4.4). In all the gelatin solutions of low, middle and high viscosities, most of the cells were alive (green color) and only a few cells were dead (red color). The cells showed homogeneous distribution in the gelatin solutions immediately after preparation of the biphasic hydrogels. After culture for 21 days, some of the cells aggregated in the gelatin solutions. Cells cultured in all the gelatin solutions and induction media showed similar distribution and aggregation. Furthermore, no cell migration from the gelatin solution regions to the surrounding GelMA bulk hydrogel regions was observed, indicating stiff GelMA bulk hydrogels protected cell migration from viscous gelatin solutions.

4.4.3 Influence of matrix viscosity on cell proliferation

DNA amount was quantified immediately after preparation of the hMSC-laden biphasic hydrogels and after culture for 7, 14 and 21 days (Figure 4.5). The cells proliferated with culture time during 3D culture in the biphasic hydrogels. hMSCs cultured in gelatin solution of low viscosity showed the highest proliferation, while in high-viscosity solution the lowest. Cells cultured in all the three types of media showed the same trends of proliferation. A slight decrease of DNA amount after 7 days of culture should be due to cell leakage from the cutting edges of the GelMA hydrogel discs embedded with the hMSC-laden gelatin hydrogel microcubes. These results indicated that hMSCs had high viability and could proliferate in gelatin solutions. Low-viscosity gelatin solution was more beneficial for cell proliferation than high-viscosity gelatin solution.

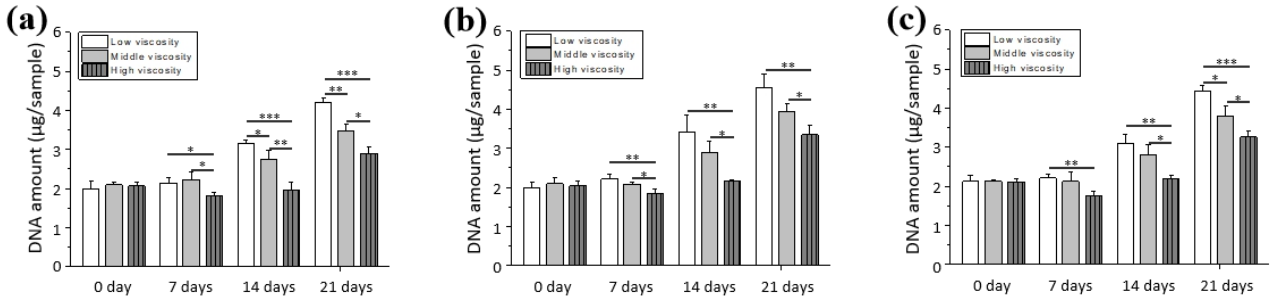

Figure 4.5 Quantification of DNA amount after culture in (a) osteogenic induction, (b) adipogenic induction and (c) mixture medium for 0, 7, 14 and 21 days. Data are shown as mean ± SD, n = 3. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

4.4.4 Influence of matrix viscosity on osteogenic differentiation of hMSCs

The hMSC-laden biphasic hydrogels were cultured in osteogenic induction medium and osteogenic differentiation of hMSCs was evaluated by quantification of alkaline phosphatase (ALP) activity, calcium deposition and expression of osteogenic marker genes, such as ALP, SP7 transcription factor (SP7), secreted

