**PHYSICAL CUES ENHANCING TISSUE ENGINEERING: FROM
CELLULAR COMMITMENT TO VASCULAR INDUCTION**

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SUMMARY

The behaviors of cells, such as survival, proliferation, differentiation, migration, can produce important physical cues, which lead to the occurrence of some important biological processes. In addition, cells are in close contact with extracellular matrix (ECM) *in vivo*, either continuously or at some important stages of development. ECM is a three-dimensional (3D) protein scaffold and the physical signals from ECM (stiffness and structure) can also affect cell behaviors.

In this thesis, we promoted vascularization of tissue constructs and cardiac differentiation of ESCs through manipulation of cell-induced or biomaterial-derived physical signals. We verified the feasibility of using PLGA nanoparticles to label and track ESC status during EB development. The unique cell viability monitoring function of the nanoparticles allowed clear presentation of live cell distribution within EBs, which had high potential to be a powerful tool to screen EBs with comparable sizes.

In addition, we successfully imitated some critical aspects of cartilage hypertrophy using *in vitro* engineered living cartilage templates and osteogenic treatment. Osteogenic treatment induced cartilage hypertrophy and creates channels for vascular invasion in LhCG constructs when implantation *in vivo*. Hypertrophic transition of LhCG constructs using osteogenic treatment may contribute to the establishment of an *in vitro* cartilage hypertrophy model that mimic initial stage of endochondral ossification.

Besides manipulation of cell behavior-origin physical signals, stiffness and

topography of biomaterials were also controlled. Gelatin-dopamine was synthesized for PDMS surface coating. Compared with gelatin, gelatin-dopamine coating reduced hydrophobicity and increased protein adsorption on the PDMS surface. In addition, the gelatin-dopamine coated PDMS surface greatly improved ESC adhesion, proliferation and pluripotency maintenance as compared to gelatin-coated surfaces. ESC-derived EBs also showed increased adhesion and facilitated myocardial differentiation on the gelatin-dopamine coated PDMS surface. These results demonstrated that gelatin-dopamine coating could effectively improve PDMS surface properties for the long-term ESC culture and enhanced ESC myocardial differentiation. Following the establishment of coating strategy, the influence of PDMS substrate stiffness on ESC pluripotency maintenance and cardiac differentiation was further investigated. Different PDMS substrates exhibited different profiles of substratum properties, which affected the behaviors of ESCs and EBs on PDMS surfaces. ESC adhesion and proliferation, as well as EB attachment, were improved on the 40:1 PDMS substrate as compared to the other PDMS substrates. Meanwhile, compared with the other PDMS substrates, ESC pluripotency preservation was improved on the 5:1 PDMS substrate, while the 40:1 PDMS substrate encouraged ESC cardiac differentiation. These results were successfully applied in fabrication of biochips for ESC pluripotency maintenance and cardiac differentiation. The use of this simple surface modification strategy to stabilize the adhesion of ESCs and EB while enhance their pluripotency and differentiation, as well as substrate stiffness optimization, can possibly contribute to the development of a real heart-on-a-chip for high throughput drug screening in the future.

Finally, the effects of pore density and pore sizes on hydrogel vascularization were studied both *in vitro* and *in vivo*. An increase of pore density greatly enhanced the proliferation and endothelial differentiation of encapsulated EPOCs in Gel-MCG constructs. In particular, compared to MS:Gelatin (1:1) counterpart, MS:Gelatin (2:1) constructs with higher pore density allowed the interconnected vascular network formation within the whole constructs upon the degradation of thinner gel walls among neighboring pores. Extensive vascular invasion was observed within the MS:Gelatin (2:1) constructs when implanted *in vivo*. These findings demonstrated that the vascularization of Gel-MCG constructs could be improved by increasing cavitory density. The effects of pore sizes on hydrogel vascularization by EPOCs were also evaluated *in vitro* and *in vivo*. Differentiation of EPOCs and hydrogel vascularization were promoted by middle pores as compared to large or small pores. When implantation *in vivo*, hydrogels with large pores induced more red blood cell-containing vessels and vascular tissues than hydrogels with small or middle pores. These results indicated that middle pores promoted hydrogel vascularization *in vitro*, while hydrogel vascularization *in vivo* was improved by large pores. These studies revealed the importance of pore densities and pore sizes in hydrogel vascularization both *in vitro* and *in vivo* and these findings can help design an optimal pore density and size for hydrogel constructs to enhance their vascularization.

In summary, the studies in this thesis prove that physical signals can be utilized to improve performances of tissue engineered constructs. By utilization of physical signals from cells, we develop a new tool to monitor ESC viability during EB

development to screen EBs with homogenous sizes and vascularize chondrocyte-derived cartilage templates with hypertrophic induction. Through manipulation of stiffness and topographic cues of biomaterials, we improve efficiency of ESC cardiac differentiation and hydrogel vascularization.

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Chapter 1: Introduction

1.1 Importance of physical cues to cell behaviors

The behaviors of cells themselves, such as survival, proliferation, differentiation, migration, can produce important physical cues, which lead to the occurrence of some important biological processes. In addition, cells are in close contact with extracellular matrix (ECM) in vivo, either continuously or at some important stages of development [1]. ECM is a three-dimensional (3D) protein scaffold and the physical signals from ECM (stiffness and structure) can also affect cell behaviors [2, 3].

1.1.1 Biological events triggered by physical signals due to cell behaviors

Some biological events that occur in vivo is triggered directly by physical cues generated by cells themselves. During embryonic development, rapid cell proliferation, as well as low level of oxygen in embryo, always lead to hypoxic microenvironment at the center of cells, which then promote the development of embryo [4]. For example, embryo cavitation at the early stage of embryo development is likely to be caused by hypoxia condition at the center of inner cell mass of embryo. Meanwhile, chondrocyte hypertrophy due to hypoxia microenvironment in the center of cartilage template plays

an essential role in endochondral ossification during embryo development.

1.1.1.1 Cavitation of embryoid bodies (EBs)

During early embryogenesis, a cavity, called proamniotic cavity, will form in the inner cell mass of an embryo when its implantation [5]. The formation of the proamniotic cavity is due to cell death within the core of the inner cell mass [6]. A central cavity can also be developed in embryonic stem (ESC)-derived embryoid bodies (EBs) [7]. EBs are formed by ESCs when cultured as aggregates and have structures that recapitulate early stages of embryo development [8]. Using EBs as a model to study proamniotic cavitation, physical cues has been recently identified as important factors to affect cavitation of EBs, apart from biochemical factors [6, 9-11] and intercellular signaling [5]. Due to the size of EBs, the partial pressure of oxygen at the center of EBs is quite lower than the surface of EBs [12]; the diffusion of soluble biochemical factors from the surface to the center of EBs is also reduced [13]. Therefore, the cavitation of EBs may be trigger by low concentration of oxygen at the core of EBs, which further results in cell death.

1.1.1.2 Endochondral ossification

Endochondral ossification is a process by which bone forms in cartilage intermediates [14]. Chondrocytes derived from condensation of mesenchymal

progenitors first proliferate and then synthesize matrix rich in type II collagen and aggrecan to generate cartilaginous template for the future bone. After that, chondrocytes in the center of the cartilage undergo chondrocyte hypertrophy, which show increased volume and express type X collagen and matrix metalloproteinase-13 (MMP-13) [15, 16]. Hypertrophic chondrocytes play a critical role in endochondral ossification. For example, MMP-13 synthesized by hypertrophic chondrocytes is important in degradation of cartilaginous matrix rich in collagen type II and aggrecan [17]. Hypertrophic chondrocytes also secrete growth factors, cytokines, and other signaling molecules to attract blood vessels and recruit osteoclasts and osteoblasts [18, 19]. Osteoblasts from differentiated adjacent perichondrial cells and delivered by blood vessels can produce bone tissues to replace cartilaginous matrix [14, 19]. Hypertrophic chondrocytes can further transdifferentiate into osteoblasts, which participate bone tissue formation together with osteoblasts from other sources [20-22]. During endochondral ossification, physical signal is also important. Hypoxia in the center of cartilage intermediates may lead to the expression of hypoxia-inducible factors (HIFs) [23, 24], which regulate chondrocyte hypertrophy, cartilage degradation, and vascularization [25]. In addition, channels in cartilage intermediates due to cartilaginous matrix degradation is also essential for blood vessel invasion [14].

1.1.2 Physical signals from ECM

Physical properties of ECM, such as stiffness and topography, are also regulators of

cell behaviors. Cells sense these physical signals through specialized transmembrane receptors [26]. Integrins is a such cell surface receptor, which can recognize and bind to specific amino sequences on ECM proteins and at the same time connect with cytoskeleton [27]. Physical signals from ECM first transduce into cells via ECM receptors and then transmit within cells through cytoskeleton, which lead to the alteration in cell phenotypes [3]. Therefore, the survival, proliferation, migration, and differentiation of cells all can be affected by physical properties of ECM [28].

1.1.2.1 The influence of ECM stiffness on cells

The stiffness of ECM is defined as the ability of ECM to undergo non-permanent deformation [29]. ECM that can easily deform at low stress are soft, whereas hard ECM show little deformation under the same stress [30]. The mechanical properties of tissues in our body vary greatly, from tissues as soft as brain to rather rigid and calcifying bone. Such ECM variations in mechanics can influence many cellular functions. For example, apoptosis of fibroblast cells, mammary gland cells and kidney epithelial cells is shown to increase on a softer substrate [31, 32]. However, another study shows apoptosis of valve interstitial cells can only be induced on stiffer matrices [33]. These findings demonstrate the importance of ECM stiffness on cell survival but the results are not consistent. The influence of ECM on cell viability may depend on the cell type. If cultured on substrates with rigidity different from the tissues surrounding cells in vivo, cells may reveal decreased viability.