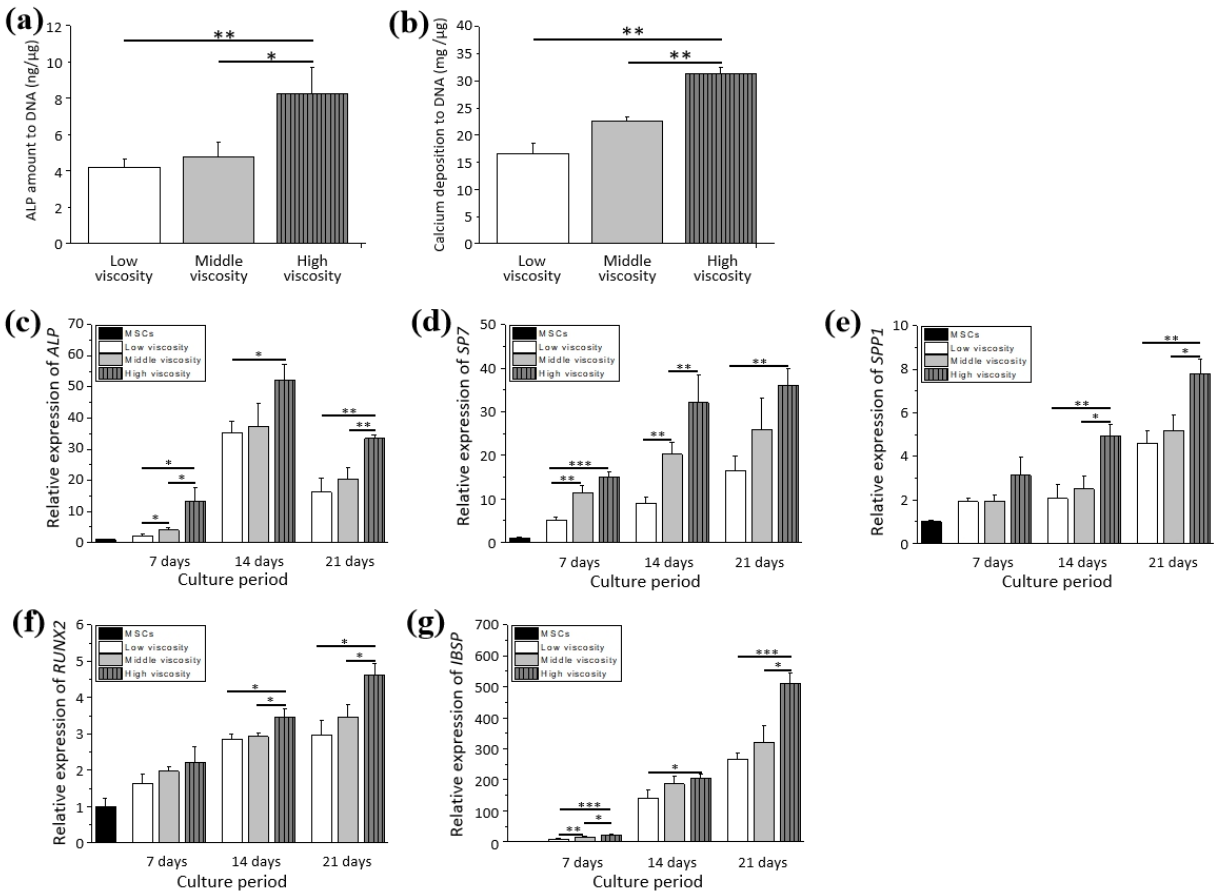

Figure 4.6 Influence of matrix viscosity on osteogenic differentiation of hMSCs cultured in osteogenic induction medium. Quantitative analysis of (a) ALP activity after culture for 7 days and (b) calcium deposition after culture for 21 days. Gene expression of (c) ALP, (d) SP7, (e) SPP1, (f) RUNX2 and (g) IBSP. Data were normalized by gene expression levels measured in P5 hMSCs. Data are shown as mean ± SD, n = 3. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

phosphoprotein 1 (SPP1), runt-related transcription factor-2 (RUNX2) and bone sialoprotein 2 (IBSP) (Figure 6). ALP is an early stage marker and calcium deposition is a late stage marker of osteogenic differentiation. After culture for 7 days, ALP activity was evaluated (Figure 4.6a). ALP activity increased with gelatin solution viscosity. Calcium deposition was analyzed after culture for 21 days (Figure 4.6b). Calcium deposition also increased with gelatin solution viscosity. Furthermore, expression of genes encoding ALP, SP7, SPP1, RUNX2 and IBSP was quantified by real-time polymerase chain reaction (Real-Time PCR) after culture for 7, 14 and 21 days (Figure 4.6c, d, e, f and g). Expression of SP7, SPP1, RUNX2 and IBSP genes increased with culture time and gelatin solution viscosity. Expression of ALP increased with solution viscosity and was the highest after 14 days of culture. These results indicated that high viscosity was beneficial for osteogenic differentiation.

4.4.5 Influence of matrix viscosity on adipogenic differentiation of hMSCs

The hMSC-laden biphasic hydrogels were cultured in adipogenic induction medium to induce adipogenic differentiation. The influence of gelatin solution viscosity on adipogenic differentiation of hMSCs was investigated by Oil Red O staining and expression analysis of adipogenic differentiation-related