Cell proliferation always increases with the increase in substrate stiffness. For example, the proliferation of chondrocyte is promoted in stiffer matrix [34]. As stiffer matrix is similar to the rigidity of cartilage, it may lead to the faster proliferation of chondrocytes in rigid matrix. However, in pathological conditions, such as tumor or fibrosis, stiffer ECM due to uncontrolled proliferation of tumor cells or fibroblast cells seem to promote the proliferation of cells in reverse [35-37], which indicate the importance of cell-matrix interaction in cell growth.

In addition, ECM stiffness regulates cell migration. Compared with cells on rigid substrates, fibroblast and kidney epithelial cells on flexible substrates show increased rates of migration, which is probably due to reduced cell spreading on the softer substrate [38]. Direction of cell migration can also be guided by ECM rigidity [39]. When cells encounter interface between materials of different stiffness, cells on softer substrates migrate to stiffer substrate, whereas cells on stiffer substrates don't move, which show the preference of cells for stiffer substrates [40]. Cell migration in response to ECM stiffness depends on integrin-based focal adhesions, which sense stiffness by applying fluctuating pulling forces to the ECM [41].

More importantly, cell fate can be determined by ECM rigidity. ECM stiffness can affect the differentiation of both embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs). Genes associated with early mesendoderm specification are upregulated in cell cultures on stiffer substrates [42], while softer substrates elicit the differentiation of ESCs towards endodermal lineage[43]. Osteogenic differentiation of ESCs is enhanced on stiff substrates as compared to soft substrates [44]. The differentiation of

MSCs is also sensitive to substrate elasticity and can be committed to neurogenic, myogenic, or osteogenic lineage on matrix with increased elasticity respectively. These findings demonstrate that the commitment of stem cells in vitro seems to be improved on the substrate with the same stiffness as ECM of cell types which differentiate in vivo [45].

1.1.2.2 The effects of EMC topography on cell behaviors

ECM contains structures with specific size, shape and periodicity [46]. For example, collagen network in heart is highly-ordered [47]. Such topographic features of ECM are quite important and can directly influence many cellular functions. The adhesion of fibroblasts and endothelial cells can be improved on surface with nano-structures as compared to smooth surfaces of similar chemistry [48, 49]. In addition, the increased adhesion has been found to be size-dependent [50]. 35 nm nano-pits cannot induce filopodia from cells, whereas filopodia produced by cells in response to pits can be observed on 75 nm and 120 nm nano-pits [51]. Sub-nano-structures (<1 nm) is insufficient to induce evident cell spreading and cytoskeleton rearrangement and only structures with nano- (~10 nm) or sub-micron (~100 nm) scales can increase cell adhesion. Similar as stiffness sensing by cells, integrins are response for recognition and transduction of such signals and can be activated by topography on the surface of substrates [52].

Cell morphogenesis can also be affected by topography of ECM. For instance,

collagen fibrils are regularly arrayed in the annulus fibrosus of intervertebral discs, which orient chondrocytes in the same directions as collagen fibrils [53]. Pore structure of a scaffold can induce neovascularization by host tissues, with a large pore size ($>400\text{ }\mu\text{m}$) beneficial for the ingrowth of blood vessels and a pore smaller ($<400\text{ }\mu\text{m}$) limiting blood vessel invasion [54]. This phenomenon can also be observed in vitro. Endothelial cells spontaneously organize into tubes with lumens when cultured within $50\text{ }\mu\text{m}$ wide channels filled with collagen gel for 24-48 hours [55]. In addition, such signals should be strong enough to be sensed by cells and cells cannot align in response to weak signals. The beneficial effects of topography disappear, when the size of topographic features becomes smaller and the substrates approach a non-patterned surface [56]. MSCs cultured on 1400 or 4000 nm wide grooves show obvious elongation and alignment, whereas 400 nm wide grooves exert negligible influences on cells cultured above [57]. Sometimes signals from topography of ECM are even stronger than chemical signals. When presented with both micro-patterned grooves and bio-adhesive protein, cells still align according to the topography signals [58].

Topographic features of ECM may also influence cell proliferation and the results are not consistent with each other. One study shows that surfaces with micro-patterned channels support a higher fibroblast proliferation than smooth surfaces and the cell proliferation is more rapid in $150\text{ }\mu\text{m}$ wide channels than smaller or wider channels [59]. However, no significant influence of nano-scale topography on osteoblast proliferation can be found [60]. Another study also demonstrates that cell proliferation on surface with nano-scale (500 nm) or micro-scale ($2\text{ }\mu\text{m}$) pits or islands is not

improved, even though cell adhesion is enhanced on these patterned surfaces [61]. Even micro-grooves as wide as 10 μm cannot have an effect on the cell proliferation [62]. It is highly possible that cells can only recognize topographic structures within some range for increased cell proliferation. Neither too small nor too large structures can stimulate cell proliferation.

The influence of ECM topography on cell differentiation has also been extensively explored. For example, MSCs on surface with sparse nano-posts show adipogenic differentiation, while dense nano-posts support MSC osteogenesis. The shape of MSC in response to different densities of nano-topographical cues can regulate intracellular cytoskeletal networks, thereby priming the commitment of MSCs. This finding indicates that cell shape is essential in cell fate determination [63, 64]. Cell shape as well as cytoskeleton can also be changed via topographical signals presented in other forms. The width of micro-patterned strips can affect the differentiation of MSCs, with improved myogenic differentiation on strips ranging from 20 μm to 200 μm as compared to wider micro-strips and non-patterned surfaces [65]. Ordered lattice micro-patterns with 3 μm interval is more effective in enhancing the osteogenic differentiation of MSCs than smaller or larger intervals [66]. In addition to MSCs, topography influences the maintenance of ESCs as well. ESCs cultured in micro-wells with a 300 μm lateral dimension and 120 μm depth show a higher level of Wnt signaling which is related to differentiation when they form EBs in suspension conditions [67].

1.2 Application of physical cues in tissue engineering

Both cell-generated physical cues and physical properties of biomaterials can affect cell phenotypes and morphogenesis. Manipulation of these physical signals can control the behaviors of cells. In the past decades, even though the importance of physical cues on cells have attracted more and more attention, the application of physical signals in tissue engineering is not as much as biochemical molecules. In combination with biochemical stimulation that is widely-used in tissue engineering [68], manipulation of physical signals from both cells and biomaterials can help engineer tissue constructs truly-usable in regenerative medicine.

1.2.1 Manipulation of cell grow in vitro

Cells can secrete many cytokines in response to stimuli in the microenvironment to maintain their normal functions in vivo. However, the application of cytokines in vitro is extremely difficult because the type and concentration of cytokines are impossible to be exactly known. In contrast, another strategy is to control the initiator of cytokine release and allow cells to do the rest by themselves. Unlike complex biochemical signal communication networks, some initiator factors in vivo are just changes in physical conditions, which, however, can be easily controlled in vitro. By regulation of physical cues from cells or ECM, cell behaviors can be controlled.

1.2.1.1 EB size manipulation

During embryonic development, hypoxia in the center of inner cell mass is as an initiator of embryo development. As hypoxia is caused by the reduced oxygen diffusion from the surface to the center, the size of cell aggregates is responsible for such reduction. Similar development process can also be observed in EBs in vitro and the size of EBs has been shown to influence their differentiation in vitro [69, 70]. For example, larger EBs support neuronal and cardiomyocyte differentiation more than smaller EBs [71]. 500 μm EBs that are formed in micro-wells can be differentiated into endothelial cells more efficiently than 300 μm or 1000 μm EBs [72]. 450 μm embryoid bodies support cardiac differentiation, while 150 μm embryoid bodies improve endothelial differentiation [73]. Expression of WNT5a is more highly expressed in smaller EBs, which enhance differentiation of endothelial differentiation. Increased expression of WNT11 in large EBs is responsible for enhanced cardiac differentiation [70].

Various methods have been developed to generate EBs with controlled sizes. Micropatterned extracellular matrix protein can result in the formation of size-specified EBs [69]. However, microcontact printing on substrates can only control the initial size of EBs. V-bottom 96 plates combined with centrifugation can also form larger EBs with high homogeneity [74, 75], but seeding cells into individual wells is time-consuming. Micro-wells allow straightforward and scalable formation of EBs with defined sizes [67, 73, 76]. For example, agarose micro-well plates with V bottoms are used for standardized ESC aggregation and EB formation [77]. Meanwhile, micro-wells with

lateral dimensions from 100-500 μm can generate uniform EBs with sizes corresponding to micro-wells [78]. However, bottoms of these micro-wells are not round, which is inconsistent with contour of EBs. Therefore, concave micro-wells are developed. EBs with uniform sizes can be generated with concave micro-wells and the size of generated EBs can be regulated by diameters of concave micro-wells [71, 72]. Round-bottom micro-wells fabricated from agarose hydrogels also have been shown to generate homogeneous embryoid bodies EBs of defined sizes [79]. Although EBs with controlled size can be formed readily by micro-wells, the number of EBs obtained from micro-wells is still limited due to a two-dimensional setting. Three-dimensional scaffolds are then explored to generate EBs with controlled sizes in large quantity. Alginate scaffolds with uniform pores can generate large numbers of homogenous EBs rapidly and removal of alginate can release the generated EBs. In addition, the size of EBs can be regulated by culture time and pore size in scaffolds [80]. Other methods, such as microfluidic devices, have been investigated as well. Using microfluidic channels, ESCs in aqueous liquid can be encapsulated by alginate shell to generate microcapsules, which will develop into EBs within 7 days [81].