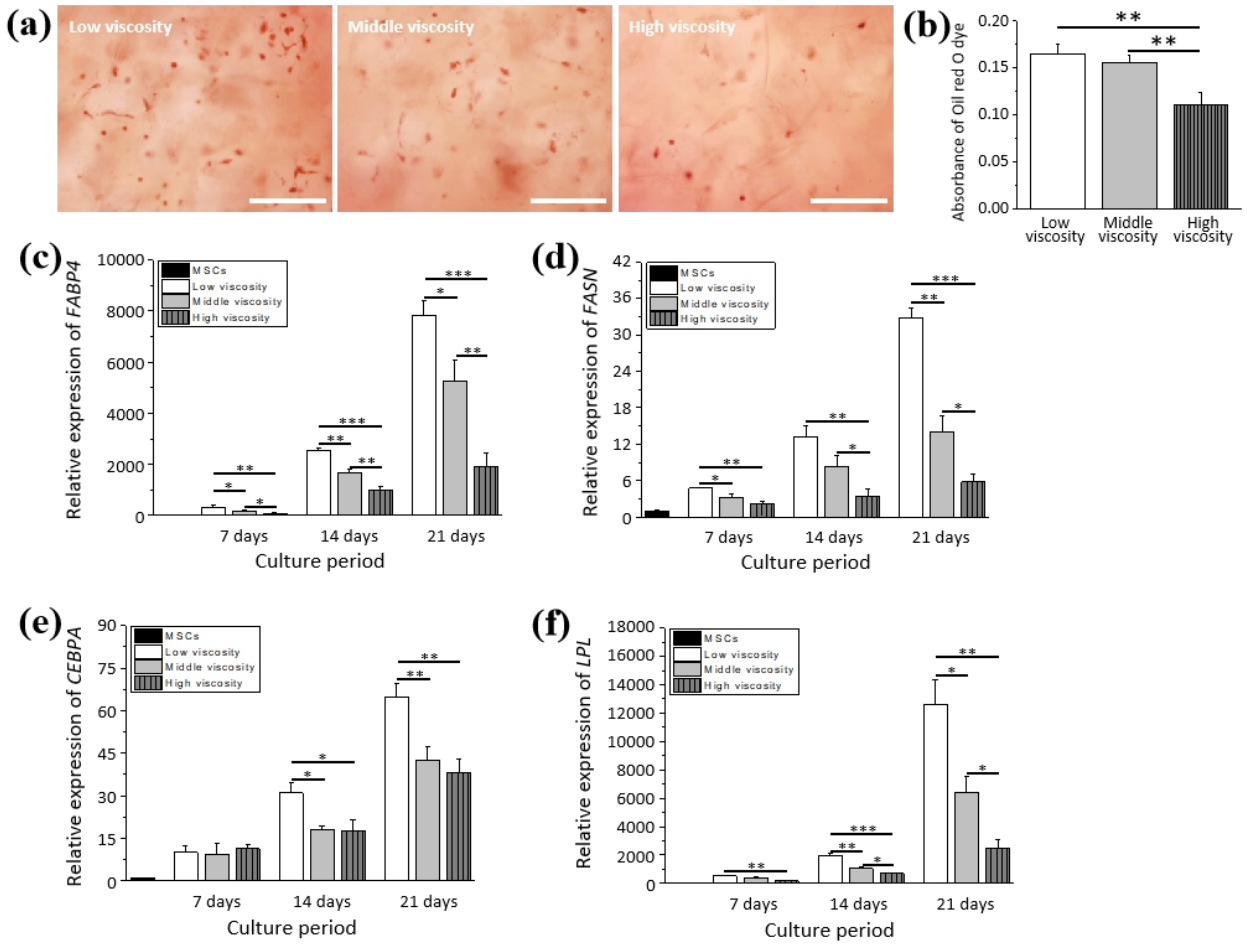

Figure 4.7 Influence of matrix viscosity on adipogenic differentiation of hMSCs cultured in adipogenic induction medium. (a) Oil Red O staining after culture for 14 days and (b) absorbance of Oil Red O dye extracted from the stained cells at 540 nm. Scale bar: 500 µm. Gene expression of (c) FABP4, (d) FASN, (e) CEBPA and (f) LPL. Data were normalized by gene expression levels measured in P5 hMSCs. Data are shown as mean ± SD, n = 3. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

genes, such as fatty acid binding protein 4 (FABP4), fatty acid synthase (FASN), CCAAT/enhancer binding protein (CEBPA) and lipoprotein lipase (LPL) (Figure 4.7). Oil Red O staining showed that oil droplets were detected after 14 days of culture (Figure 4.7a). Quantification of the stained oil droplets showed that low-viscosity gelatin solution showed the highest amount of oil droplets, while high-viscosity gelatin solution showed the lowest amount (Figure 4.7b). Gene expression of adipogenic markers was quantified by Real-Time PCR after culture for 7, 14 and 21 days (Figure 4.7c, d, e and f). Expression of FABP4, FASN, CEBPA and LPL increased with culture time. hMSCs cultured in low-viscosity gelatin solution showed higher expression of these genes than did the cells cultured in high-viscosity gelatin solution. The results indicated that low viscosity was beneficial for adipogenic differentiation.

4.4.6 Influence of viscosity on competing osteogenic and adipogenic differentiation of hMSCs

To investigate how solution viscosity affects the competition of osteogenic and adipogenic differentiation of hMSCs, the hMSC-laden biphasic hydrogels were cultured in a mixture medium of osteogenic and adipogenic induction media. A mixture medium of osteogenic and adipogenic induction media at a ratio of 7:3 v/v was used to stimulate the simultaneous osteogenic and adipogenic differentiation as previously reported [29]. The osteogenic and adipogenic differentiation capacities of hMSCs were investigated by analysis of osteogenic and adipogenic markers (Figures 4.8 and 4.9). Both ALP activity and

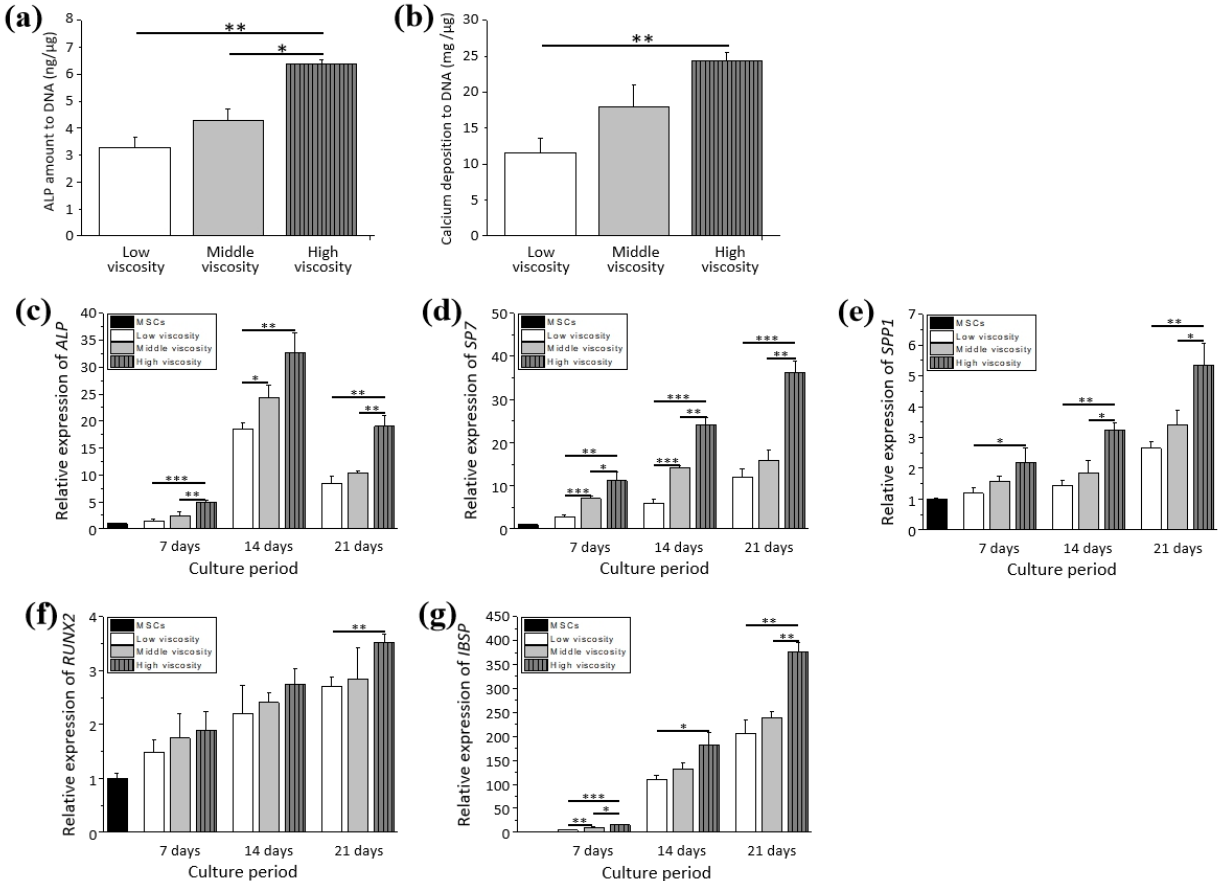

Figure 4.8 Influence of matrix viscosity on osteogenic differentiation of hMSCs cultured in mixture medium. Quantitative analysis of (a) ALP activity after culture for 7 days and (b) calcium deposition after culture for 21 days. Gene expression of (c) ALP, (d) SP7, (e) SPP1, (f) RUNX2 and (g) IBSP. Data were normalized by gene expression levels measured in P5 hMSCs. Data are shown as mean ± SD, n = 3. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