Even though numerous technologies have been developed, methods that can track EB development in real time are few. An approach that can monitor viability, proliferation, differentiation of ESCs during EB development can allow us to screen suitable EBs for applications.

1.2.1.2 Mimicking endochondral bone formation in vitro

Endochondral ossification is initiated due to hypoxia in the center of a cartilage template developed from chondrogenesis of condensed MSCs [14]. A similar process can be mimicked in vitro. Human adult bone marrow-derived MSCs (BM-MSCs) can be used as a cell source for endochondral ossification in vitro. BM-MSCs has signaling pathways consistent with MSCs for endochondral bone formation during embryonic development and can execute an endochondral program to generate ectopic bone [82, 83]. Chondrogenesis of MSCs can be primed using well-defined chondrogenic medium [84, 85]. Hypoxia can be induced in vitro simply by culturing MSCs in high density in a three-dimensional environment [86, 87]. Hypoxia-induced hypertrophy is crucial for endochondral ossification. Bone tissues can only be formed when MSC-derived cartilage templates become hypertrophic and the enhanced hypertrophy of cartilage template can accelerate bone formation [82]. A cartilage intermediate that is developed from chondrogenesis of MSCs contains all necessary signals to initiate bone tissue formation, vascular tissue invasion, remodeling, and bone marrow establishment [21, 22, 25]. Subsequent development of hypertrophic cartilages in vivo can result in a fully functional bone formation with mature blood vessels and bone marrow [88].

Many groups have explored strategies of following endochondral bone development stages for bone tissue engineering. Pellet culture is developed to form three-dimensional aggregates of MSCs to mimic MSC condensation during endochondral ossification [85, 89, 90]. MSCs are first cultured in monolayer conditions and then transferred into conical tubes. After centrifugation, MSC pellets are formed,

which then cultured chondrogenic medium. Cartilaginous matrix can be generated by cells after culture in vitro for 7 days and hypertrophic markers can be expressed after culture in vitro for 14 days [91]. Because hypoxia conditions in the center of pellets provide important physical signals to initiate chondrogenesis of MSCs, external growth factor stimulations can be avoided [92]. Pre-vascularization of chondrogenic primed MSC pellets can enhance vessel formation and increased mineralization within pellets following in vivo implantation [90]. In addition, MSC-derived cartilage pellets have been shown to repair bone defects of mice and the implanted cartilage pellets are transformed into vascularized bone tissue after repair [93]. Another high-density cell culture system is micro-mass [94, 95]. Cell suspensions of high cell density (20×10^6 cells/mL) are directly plated on tissue culture plate and 24 hours later cells fuse into a sphere. Chondrogenesis occur within 3 days and extensive cellular hypertrophy is observed within 7 days [94]. Compared with pellet culture, micro-mass is larger and showed more cartilaginous matrix while decreased chondrocyte hypertrophy [95]. As no additional force is used to condense micro-mass, the organization of micro-mass is less dense than pellets and mass transfer is thus better. As a result, hypoxia-induced cartilage hypertrophy in micro-mass is not so obvious as pellet. Besides, seeding high densities of MSCs into a transwell can also be used to engineer hypertrophic cartilage [82]. However, without support from scaffolds, MSC-derived cartilage templates is not large enough for real bone regeneration in clinical settings. Biomaterials combined with high cell density seeding are then investigated to engineer scalable hypertrophic cartilages. Seeding high density of MSCs (70×10^6 cells/mL) onto collagen meshes can

result in the formation of large-scale hypertrophic cartilages after chondrogenic priming. After subcutaneous implantation, the resultant hypertrophic cartilage can generate bone tissues with a size comparable to native bones [96]. Due to the large size of hypertrophic cartilages that are developed from collagen-based scaffolds and hypertrophic MSCs, such hypertrophic cartilage can be used to repair critical-sized bone defects of rats [97].

Although endochondral ossification can be recapitulated in MSC-derived cartilages in vitro, the heterogeneity of MSC differentiation cannot ensure all MSCs have similar chondrocytic phenotypes after chondrogenic differentiation [85]. Mature chondrocytes with homogenous phenotype in adult cartilage tissues may be an alternative cell source for endochondral ossification in vitro. It has been proved that chondrocytes in articular cartilage explants and chondrocytes cultured in monolayer can be induced into a hypertrophic status [98, 99]. Chondrocytes in vivo can also become hypertrophy [100]. However, whether engineered cartilage template can be hypertrophic remains unknown. Meanwhile, whether implantation of such hypertrophic artificial cartilages can result in bone formation in vivo also needs to be investigated.

1.2.2 Manipulation physical properties of biomaterials

One requirement for cells to sense physical cues of biomaterials is that cells can attach to the surface of materials. Therefore, adhesion sites need to be incorporated into scaffolds. For example, cells can recognize short peptide Arginine-Glycine-Aspartic acid (or RGD) to bind with ECM [101]. Attachment is prerequisite for anchorage-

dependent cells, such as MSCs, to survive and function. Thus, RGD peptide modified poly(ethylene glycol) (PEG) hydrogel can support a high level of MSC viability, while the viability of MSCs encapsulated in unmodified PEG decreases over time [102]. Simply coating bio-inert materials with ECM proteins is also useful. Laminin coating improves the adhesion of neural cells onto polystyrene-based scaffolds [103] and bone-like ECM deposited on the surface of titanium enhances osteogenic differentiation of MSCs [104]. Only after biomaterial functionalization with cell adhesive sites, the optimization of biomaterial stiffness and topography is meaningful. Since stiffness and topography of biomaterials can affect cell behaviors [1], these physical properties of scaffolds need to be rationally-designed to engineer tissue constructs that are functional and can be truly used for regenerative therapies in clinical settings.

1.2.2.1 Application of substrate stiffness in tissue engineering

Self-renewal capacity of stem cells can be supported by a soft substrate. For example, ESCs on soft polyacrylamide can express higher pluripotent markers as compared to rigid tissue culture plates [105]. The pluripotency of ESCs can even be maintained on soft polyacrylamide without exogenous leukemia inhibitory factor (LIF), which is commonly used to inhibit spontaneous differentiation of ESCs [105]. Furthermore, ESC pluripotency increase with the decrease of substrate stiffness [106]. ESC pluripotency inversely relates to cell stiffness [107]. High stiffness due to increased F-actin expression can activate STAT3 signaling and thereby induce ESC

differentiation [108]. On substrates with same rigidity, differentiated ESCs show a higher cytoskeleton contractility than in differentiated ESCs [109]. Hence, reduced cell-substrate tractions and stiffness of ESCs on a softer substrate may lead to well-preserved ESC pluripotency [105].

On the other hand, stiffness of substrate can be used to promote differentiation of stem cells. When ESCs are cultured on polydimethylsiloxane (PDMS) substrates coated with fibronectin, mesodermal differentiation of ESCs is increased on stiffer substrates [42]. Mesendodermal and osteogenic differentiation of ESCs on stiff PDMS coated with collagen type I are enhanced as compared to soft PDMS, probably due to increased cell spreading on a stiffer substrate [44]. In addition, substrates with stiffness near the physiological stiffness of brain tissues are used to improve differentiation of neural stem-cells into neurons and subsequent maturation of the specified neurons [110-112]. Myosin in myotubes that are fused by myoblasts striates only on hydrogels with stiffness comparable to normal muscle tissues [113]. Muscle stem cells that cultured on soft hydrogel substrates with the elasticity of muscle tissues can still self-renew in vitro and repair damaged muscles when implanted in vivo; while muscle stem cells on rigid plastic dishes lose capacity of self-renewal generating offsprings with greatly diminished regenerative potential [114].

Besides, stiffness of substrate can be used to regulate stem cell lineage commitment. MSCs on polyacrylamide gels that have different elasticities and the same collagen I coatings can be specified into different lineages in identical culture conditions. On soft substrates, MSCs can be differentiated into neuron-like cells; On stiffer

substrates, MSCs can be differentiated into myoblast-like cells; on even stiffer substrates, MSCs can be differentiated into osteoblast-like cells [45]. Myosin and focal adhesions are involved in sensing matrix elasticity [45]. In addition, stiff polyacrylamide hydrogels coated with collagen I stimulate osteoblast differentiation of MSCs, whereas soft polyacrylamide hydrogels with the same coating promotes differentiation of MSCs into adipocytes [39]. Further studies show that collagen anchoring densities on soft polyacrylamide hydrogels lower than stiff polyacrylamide hydrogels. Therefore, MSCs exert a lower mechanical force to pull loosely-bound collagen on the soft polyacrylamide hydrogels, which leads to adipogenic differentiation of MSCs. In contrast, MSCs use a higher mechanical force to pull tightly-bound collagen on the stiff polyacrylamide hydrogels, which results in osteogenic differentiation of MSCs [115]. The influence of substrate stiffness on cell fate determination is also proved on substrates with geometry-induced rigidity. On PDMS micro-posts coated with fibronectin, osteogenic specification is favored on rigid micro-post arrays; while adipogenic differentiation is enhanced on soft ones [116, 117]. MSCs that undergo osteogenic differentiation demonstrate higher traction forces than cells differentiated into adipocytes [116]. Cytoskeletal contractility may be central in fate decisions of stem cells.