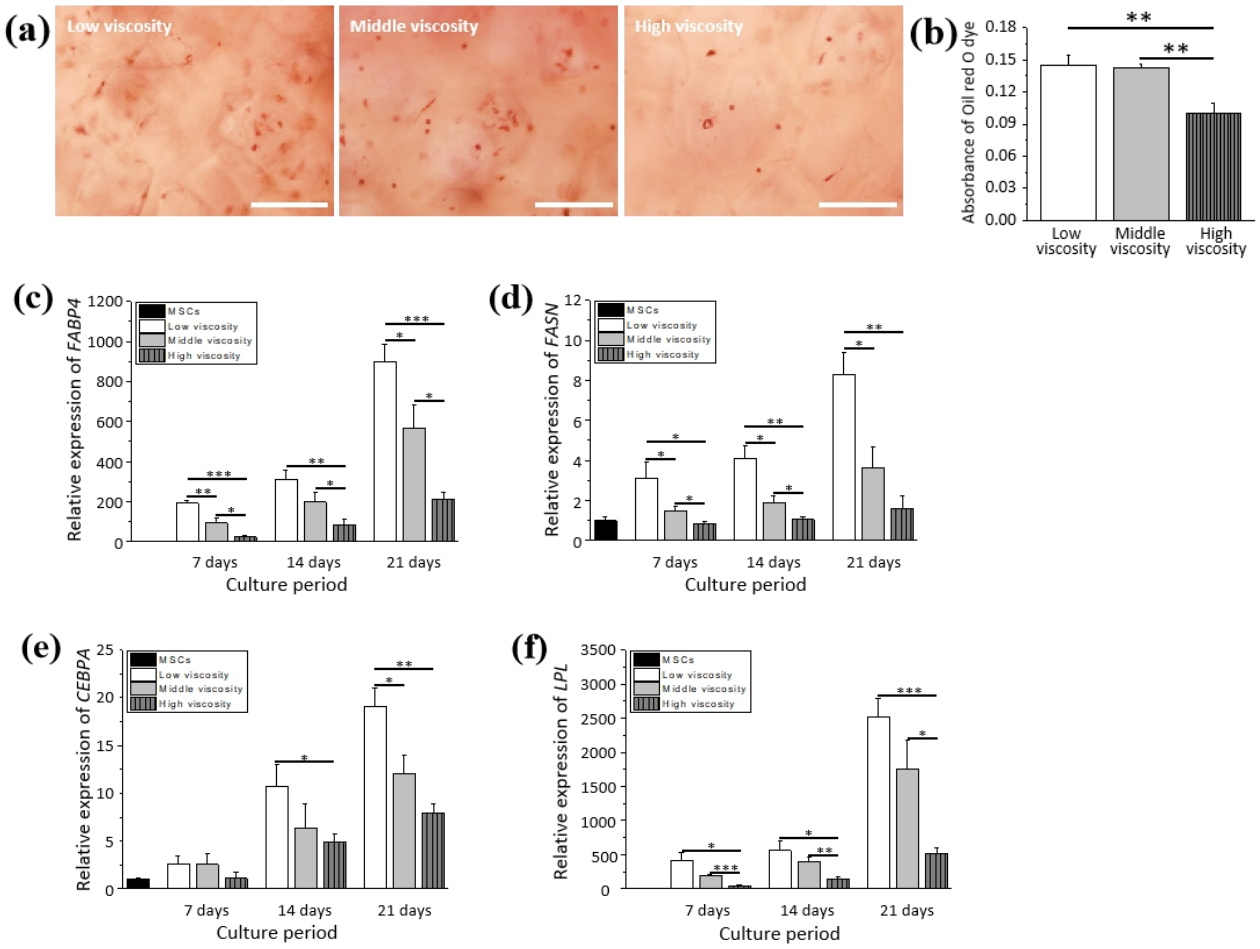

Figure 4.9 Influence of matrix viscosity on adipogenic differentiation of hMSCs cultured in mixture medium. (a) Oil Red O staining after culture for 14 days and (b) absorbance of Oil Red O dye extracted from the stained cells at 540 nm. Scale bar: 500 μm. Gene expression of (c) FABP4, (d) FASN, (e) CEBPA and (f) LPL. Data were normalized by gene expression levels measured in P5 hMSCs. Data are shown as mean ± SD, n = 3. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

calcium deposition increased with viscosity (Figure 4.8a and b). Expression level of osteogenic marker genes of ALP, SP7, SPP1, RUNX2 and IBSP also increased with viscosity and culture time (Figure 4.8c, d, e, f and g). Expression of ALP increased for the first 14 days, while decreased after 21 days of culture (Figure 4.8c). These trends were the same as the gene expression patterns of hMSCs cultured in the single osteogenic induction medium, although their levels were lower, when the data in Figures 4.6 and 4.8 are compared. Oil Red O staining confirmed the presence of oil droplets (Figure 4.9a). Quantification of the stained oil droplets indicated more oil droplets were detected in hMSCs cultured in low-viscosity gelatin solution (Figure 4.9b). Expression of adipogenic differentiation-related genes, FABP4, FASN, CEBPA and LPL, decreased with viscosity, while increased with culture time (Figure 4.9c, d, e and f). All the data of oil droplet formation and expression levels of adipogenic differentiation-related genes showed the same trends as those of hMSCs cultured in the single adipogenic induction medium. However, their levels were lower than those of hMSCs cultured in the single adipogenic induction medium, which is shown in Figure 4.7c, d, e and f. These results also indicated that high-viscosity gelatin solution was good for osteogenic differentiation, while low-viscosity solution for adipogenic differentiation of hMSCs even in mixture medium.