1.2.2.2 Manipulation of substrate topography in tissue engineering

Cell morphologies and spreading, which are closely associated with stem cell

differentiation[118, 119], can be influenced by topography of biomaterials through interactions between ECM proteins and their specific receptors on cell membranes [120]. Therefore, geometric cues of biomaterials can enhance differentiation of stem cells or directly determine stem cell fate [119, 121-123]. For instance, differentiation of ESCs into neural lineages is improved on nanofiber-based scaffolds [122]. Nanoscale topography can both effectively and rapidly induce differentiation of ESCs into a neuronal lineage [124] and stimulate MSCs to produce bone mineral in vitro without any exogenous biomolecule [125]. As no cytokines are used, concentration optimization of these agents for efficient differentiation and their side effects can be avoided. Further studies show that such geometry induced-differentiation is size dependent. 30 nm diameter nanostructures promote adhesion without noticeable differentiation, while larger ones (from 70 to 100 nm in diameter) can elicit a dramatic stem cell elongation, which results in cytoskeleton deformation and following osteogenic differentiation [126]. Neural stem cells preferentially differentiate into oligodendrocytes on thin nanofibers (283 nm fibers) and favor neuronal differentiation on wide nanofibers (749 nm fibers) [127]. Besides nanoscale topography, microscale geometric cues can also affect differentiation of stem cells. In vivo osteogenic differentiation of MSCs in β -tricalcium phosphate scaffolds with middle pores is improved as compared to ceramics with large or small pores [128]. A pore size ranging from 370 to 400 μm supports chondrogenic differentiation of adipose stem cells in polycaprolactone scaffolds than other pore sizes [129]. Chondrogenic differentiation of MSCs in collagen-hyaluronic acid scaffolds is also enhanced by pores with comparable

sizes (300 μm) [130].

In addition, substrate topography can provide directional cues to guide cell morphogenesis and migration in vitro. Neural stem cells can grow in parallel with the direction of aligned PLLA fibers [131]. When seeding ESC-derived cardiomyocytes into hydrogel constructs that contain parallel channels and micrometer-sized pores, cardiomyocytes organize themselves only within scaffold channels rather than micrometer-sized pores and proliferate to reach a cell density comparable to an adult heart [132]. Furthermore, such topography-induced cellular migration is size-dependent. In scaffolds with small pores (from 85 to 120 μm), cells only aggregate at surfaces and cannot migrate into the center of the scaffold; while large pores (325 μm) improve cell migration toward the center of scaffolds [133]. On the other hand, topographic cues can also regulate host responses to scaffolds. Pores structures support rapid and extensive angiogenesis throughout the scaffold [134]. Meanwhile, host responses to pores varying in sizes are different. Small vessels with poor penetration are supported in scaffolds with small pores (< 200 μm); while large blood vessels with deep penetration are encouraged in with large pores (> 200 μm) [135]. Another study using porous poly(ethylene glycol) (PEG) hydrogels shows more extensive invasion of vascularized tissues in vivo in hydrogels with large pores as compared to hydrogels with small pores [136].

1.3 Objectives of this thesis

In this thesis, we aim to manipulate physical signals either generated by cells themselves or from biomaterials to improve vascularization of tissue constructs or cardiac differentiation of ESCs. The specific aims are as follows:

Specific aim 1: Develop a real-time tracking system to monitor EB development, which can be used to screen EBs with homogenous sizes.

Specific aim 2: Engineer a vascularized hypertrophic cartilage template to mimic initial stage of endochondral ossification in vitro.

Specific aim 3: Optimize substratum stiffness to improve ESC pluripotency maintenance and cardiac differentiation.

Specific aim 4: Investigate the effects of pore density and pore sizes on hydrogel vascularization both in vitro and in vivo.

1.4 Organization of this thesis

This thesis is organized into 7 chapters.

Chapter 1 (current chapter) highlights the importance of physical cues to cell behaviors. Physical cues can either be generated by cells or come from ECM where cells reside. Hypoxia due to mass transfer limitation in the center of cell aggregation initiates a lot of complex biological processes. Meanwhile, stiffness and topography of ECM are main physical factors that regulate cell behaviors. Application of these physical signals can greatly improve performance of fabricated tissue constructs.

Finally, this chapter defines aims and structures of this thesis.

Chapter 2 investigates the use of poly (lactic-co-glycolic acid) (PLGA)-based nanoparticles containing calcein acetomethoxy (CAM) to monitor cell viability during EB development. Due to hypoxia-induced cell death in the core of EBs, PLGA-nanoparticle-labeled ESCs gradually lose their signals. As cell death increases with EB sizes, we can screen EBs with homogenous sizes by quantification of signal intensity within EBs. EBs with comparable sizes can be differentiated into myocardiocytes with minimal variations.

Chapter 3 develops a chondrocyte-derived hypertrophic cartilage template that mimics initial stage of endochondral ossification. Cartilage hypertrophy induction is important in this process, which provides channels for host vascular tissue ingrowth through matrix remodeling.

Chapter 4 demonstrates the use of gelatin-dopamine to improve ESC adhesion on polydimethylsiloxane (PDMS). Highly hydrophobic surfaces of unmodified PDMS discourage stable ECM protein adsorption and thus show ineffective cell adhesion. Grafting gelatin with dopamine greatly increases ESC adhesion, proliferation and efficiency of cardiac differentiation.

Chapter 5 further optimizes substratum stiffness to improve ESC pluripotency maintenance and cardiac differentiation based on PDMS coating strategy illustrated in Chapter 4. Biochips using optimized substratum stiffness are fabricated respectively for ESC pluripotency maintenance and cardiac differentiation.

Chapter 6 reports that hydrogel vascularization is enhanced by an increase of pore

densities both in vitro and in vivo. Dissolvable gelatin microspheres can create pore structures in hydrogels following their dissolution in water at 37 °C. By increasing ratios of gelatin microspheres to hydrogel precursor solutions, pore densities in hydrogels can increase accordingly.

Chapter 7 investigates the effect of pore sizes on hydrogel vascularization both in vitro and in vivo. Pore sizes in hydrogels can be simply controlled by gelatin microspheres varying in sizes. Vascularization in vitro is improved in hydrogels with middle pores as compared to hydrogel with large or small pores, while large pores supported hydrogel vascularization in vivo better than middle or small pores.

Chapter 8 summarizes current work and suggests possible future work.

Chapter 2: Real-time and Non-invasive Monitoring of Embryonic Stem Cell Survival during the Development of Embryoid Bodies with Smart Nanosensor

2.1 Introduction

Embryonic stem cells (ESCs) are a population of self-renewing, pluripotent cells that are able to differentiate into cell types representing all three germ layers [137, 138]. ESCs-derived embryoid bodies (EBs, spherical aggregates of ESCs in suspension) recapitulate critical events during early embryo development, which offers a perfect *in vitro* platform to study cell localization, distribution, viability and lineage commitment during embryonic development [139, 140].

The distribution pattern of viable ESCs within EBs is closely related with the maturation of EBs. With the progress of ESC differentiation, programmed cell death (apoptosis) gradually occurs in the core of EBs [11, 141]. Additionally, several growth factors commonly used to promote ESC differentiation, such as bone morphogenetic protein (BMP) [6, 10, 142] and fibroblast growth factor (FGF) [143, 144], are also reported to be involved in inducing cell apoptosis in EBs. Therefore, noninvasive and real-time monitoring of ESC viability distribution in EBs would allow researchers to optimize the culturing condition in time during EB development and to select the

suitable EBs for subsequent applications.

To monitor the *in vitro* cell viability continuously and non-invasively, fluorescent labels are always preferred due to the low-cost and convenience. For example, lipophilic carbocyanine dyes (e.g. DiO) can selectively label the plasma membrane [145] and has been utilized for the visualization of cell migration and proliferation [146-149]. Unfortunately, carbocyanine dyes cannot provide any information about the cell properties like viability. Another approach is to introduce fluorescent reporter genes (e.g. sequences coding green fluorescent protein (GFP)) into cells of interest [150]. Only if the cells are viable, there are the fluorescent proteins that can be used as the markers for tracking the cell migration and survival [151, 152]. For example, transplantation of GFP-expressing tumor cells into nude mice allowed tumor cell invasion and metastasis to be visualized *in vivo* for the first time [153, 154]. The only problem about the reporter gene-based strategy is the low non-viral transfection efficiency in primary cells and stem cells (usually <10%) [155, 156]. In addition, there might be mutations in genetically modified cells as a result of exogenous gene insertion [157, 158].

Previously, we have designed a nanoparticle-based sensor platform to track real-time expression of specific biomarkers that correlate with cell status and functions [159, 160]. Based on this platform, we developed a viability nanosensor by incorporating non-fluorescent calcein acetomethoxy (CAM) into poly (lactic-co-glycolic acid) (PLGA) nanoparticles [161]. When internalized by cells, PLGA nanoparticles gradually degrade within the cytoplasm and continuously release

encapsulated CAM, which are then converted by esterases in living cells into fluorescent calcein molecules. As a result, CAM nanosensors can be used not only for cell labeling but also for monitoring cell viability.