4.5 Discussion

Numerous studies have been conducted to investigate the influence of mechanical cues on cell functions and demonstrated that mechanical cues play a pivotal role in controlling cell functions, such as stem cell differentiation [30-32]. Although there have been intensive studies on the influence of matrix elasticity and viscoelasticity on stem cell differentiation, influence of viscous property of ECM on cell differentiation remains elusive, especially in 3D microenvironment. Therefore, in this part, a 3D biphasic solution/hydrogel culture system having viscous gelatin solution embedded in the chemically crosslinked GelMA hydrogel was used to investigate the influence of solution viscosity on the competing osteogenic and adipogenic differentiation of hMSCs during 3D culture. Gelatin derived from thermal denaturation of collagen has thermosensitive properties which show reversible sol-gel phase transition [33, 34]. Such intrinsic property of gelatin enabled cells to be encapsulated in gelatin hydrogel at low temperature (4 ºC), which became an aqueous solution during culture at 37 ºC with an adjustable viscosity (Figure 4.1). In addition, gelatin can be modified with methacrylic anhydride to prepare chemically crosslinked GelMA hydrogels by UV irradiation [35]. In this way, hMSCs were first embedded in the physically crosslinked gelatin hydrogel and then the cell-laden physical gelatin hydrogel microcubes were incorporated in the chemically crosslinked GelMA hydrogel (Figures 4.2 and 4.3). During culture at 37 ºC, the physically crosslinked gelatin hydrogel microcubes were changed to gelatin solution droplets while the chemically crosslinked GelMA hydrogel remained as hydrogels to embed the cell-laden gelatin solution for 3D culture. Live/dead staining confirmed high viability of the embedded hMSCs and the cells were confined in the gelatin solution droplet regions (Figure 4.4). Thus, the biphasic hydrogel culture system could provide a 3D viscous microenvironment for hMSCs during cell differentiation culture. hMSCs cultured in the biphasic hydrogels proliferated with culture time. Cell proliferation was faster in the low-viscosity gelatin solution (Figure 4.5). The results were in good agreement with other studies reporting that soft hydrogels are good for cell proliferation [36-38]. Cell can spread more in low-viscosity solution than in high-viscosity solution [28]. Restricted cell spreading can inhibit cell proliferation [39]. Influence of gelatin solution viscosity on osteogenic and adipogenic differentiation of hMSCs was compared. At first, hMSCs laden in the biphasic hydrogels were cultured in a single induction medium of osteogenesis or adipogenesis. The high-viscosity gelatin solution promoted osteogenic differentiation of hMSCs because ALP activity, calcium deposition and expression of osteogenic differentiation-related genes (ALP, SP7, SPP1, RUNX2 and IBSP) in the high-viscosity solution were higher than those in the low-viscosity solution when hMSCs were cultured in osteogenic induction medium (Figure 4.6). On the other hand, hMSCs cultured in the low-viscosity gelatin solution formed more significant amount of lipid vacuoles and expressed a higher level of adipogenic differentiation-related genes (FABP4, FASN, CEBPA and LPL) than did in the high-viscosity solution when hMSCs were cultured in adipogenic induction medium (Figure 4.7). It has been reported that osteogenic differentiation and adipogenic differentiation have an inverse correlation and their competing differentiation can be controlled by culturing hMSCs in different ratio of osteogenic and adipogenic induction media [21, 29, 40, 41]. A mixture medium of osteogenic and adipogenic induction media at a ratio of 7:3 v/v had a good balance between the two differentiations of hMSCs [29]. When the hMSC-laden biphasic hydrogels were cultured in the mixture medium, hMSCs underwent competing differentiation to both osteogenic and adipogenic lineages where osteogenic differentiation was promoted in the high-viscosity gelatin solution (Figure

4.8), while adipogenic differentiation was promoted in the low-viscosity gelatin solution (Figure 4.9). The influence of solution viscosity on adipogenic and osteogenic differentiation of hMSCs was similar to the previously reported influence of matrix stiffness [21]. It has been reported that murine MSCs cultured in 3D alginate hydrogels are predominantly induced to osteogenic differentiation in stiff alginate hydrogel, while to adipogenic differentiation in soft alginate hydrogel. Cells might sense and transduce the mechanobiological signals from both viscous solution and stiff matrices through the same integrin adhesion complexes [42]. Considering the numerous studies of elastic and viscoelastic properties on stem cell differentiation and very limited systems for investigation of viscous solution on cell functions, the 3D biphasic solution/hydrogel culture system in this part worked as an useful system for the elucidation of viscosity on stem cell differentiation in a more biomimetic 3D microenvironment. The competing osteogenic and adipogenic differentiation of MSCs could be significantly affected by the viscosity of culture solutions. The biphasic culture system can be used not only for 3D cell culture as shown in present study, but also for 3D culture of tissues in viscous microenvironment.