Here, we report the use of viability nanosensors for highly-efficient ESC labeling and non-invasive monitoring of ESC survival and distribution during EB development (**Fig. 2.1**). As comparisons, lipophilic carbocyanine dye (i.e. DiO) and GFP reporter gene are used in parallel. Following the homogenous and highly-efficient internalization, intracellular nanosensors gradually release the non-fluorescent molecules that become fluorescent only in viable cells. While signals from these nanosensors show a steady decrease from day 1 to day 7 due to the rapid cell division within EBs, the unique cell viability monitoring function of nanosensors allows a clear presentation of living cell distribution within the EBs. The analysis of ESC proliferation rate, pluripotency maintenance and differentiation capacity are used for proving the biosafety of the nanosensors.

Fig. 2.1 Schematic illustration of nanosensor labeling and monitoring ESCs during EB formation.

2.2 Materials and Methods

All chemicals except mentioned specifically were all purchased from Sigma-Aldrich and used without further purification. All cell culture reagents and supplements were obtained from Life Technologies (USA), unless otherwise stated.

2.2.1 Viability nanosensor synthesis

100 mg of PLGA (lactide:glycolide = 50:50) was dissolved in 2 mL chloroform at 4 °C and then mixed with 250 µg of CAM (Life technologies) in 250 µL dimethyl sulfoxide (DMSO). The mixture was added dropwise into 3% (w/v) poly (vinyl alcohol) (PVA) solution followed by homogenization (Tissue Master 125, Omni International) at 24,000 rpm for 1 min. The resulted emulsion was then placed in chemical hood overnight for the evaporation of chloroform. Finally, nanosensors were collected by centrifuging at 6,000 rpm for 5 minutes, washed thrice with double-distilled water, and freeze-dried (-80 °C) before being stored in -20 °C prior to usage.

2.2.2 Murine embryonic stem cell (mESC) culture

mESC (E14TG2a, American Type Culture Collection (ATCC)) were maintained on tissue culture flasks (Falcon) pre-coated with 0.1% (w/v) gelatin. The culture

medium was Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 15% fetal bovine serum (FBS, PAA Laboratories), 0.1 mM nonessential amino acids (NEAA, Gibco), 0.1 mM 2-Mercaptoethanol (2-ME) and 1000 units/mL leukemia inhibitory factor (LIF, Millipore). Upon reaching 80% confluence, the cells were trypsinized and used for subsequent experiment. Only cells with passage number of 6 to 10 were used for experiments in this study.

2.2.3 Cell labeling with nanosensors

1 mg nanosensors were placed in 100 μ L of 0.1% (w/v) poly-L-lysine (PLL) aqueous solution for approximately 20 minutes. Then, PLL-coated nanoparticles were purified through centrifugation and re-dispersed in 1ml mESC culture medium. mESCs were plated on the gelatin-coated flask overnight before being incubated with nanosensor-containing medium. 12 hours later, the nanosensor-containing medium was then replaced with normal mESC culture medium.

2.2.4 Cell labeling with DiO

2 μ L DiO cell-labeling solution was added to 1 ml mESC culture medium to prepare the 0.2% (v/v) working solution. Then mESCs were plated on the gelatin-coated flask overnight before being incubated with the working solution for 4 hours. The DiO-containing medium was then replaced with normal mESC culture medium.

2.2.5 Cell labeling with enhanced GFP (EGFP) plasmid

400 ng pEAK12-EGFP plasmid (promoter: cytomegalovirus, Clontech, USA) was mixed with 1 μ L Lipofectamine 2000 (Invitrogen) and incubated at room temperature for 20 minutes before being added to 500 μ L mESC culture medium to prepare the working solution. mESCs were plated on the gelatin-coated flask overnight before being incubated with the working solution. 6 hours later, the Lipofectamine-containing medium was replaced with normal mESC culture medium.

2.2.6 Fluorescence imaging

All fluorescent images were captured with Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus). Then fluorescent positive population percentage and average cellular fluorescent intensity were calculated. First, fluorescent area and average fluorescent intensity in one fluorescent image were quantified with ImageJ. Cell-covered area in according phase contrast image was also calculated with ImageJ. Then fluorescent positive population percentage and average cellular fluorescent intensity were achieved by normalizing the fluorescent area and average fluorescent intensity to the cell-covered area, respectively. For each group, at least 6 images of each group were captured and analyzed.

2.2.7 Flow cytometry analysis

Cells were dissociated from culture plate using 0.25% trypsin (Gibco) and fixed

in 4% (w/v) paraformaldehyde at room temperature for 20 minutes. After being washed with PBS thrice, 1 million cells from each group were re-suspended in 0.5% (w/v) bovine serum albumin (BSA) solution. Flow cytometry analysis was performed on LSRFortessa X-20 (BD Biosciences) and analyzed with the BD FACSDiva Software (BD Biosciences) and FlowJo (Tree Star).

2.2.8 Cell proliferation assay

mESCs were seeded at a density of 5.0×10^4 cells/well onto a 24-well tissue culture plate and labeled as above mentioned. Following labeling, the cells were continuously cultured for 7 days. At various time points (day 1, 3 and 7), the culture medium was removed, and the cells were incubated with 500 μ L culture medium supplemented with 50 μ L of WST-1 reagent (Roche) for 3 hours. The supernatant was then collected and examined (absorbance: 450 nm; reference: 620 nm) with Multiskan Spectrum Microplate Photometers (ThermoScientific, Finland). To obtain proliferation rate, readings at different time points were normalized against that of day 1 for each group. At least three replicates (n=3) from each group were assessed.

2.2.9 EB formation assay and ESC three-germ layer differentiation

To initiate EB formation, mESCs were dissociated into single cells using 0.25% trypsin (Gibico) and cell suspension droplets consisting of 1000 cells in 20 μ L of

differentiation medium were hung on the lids of petri dishes to make “hanging drops” [162]. The differentiation medium composed of Iscove’s modified Dulbecco’s medium (Gibco) supplemented with 15% FBS, 0.1 mM NEAA and 0.1 mM 2-ME. 48 hours later, the EBs were transferred onto non-adherent petri dishes. For EB formation assay, EBs cultured in suspension conditions for another 5 days. For ESC three germ layer differentiation, EB cultured in suspension conditions for 3 days and were seeded onto 0.1% gelatin-coated 24-well tissue culture plate on day 5. From day 5 to day 14, differentiation medium was changed every other day.

2.2.10 Confocal imaging

EBs were fixed in 4% (w/v) paraformaldehyde solution at 4°C and washed thrice with PBS before imaging. Samples were imaged with confocal microscope LSM710 (Zeiss). Florescence intensity distribution across EBs developed from ESCs labeled with DiO or nanosensor on day 7 was also analyzed with the ImageJ software. The center of EB was designated as ‘0’ and used as reference to normalize all other positions.

2.2.11 Gene expression analysis

Gene expression analysis was performed by using quantitative real-time polymerase chain reaction (qPCR). At various time points, mESCs or EBs were dissolved in TRIzol (Gibco, ThermoScientific) for extraction of RNA, which was then

converted into cDNA via reverse transcription prior to gene expression evaluation through qPCR. All the reverse transcription reagents used were obtained from Promega (USA). Subsequently, qPCR was done using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Singapore) and the CFX Connect Real-Time System (Bio-Rad, Singapore). Gene markers analyzed in this study include OCT4 and Nanog for stem cell pluripotency; Sox17 and GATA4 for endoderm differentiation; Brachyury (T) and PDGFR α for mesoderm differentiation; Sox1 and OTX2 for ectoderm differentiation. **Table 2.1** listed primers (AIT Biotech, Singapore) used in this experiment. For analysis, target gene expression was first normalized to housekeeping gene β -actin in the corresponding sample. Then the relative target gene expression was further normalized to the relative target gene expression in day 1 sample from positive control group. Three replicates from each different modified PDMS substrate (n=3) were used to determine each target gene expression.

Table 2.1 qRT-PCR primer sequences for mouse gene markers: forward (F) and reverse (R).

Gene	Accession Number	Primer sequence (both 5'-3')	Annealing temperature (°C)	Product size (bp)
OCT4	NM_013633	F: AAAGCAACTCAGAGGGAACCTCCT R: TAGCTCCTTCTGCAGGGCTTTCAT	58	122
Nanog	AF507043	F: TGGTGTCTTGCTCTTTCTGTGGGA R: AACTCATGTCAGTGTGATGGCGA	58	180
Sox17	NM_001289464	F: TGTAAGGTGAAAGGCGAGGTGGT R: GCATCTTGCTTAGCTCTGCGTTGT	58	182
GATA4	NM_008092	F: TCAAATTCCTGCTCGGACTTGGA R: GTTTGAACAACCCGGAACACCCAT	58	163
Brachyury (T)	NM_009309	F: TCTGGTCTGTGAGCAATGGTA R: CCGTGTCTACTGGCTGTCA	58	210
PDGFR α	NM_011058	F: CCTCGTGCTTGGTCGGATT R: TAACTTCTTGCTCTCGTCTG	58	384
Sox1	NM_009233	F: ATGCACAACCTCGGAGATCAG R: GTAATCCGGGTGTTCCCTTCAT	58	129
OTX2	NM_001286481	F: GAAGGGAGAGGACGACATTTAC R: CTTCCCTCATGAAGATGTCTGG	58	93
β -actin	NM_007393	F: TCATGAAGTGTGACGTTGACATCCGT R: CCTAGAAGCATTGCGGTGCACGATG	58	285

2.2.12 Immunohistochemistry staining

mESCs or EBs were fixed in 4% (w/v) paraformaldehyde at 4°C until staining.