4.6 Conclusions

A 3D biphasic culture system was used to investigate the influence of matrix viscosity on competing osteogenic and adipogenic differentiation. The viscous gelatin solutions promoted hMSCs proliferation and their promotive effects decreased with increasing viscosity. hMSCs differentiation was dependent on solution viscosity where high-viscosity solution was beneficial for osteogenic differentiation, while low-viscosity solution beneficial for adipogenic differentiation. The results suggested solution viscosity played an important role in controlling stem cell differentiation.

4.7 References

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Chapter 5

Conclusions and future perspective

5.1 Conclusions

This dissertation describes the influence of solution viscosity on cell functions by using 3D biphasic solution/hydrogel culture models. Gelatin solutions with three different concentrations and viscosities were embedded in chemically crosslinked GelMA hydrogels. Chondrocyte functions including proliferation, morphology, ECM production and phenotype were regulated by gelatin solution of different viscosities during 3D culture. Furthermore, solution viscosity could regulate chondrogenic, osteogenic and adipogenic differentiation of hMSC in the presence of respective induction factors.

In chapter 1, a general introduction is presented to elucidate how cells response to mechanical cues and how mechanical cues affect various cell functions. Previous studies dealing with influence of mechanical cues on cell functions are introduced and their limitations (e.g. elastic matrix) are discussed. In addition, the progression of recent studies (e.g. viscoelastic and viscosity matrix) is introduced and advanced models are discussed. According to the summary and discussion, the motivation and originality of this study are mentioned.

In chapter 2, a preparation method of biphasic gelatin solution/hydrogel culture models for 3D culture of chondrocytes and their viscosity influence on cell functions are described. These biphasic gelatin solution/hydrogel culture models were composed of cell-laden gelatin solutions with different viscosites as an inner part and chemically crosslinked GelMA hydrogels as an outer part. The viscosity of gelatin solution was adjusted by altering its concentration where viscosity was proportional to concentration. Low viscous solution showed fast cell proliferation and a more elongated cell morphology but less ECM production and cartilaginous gene expression, whereas high viscous solution enhanced cartilaginous gene expression and ECM production but showed slower cell proliferation.

In chapter 3, hMSCs were cultured in biphasic gelatin solution/hydrogel culture models in the absence or presence of chondrogenic induction factors. Cells cultured in viscous gelatin solutions showed high cell viability regardless of viscosity. Low viscous solution promoted hMSC proliferation but suppressed ECM production, whereas high viscous solution enhanced ECM production but showed slower hMSC proliferation. In particular, in the presence of chondrogenic induction factors, high-viscosity gelatin solution had a higher promotive effect on chondrogenic differentiation of hMSCs than did low- viscosity gelatin solution.

In chapter 4, solution viscosity was used to simultaneously regulate osteogenic and adipogenic differentiation of hMSCs at the same culture condition. hMSCs embedded in gelatin solution with different viscosities were cultured in a single induction medium of osteogenesis, adipogenesis and their mixture medium. Gelatin solution viscosity promoted hMSC proliferation, but it promotive effect decreased with increasing viscosity. Furthermore, hMSC differentiation showed viscosity-dependent results where low-viscosity gelatin solution was beneficial for adipogenic differentiation, while high viscous solution was beneficial for osteogenic differentiation.

In conclusions, 3D biphasic solution/hydrogel models were prepared to investigate the influence of solution viscosity on cell functions during 3D culture. Solution viscosity was easily controlled by altering solution concentrations. These preparation strategies enabled cells to be cultured in a 3D viscous microenvironment which is close to the 3D native ECM microenvironment. Gelatin solution viscosity regulated chondrocyte functions including cell proliferation, morphology, ECM production and cartilaginous gene expression. Furthermore, hMSC differentiation including chondrogenic, osteogenic and adipogenic differentiation was regulated by gelatin solution viscosity in the presence of respective induction factors. Taken together, these results suggested that solution viscosity was one of the key parameters affecting various cell functions.