Samples were washed thrice with PBS prior to immunostaining. Nanog antibody (rabbit polyclonal IgG, sc-33760, Santa Cruz Biotechnology) and OCT4 antibody (goat polyclonal IgG, sc-8629, Santa Cruz Biotechnology) were used for pluripotency evaluation. Sox-17 antibody (goat polyclonal IgG, sc-17356, Santa Cruz Biotechnology) and GATA4 antibody (goat polyclonal IgG, sc-1237, Santa Cruz Biotechnology) were used for endoderm differentiation assessment. Brachyury antibody (goat polyclonal IgG, AF2085, R&D Systems) and PDGFR α antibody (goat

polyclonal IgG, AF1062, R&D Systems) were used for mesoderm differentiation assessment. OTX2 antibody (goat polyclonal IgG, sc-30659, Santa Cruz Biotechnology) and Sox-1 antibody (goat polyclonal IgG, sc-17318, Santa Cruz Biotechnology) were used for ectoderm differentiation assessment. Samples were incubated with according primary antibody diluted at 1:200 in 10% (v/v) horse serum and 0.4% (v/v) Triton X-100 at 4 °C for overnight. After that, samples were washed with PBS and incubated with Goat anti-Rabbit IgG (H+L) Secondary Antibody (Alexa Fluor 546, A-11010, Invitrogen) or Rabbit anti-Goat IgG (H+L) Secondary Antibody (Alexa Fluor 546, A-21085, Invitrogen) diluted at 1:200 in 10% (v/v) horse serum and 0.4% (v/v) Triton X-100 at room temperature for 1 hour in the dark. Nuclei were counterstained by 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Stained samples were imaged with Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus).

2.2.13 Statistical Analysis

All results were presented as mean $\pm$ standard deviation. One-way ANOVA was used for comparisons assuming equal variances followed by least significant difference (LSD) test. $p < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 ESC labeling using nanosensors

The nanosensors were synthesized according to our previous studies [161]. Nanosensors used in this study was spherical in shape and $\sim 1 \mu\text{m}$ in size (Fig. S2.1a). Right after synthesis, nanosensor was almost neutral (-0.1 mV). However, PLL coating resulted in positively charged nanosensors ($+15.5 \text{ mV}$), which assists in their adsorption to cell membrane and subsequently internalization. When incubated in cell culture medium with 10% FBS at 37°C , only 10% of encapsulated CAM was released over the first 24 hours (Fig. S2.1b). In addition, when placed in cell culture medium with 10% FBS at 37°C , the average size of nanosensor only increased by 10% after 24 hours of serum exposure and 20% after 96 hours (Fig. S2.1c). These results showed good stability of the nanosensor during serum exposure, appropriate for cell labeling which occurs on the first 24-hour.

The ESCs were labeled with nanosensors through the simple incubation. One key parameter in this step was the concentration of nanosensors. We screened 5 concentrations (0.1, 0.5, 1, 2, 4mg/mL) and found out that a higher concentration of nanosensors resulted in a high cellular fluorescence. However, we observed significant loss of cell viability when labeling concentration exceeds 1 mg/mL. The low cellular fluorescence in 4mg/mL labeling group is also likely a result of poor cell viability. Therefore, to achieve a balance between labeling efficiency and cell viability, ESCs were labeled with 1 mg/mL nanosensors for all the following

experiments. The nanosensors at this concentration showed comparatively-strong signal intensity while the minor effect on ESC viability.

As comparisons, ESCs were also labeled with DiO, a lipophilic carbocyanine dye that inserts into cell membrane and GFP plasmid transfection through Lipofectamine. As shown in **Fig. 2.2a**, nanosensors showed the highest labeling efficiency, while there were only a few fluorescent cells for GFP plasmid transfection group. Flow cytometry analysis (**Fig. 2.2b**) revealed that nanosensors labeled 99.8% cells. Meanwhile, DiO labeled 92% cells and only 7.52% cells expressed GFP in the plasmid transfection group. Thus, nanosensor labeling outperformed other two methods in terms of labeling efficiency.

Fig.2.2 Labeling efficiency of nanosensors on ESCs. (a) Florescent images of ESCs labeled by nanosensors, DiO and GFP transfection 24 hours later. (b) Flow cytometry analysis of labeled cells in nanosensor group, DiO group and GFP group. Green color indicates labeled cells.

2.3.2 Dilution of nanosensors in ESCs upon cell divisions

One challenge for labeling cells with nanoparticles is the dilution of intracellular nanoparticles, which is due to the exocytosis of nanoparticles and division of cells [163]. To study the signal dilution of nanosensors in ESCs, labeled ESCs were continuously observed for 7 days, since EB period in routine ESC differentiation protocols usually lasts 5-7 days [164].

As showed in **Fig. 2.3a**, fluorescent signals in nanosensor-labeled ESCs decreased significantly from day 1 to day 3, but subsequently remained relatively constant until day 7. Signal dilution was also observed in DiO group, though the attenuation was less compared to the nanosensor group. On the contrary, the signal of GFP-expressing cells increased gradually over the 7 days of culture, most likely due to the accumulation of GFP within individual cells. Clearly, if it were not for the risk of mutagenesis and extremely low transfection efficiency of non-viral transfection, the GFP-labeling would be the best choice for tracking ESCs in the EB development.

Fluorescent signal dilution was further analyzed by quantifying fluorescent-positive population and average cellular fluorescent intensity of each group (**Fig. 2.3b and c**). Both DiO and nanosensor groups showed a reduction in fluorescent-positive population. Nanosensor group had 85.21% fluorescent-positive population on day 1, 75.61% fluorescent-positive population on day 3, and 42.16% fluorescent-positive population on day 7. DiO group showed lower fluorescent-positive population, with 59.18% fluorescent-positive population on day 1, 51.52% fluorescent-positive population on day 3, and 46.37% fluorescent-positive population on day 7. In

contrast, GFP had a constant but quite low fluorescent-positive population, with 0.69% fluorescent-positive population on day 1, 1.86% fluorescent-positive population on day 3, and 0.63% fluorescent-positive population on day 7 (**Fig. 2.3b**).

Average cellular florescence intensity also decreased in both nansensor and DiO groups. Nanosensor group showed 41.94 cellular florescence intensity on day 1, 14.38 cellular florescence intensity on day 3, and 9.48 cellular florescence intensity on day 7. DiO group showed initial low while steadily decreased cellular florescence intensity, with 23.82 cellular florescence intensity on day 1, 16.47 cellular florescence intensity on day 3, and 14.37 cellular florescence intensity on day 7. Though quite low in cellular florescence intensity, GFP group showed an increasing and subsequently decreasing signals intensity, with with 0.24 cellular florescence intensity on day 1, 0.97 cellular florescence intensity on day 3, and 0.27 cellular florescence intensity on day 7 (**Fig. 2.3c**).

Fig. 2.3 Tracking of nanosensor-labeled ESCs for 7 days in vitro. (a) Florescent images of ESCs labeled by nanosensors, DiO and GFP transfection from day 1 to day 7. (b) Fluorescent-positive population in nanosensor group, DiO group and GFP group from day 1 to day 7. (c) Fluorescent intensity in nanosensor group, DiO group and GFP group from day 1 to day 7. Green color indicates labeled cells; The data are presented as the mean $\pm$ standard deviation (n=6). ** indicates $p < 0.01$.

2.3.3 The development of EBs with nanosensor-labeled ESCs

EBs are cell aggregates which are formed due to ESCs proliferation and differentiation in suspension condition. During the development of EBs, ESCs labeled with nanosensors, DiO or GFP were suspended in differentiation medium and then hung on the lids of petri dish to make “hanging drops”. EBs were well-formed 24 hours after hanging drop fabrication.

The formed EBs were maintained in culture medium and imaged for 7 days (**Fig. 2.4a**). On day 1, both nanosensor and DiO EB groups showed a strong and homogenous fluorescent signals through the whole cell aggregates. In contrast, the fluorescent signal in GFP EB group had an imbalance distribution.

As EBs developed from day 2 to day 7, all three groups lost signals gradually (**Fig. 2.4b**). The signal attenuation of DiO was the slowest and the fluorescence was still pretty strong on day 7 (**Fig. 2.4b**). Nanosensor group lost the signal in a faster way (**Fig. 2.4b**). The performance of GFP group was the worst and the fluorescent signal was completely lost on day 7 (**Fig. 2.4b**).

One thing to note is the dark core of EBs in the phase contrast images in all three groups on day 5 and day 7 (**Fig. 2.4a**), which were necrotic center developed in EBs because of gradually-increasing EB size beyond nutrition diffusion limitation. As CAM released from nanosensors could only label living cells, we then evaluated whether the observed signal decrease was due to the loss of cell viability in the necrotic core of EBs.

Fig. 2.4 The development of EBs with nanosensor-, DiO-, or GFP-labeled ESCs from day 1 to day 7. Green color indicates labeled cells; insets indicate phase contrast images of EBs.