5.2 Future perspective

In this study, cells were cultured in a 3D viscous microenvironment, which is originality of this study. To provide cells with viscous microenvironment during 3D culture, cells were laden in gelatin solution. Gelatin solution could provide cells with suitable biomimetic microenvironment and allowed cells to be embedded in the chemically crosslinked GelMA hydrogels due to its intrinsic gelation property. However, in spite of such advantages, gelatin has some limitations. For example, altering concentration of gelatin solution may affect cell functions through non-viscous parameters, such as osmotic pressure, volume exclusion and cell interaction ligands. In addition, the viscosity range of gelatin solution used in the study was not broad due to the preparation method.

In order to prepare better 3D viscous culture system, synthetic materials with a broad range of viscosity may be suitable candidates. Synthetic materials do not present cell adhesion ligands and are not degradable by mammalian enzymes. In addition, the viscosity of synthetic materials controlled by changing their molecular weight can provide a broad range of viscosity. However, since such synthetic materials cannot provide cells with cell-friendly microenvironment, use of the mixture of synthetic materials and biological matrices for cell culture will be a good strategy.

The 3D biphasic solution/hydrogel model has shown a great potential in regulating various cell functions related to tissue regeneration. This study can be extended to cancer research. In fact, mechanical cues can affect and regulate functions of both normal and cancer cells. So far, it has been reported that various mechanical cues (e.g. osmotic pressure, elastic matrix and viscoelastic matrix) can regulate functions of cancer cells. Indeed, in our body, the increased tissue stiffness is associated with cancer development and progression, and hydrostatic interstitial fluid pressures mostly increase in tumors. Furthermore, enhanced extracellular fluid viscosity affects cell alignment and cancer cell migration. Nevertheless, the influence of solution viscosity on cancer cell behaviors is still in its infancy. Therefore, culturing cancer cells in a broad range of viscous microenvironment will provide useful information about not only cell response to viscosity but also cancer cell behaviors.

List of publications and awards

Publications:

1. **Kyubae Lee**, Yazhou Chen, Toru Yoshitomi, Naoki Kawazoe, Yingnan Yang and Guoping Chen. Osteogenic and adipogenic differentiation of mesenchymal stem cells in gelatin solutions of different viscosities. Advanced Healthcare Materials. 2020, 9(23): 2000617.

2. **Kyubae Lee**, Yazhou Chen, Xiaomeng Li, Naoki Kawazoe, Yingnan Yang and Guoping Chen. Influence of viscosity on chondrogenic differentiation of mesenchymal stem cells during 3D culture in viscous gelatin solution-embedded hydrogels. Journal of Materials Science & Technology. 2021, 63: 1-8.

3. **Kyubae Lee**, Yazhou Chen, Xiaomeng Li, Yongtao Wang, Naoki Kawazoe, Yingnan Yang and Guoping Chen. Solution viscosity regulates chondrocyte proliferation and phenotype during 3D culture. Journal of Materials Chemistry B. 2019, 7(48), 7713-7722.

4. Yazhou Chen, **Kyubae Lee**, Naoki Kawazoe, Yingnan Yang and Guoping Chen. ECM scaffolds mimicking extracellular matrices of endochondral ossification for the regulation of mesenchymal stem cell differentiation. Acta Biomaterialia. 2020, 114, 158-169.

5. Yazhou Chen, **Kyubae Lee**, Yingnan Yang, Naoki Kawazoe and Guoping Chen. PLGA-collagen-ECM hybrid meshes mimicking stepwise osteogenesis and their influence on the osteogenic differentiation of hMSCs. Biofabrication. 2020, 12(2).

6. Yazhou Chen, **Kyubae Lee**, Naoki Kawazoe, Yingnan Yang and Guoping Chen. PLGA-collagen-ECM hybrid scaffolds functionalized with biomimetic extracellular matrices secreted by mesenchymal stem cells during stepwise osteogenesis-co-adipogenesis. Journal of Materials Chemistry B. 2019, 7(45), 7195-7206.

7. Yazhou Chen, **Kyubae Lee**, Ying Chen, Yingnan Yang, Naoki Kawazoe and Guoping Chen. Preparation of stepwise adipogenesis- mimicking ECMs-deposited PLGA-collagen hybrid meshes and their influence on adipogenic differentiation of hMSCs. ACS Biomaterials Science & Engineering. 2019, 5(11), 6099−6108.

Awards:

1. **Oral Presentation Award**, The 19th Asian BioCeramics Symposium, Taipei, Taiwan, 2019.

2. **Best Presentation Award**, The 2nd Glowing Polymer Symposium, Tokyo, Japan, 2019.

3. **Best Presentation Award**, The 1st Glowing Polymer Symposium, Tokyo, Japan, 2018.

4. **Best Poster Award**, The International Symposium on 3rd Asian University, Seoul, Korea, 2018.

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