2.3.4 Cell viability reporting function of nanosensors during EB formation

The EBs developed from labeled ESCs were then closely examined with confocal microscope (**Fig. 2.5a**). On day 5 and day 7, positive signals were still observed on the edge of the nanosensor-labeled EBs, with the center region showing no fluorescence (thereby forming a negative core within a positive shell). In contrast, DiO group showed homogenously-distributed signals across the whole EB (a positive core within a positive shell) even until day 7. In GFP group, signals disappeared from day 5 to day 7, leaving completely-dark areas in the field. As a result, EBs with a negative core and a negative shell were formed.

Through fluorescence intensity quantification of EB cross-section images, the

difference in signal distribution between the nanosensor and DiO group was further revealed. As revealed in **Fig. 2.5b**, the fluorescence signal on two edges of EBs was clearly much stronger than that in the center in the nanosensor group. In DiO group, there was no significant difference between the edge and center of EBs. These results indicate that the decrease in signal intensity in EBs from the nanosensor group was partially due to the presence of non-fluorescent dead cells that accumulated in the core of EBs. These results also demonstrate the unique live/dead reporting function of viability nanosensor during the process of EB formation.

Fig. 2.5 Cell viability reporting function of nanosensors. (a) Confocal images of EBs developed from nanosensor-, DiO-, or GFP-labeled ESCs from day 3 to day 7. (b) Fluorescence intensity

distribution across EBs developed from ESCs labeled with DiO or nanosensors on day 7. Green color indicates labeled cells.

2.3.5 The effects of nanosensor labeling on ESC differentiation

Finally, the effects of nanosensor labeling on ESC three-germ layer differentiation were examined via qPCR and immunostaining. Two representative genes for each germ layer were analyzed in order to more accurately evaluate differentiation status of ESCs. Sox17 and GATA4 genes were used as endoderm biomarkers; Brachyury (T) and PDGFR α genes as mesoderm biomarkers; and Sox1 and OTX2 genes as ectoderm biomarkers. **Fig. 2.6** revealed that nanosensor and DiO groups expressed three-germ layers-associated genes in an increasing trend, whereas GFP group exhibited a downregulated trend along the culture period. While there were differences in the extent to which these genes were expressed between labeled and unlabeled ESCs, the protein biomarkers could be still positively stained in all the three labeling groups (red color), with nanosensor and DiO group showing more intensive color than the GFP group (**Fig. 2.7**). Only a slight difference in staining degree could be found between the nanosensor group and the control group. These data demonstrate that three-germ layers can be successfully specified in nanosensor-labeled ESCs and that nanosensor only has minor inhibitory effects on ESC three-germ layer differentiation.

Fig. 2.6 Gene expression analysis of three-germ layer differentiation markers in nanosensor group, DiO group and GFP group from day 1 to day 7. The data are presented as the mean $\pm$ standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

Fig. 2.7 Immunostaining for three-germ layer differentiation markers in nanosensor group, DiO group and GFP group 14 days after differentiation *in vitro*. Red color indicates positive staining; Blue color indicates cell nuclei.

2.4 Discussion

This study explores the utilization of viability nanosensors to label ESCs and monitor their survival during the development of EBs. These nanosensors were made through encapsulating CAM (a widely-used dye for labeling viable cells) within PLGA nanoparticles. Once internalized by the ESCs, PLGA started to degrade and CAM molecules were slowly released. Under presence of esterases, the non-fluorescent CAM was converted to the strong fluorescent calcein. As the enzymes were only present in the viable cells, the fluorescent signal could reflect the viability of cells. In comparison to GFP transfection with Lipofectamine, nanosensor labeling exhibited a high labeling efficiency (**Fig. 2.2**). The efficiency of GFP transfection for

ESCs in this study was less than 10%, which was consistent with previous reports [165-169]. In comparison to DiO that is known to label cells in non-uniform manner [170], the distribution of fluorescent signals in the nanosensor group was more homogenous. These results imply the feasibility of using CAM nanosensors as a powerful tool to label ESCs.

The dilution of nanosensor signals in ESCs was found to be quick in this study, with 10% decrease in fluorescence-positive population and 50% loss of cellular fluorescent intensity from day 1 to day 3 (**Fig. 2.3**). In our previous study, we showed that stable CAM signal could be observed from nanosensor-labeled MSCs for at least few weeks, better retained than DiO labeling [161]. The difference was probably due to the much more rapid proliferation rate of ESCs as compared to MSCs [171]. Even though the signal decrease in nanosensor group was more obvious than DiO group (**Fig. 2.3c**), the fluorescence-positive population in nanosensor group was higher than DiO group (**Fig. 2.3b**). PLGA nanoparticles can serve as a reservoir for continuous CAM release, which might be responsible for the higher fluorescence-positive population in nanosensor group [161]. For DiO group, cell division also led to a decrease in fluorescence-positive population and signal intensity (**Fig. 2.3**), as labeled plasma membrane of mother cells was allocated to two daughter cells upon division. Therefore, its retention is also quite poor in the rapidly growing ESCs. In comparison, GFP-transfected cells were mainly well-adhered (differentiated) cells with limited proliferation capacity. As a result, fluorescence-positive population and cellular fluorescent intensity increased from day to day 3 with the accumulation of GFP, even

though both fluorescence-positive population and cellular fluorescent intensity were quite low in GFP group (**Fig. 2.3b and c**).

With the progress of differentiation, the cells in the core of EBs undergo programmed cell death and cavitation [141], which resembles the amniotic cavity formation during early embryo development in vivo [10, 172]. The size of EBs extending nutrition and oxygen diffusion limit is chiefly responsible for cell death occurring within the core of EBs [11, 13]. The presence of nanosensors in the ESCs allowed us to monitor the viability of cells in this situation in real time. As shown in **Fig. 2.5**, cells in the periphery of EBs remained to be fluorescent, whereas the core of EBs lost their fluorescence, thereby forming a spheroid structure with fluorescent shell and dark core. This is consistent with the previous reports showing the occurrence of apoptotic cell death in the developed EBs [173, 174]. In contrast, there was fluorescent signal throughout the EBs formed by DiO-labeled ESCs. Although GFP-expressing ESCs in the GFP EB group presented undiluted fluorescence, both low transfection efficiency due to Lipofectamine as transfection reagent and possible transgene silencing [175, 176] resulted in the low percentage of cells expressing GFP, which hinders the visualization of all viable cells in EBs. Thus, the unique cell viability monitor function of viability nanosensors is well fulfilled in the EB formation model.

The analysis of ESC proliferation rate, pluripotency maintenance and differentiation capacity in this study proved the safety of the nanosensors. Nanosensor labeling did not affect ESC proliferation and only have minor inhibitory effects on

cell pluripotency. Meanwhile, for ESC differentiation potential, nanosensor labeling also did not cause severe inhibitions, with the presence of all three-germ layer biomarkers at the end of the differentiation experiment (**Fig. 2.7**). During the fabrication of CAM nanosensor, CAM was dissolved in DMSO prior to encapsulation. While DMSO is shown to induce ESC differentiation into multiple lineages [177, 178] and affect EB phenotypes [179], the slow degradation of nanosensors meant that we only observe minimal influences over these attributes due to nanosensor labeling.

2.5 Summary

In this study, we verified the feasibility of using viability nanosensors to label and track ESC status during EB development. Compared to DiO labeling and GFP plasmid transfection, nanosensor labeling showed homogenous and highly-efficient labeling. In particular, the unique cell viability monitoring function of nanosensors allowed clear presentation of live cell distribution within EBs. Therefore, viability nanosensors have high potential to be a powerful tool to facilitate optimization of EB culturing condition and eventual selection. Future improvements focusing on enhanced signal stability and negligible inhibitory effects of the nanosensor would allow further application on actual growing embryos.

Supplementary Information

Fig. S2.1. Nanosensor characterization. (a) Hydrodynamic diameter of nanosensors and the corresponding SEM image (inlet). (b) Release profile of CAM from nanosensors in cell culture medium with 10% FBS at 37 °C. (c) Serum stability of CAM nanosensor and blank nanosensors in cell culture medium containing 10% FBS at 37 °C.

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Chapter 3: Osteogenic Treatment Initiating a Tissue-Engineered Cartilage Template Hypertrophic Transition

3.1 Introduction

Endochondral ossification is a process by which long bones and the axial skeleton are formed during embryo development [82]. Chondrocytes derived from condensation of mesenchymal progenitors first proliferate and then synthesize matrix rich in type II collagen and aggrecan to generate cartilaginous model for the future bone. After that, chondrocytes in the center of the cartilage undergo hypertrophy, through which chondrocytes enlarge in their shape and synthesize collagen type X (Col X). Blood vessels then invade into the hypertrophic cartilage, delivering osteoblast progenitors that produce new bone matrix within it [180]. Since hypertrophic chondrocytes secrete various growth factors, cytokines, and other bio-molecules that can attract osteoblasts and endothelial cells which participate in endochondral ossification, the role of hypertrophic chondrocytes is critical in the whole process [19]. Recent studies also show that hypertrophic chondrocytes can even survive and further transdifferentiate into osteoblasts and osteocytes, which will contribute to endochondral bone formation *in vivo* together with osteogenic cells from blood vessels [181-185]. The similar complex process occurs in bone fracture repair in adult as well [186]. In order to repair bone

fracture, an avascular cartilage callus is first formed followed by vascular invasion and matrix mineralization, finally remodeling into functional bone [93]. Furthermore, hypertrophic differentiation of chondrocytes can also be observed in osteoarthritis (OA). Hypertrophic chondrocytes in OA will express Col X and MMP-13 similarly and lead to the mineralization and neovascularization of the cartilage ECM [187].

Due to the critical role that hypertrophic chondrocytes play in cartilage hypertrophy, *in vitro* monolayer chondrocyte culture model [188] and three dimensional (3D) cartilage explant model [189] have been established to mimic chondrocyte hypertrophy. However, for two dimensional chondrocyte culture model, chondrocytes in monolayer culture behave differently from native chondrocytes because of lack in a 3D environment that is required for cell-cell and cell-ECM interactions [190]. As for cartilage explant model, dense extracellular matrix (ECM) surrounding chondrocytes makes *in vitro* blood vessel invasion into cartilage difficult to occur because ECM has to be degraded prior to vascular invasion. To overcome these limitations, some groups seeded mesenchymal stem cells (MSCs) on biomaterials, such as agarose hydrogel and collagen I meshes [191, 192], and then primed fabricated constructs using chondrogenic and hypertrophic factors to generate hypertrophic cartilage. While some groups used pellet culture [193] and transwell [194] to generate scaffold-free constructs, which underwent chondrogenic differentiation followed by hypertrophic induction. Even though biomaterials can deliver biochemical and biophysical signals to cells embedded in constructs and protect cells from excessive mechanical loading upon implantation, there is a possibility that non-degradable biomaterial will affect cell-cell interactions

and also blood vessel invasion into hypertrophic cartilage. Cartilage templates engineered using pellet culture and transwell methods will be difficult to be implantable in clinical practice due to their small sizes. An *in vitro* scaffold-free and large-sized cartilage hypertrophy model is thus desired.

In our previous work, we have fabricated a scaffold-free 3D porous cartilage template, living hyaline cartilaginous graft (LhCG), which is composed of only chondrocytes and their synthesized ECM [195]. During construct fabrication, chondrocytes and gelatin microspheres that will dissolve in medium are co-encapsulated within alginate hydrogel. Cavities created by dissolved gelatin microspheres will provide space for chondrocytes to proliferate and ECM accumulation. Even though LhCG is very soft in texture upon alginate hydrogel removal, it still can maintain intact in structure because of interpenetrating ECM. After that, LhCG will gradually become condense in structure due to further cells growth and ECM accumulation [195]. LhCG is tangible and large in size, which makes it possible to be used in clinical practice. As a scaffold-free tissue engineered cartilage template, LhCG constructs closely mimic natural cartilage, free from any negative effects caused by biomaterials. It also permits the establishment of a co-culture system between chondrocytes and seeded cells, which enables a direct cell-cell interaction to occur in a 3D environment. Finally, its porous structures resulted from micro-cavities left by dissolved gelatin microspheres can provide potential channels to allow blood vessels to grow into constructs.

In this study, we will try to induce this tissue-engineered porous cartilage template

toward a hypertrophic status so as to develop an *in vitro* cartilage hypertrophy model. In order to mimic vascular invasion following cartilage hypertrophy *in vitro*, endothelial progenitor cells (EPCs) were also employed to co-culture with LhCG constructs in this study. We hypothesized that osteogenic medium used in our previous study [196] could induce hypertrophic change of LhCG construct. LhCG construct was first treated with osteogenic medium for hypertrophic induction. Thereafter, EPCs were seeded onto LhCG constructs to mimic vascular invasion, which were then further co-cultured *in vitro* or subcutaneously implanted into nude mice for *in vivo* evaluation. At designated time points, the constructs were harvested and evaluated for hypertrophic markers and vascular invasion, respectively. LhCG constructs with osteogenic medium treatment but without cell seeding were also established for parallel comparison.

3.2 Materials and methods

All chemicals used in this study were purchased from Sigma-Aldrich (Singapore) and all cell culture reagents and supplements were obtained from Invitrogen, unless otherwise stated.

3.2.1 LhCG construct establishment

The LhCG construct used in this study was based on the LhCG prototype already established in our previous works and LhCG was fabricated according to previous

protocol with modification [195, 196]. Briefly, double emulsion method (oil/water/oil) was employed to generate gelatin microspheres and these gelatin microspheres were further sieved to obtain spheres from 150 μm to 180 μm in diameter. Then 1000 units/ml penicillin and 1000 mg/ml streptomycin solution was utilized to sterile microspheres via overnight immersion following storage in phosphate buffer saline (PBS) at 4 °C. The areas on patellar articular surface of the femur from three 6-month-old domestic pigs were used to extract cartilage. PBS was used to wash all cartilage pieces, which were then cut into smaller pieces in 100 mm Petri dish. Dulbecco's Modified Eagle Medium (DMEM) and 10% (v/v) fetal bovine serum (FBS) were prepared to dissolve collagenase type II at the concentration of 1 mg/mL, which was then used to digest cartilage pieces for 14 hours. After digestion, supernatants were collected and centrifuged at 1100 rpm to remove collagenase. Cell pellet was then re-suspended and transferred into culture flasks for monolayer expansion. Only passage 1 chondrocytes were used for following the LhCG construct fabrication. The microspheres (0.3 g/ml) were mixed with porcine chondrocytes (10 million cells /ml) in 1 ml 4 °C alginate solution (1.5% w/v in 0.15 M NaCl). The mixture was then transferred to 30 mm Petri dish and enabled to spread evenly in Petri dish. Subsequently, gelation was initiated by gently adding 102 mM cold calcium chloride solution on top of mixture. The fabricated construct was cut into smaller round pieces of approximately 4mm in diameter before culturing in chondrocytes culture (CC) medium, which consisted of DMEM supplemented with 20% (v/v) of FBS, 0.1 mM nonessential amino acids (NEAA), 0.01 M 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), 0.05 mg/ml vitamin

C, 0.4 mM proline, 100 units/ml penicillin and 100 mg/ml streptomycin. Small round constructs were cultured on orbital shaker at shaking speed of 50 rpm in humidified incubator at 37 °C with 5% CO₂ for 8 h every day. Because encapsulated gelatin microspheres would dissolve into culture medium within two days at 37 °C, cavities of corresponding sizes would be created within alginate bulk. Alginate bulk would be subsequently removed using sodium citrate (55 mM in 0.15 M NaCl) solution for 10 min at 25°C after 35 days of constructs culture in medium. The twice removal of gel materials fabricated LhCG construct that was comprised of only chondrocytes and their secreted ECM and this LhCG construct was employed as a platform to explore cartilage hypertrophy *in vitro* in this study.

3.2.2 Effect of osteogenic medium on endostatin production by LhCG

After removal of alginate, LhCG constructs were still cultured in CC medium for 3 to 5 days to allow chondrocytes to further proliferate and synthesize ECM. The day on which LhCG were transferred to osteogenic medium is denoted as “Day 0”. Osteogenic medium was medium composed of CC medium supplemented with 100 nM dexamethasone, 50 mM ascorbic acid 2-phosphate and 10 mM β-glycerophosphate. To determine the quantification of endostatin generated by LhCG when cultured in osteogenic medium or in CC medium, the medium was removed 3 days prior to each specific time point and replaced with an equal volume of medium (2 ml per construct).

The medium collected after 3 days was stored at -80°C until analysis. Enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instruction (BlueGene Biotech, China). Results were expressed as percentage value compared with endostatin value on Day 2.

3.2.3 Co-culture medium selection

Co-culture medium was selected via live/dead fluorescent staining (Molecular probes, Invitrogen) that could evaluate the viability of chondrocytes and EPCs. Murine EPCs were purchased from BioChain Institute (USA) and maintained in EPC growth medium (BioChain, USA). EPCs and their derived endothelial cells (for short, generally named as "EPCs") were used in this study. EPCs were cultured in 24-well plate in a density of 1 million cells/ml (200 μ l per well) in CC medium, osteogenic medium, MCDB131 medium, which consisted of MCD131 basal medium supplemented 20% FBS, 2% bovine brain extract (Hammond Cell Tech, Healdsburg, California, USA), 0.1 mg/ml heparin, 100 units/ml penicillin and 100 mg/ml streptomycin, mixture of CC medium and MCDB131 medium in a ratio of 1 to 1, and mixture of osteogenic medium and MCDB131 medium in a ratio of 1 to 1, respectively. Medium was changed every other day. Calcein (0.5 μ l/ml) and ethidium homodimer (2 μ l/ml) were added to medium culturing EPCs on designated time points. After 30 min of incubation at 37 °C, medium was replaced with equal volume of PBS and the construct and EPCs were examined under fluorescence microscope. The dead cells were stained red while the live cells

were stained green. LhCG construct was cultured in the selected medium and the viability of chondrocytes was also evaluated using live/dead fluorescent staining as mentioned earlier.

3.2.4 Seeding of EPCs onto the LhCG construct

LhCG construct was cultured in osteogenic medium for 9 days before cell seeding. On Day 9, EPCs were suspended in MCD131 medium (1 million cells / ml) and 500µL of the cell suspension was added onto the LhCG construct. On the next day, unattached EPCs were removed and co-culture construct continued to be cultured in MCD131 medium for 27 days until Day 36. This construct was indicated as EPC. A negative control in which LhCG construct was cultured in CC medium for 36 days was indicated as CC. A positive control in which LhCG construct was cultured in osteogenic medium for 36 days was indicated as Osteo. LhCG construct which was not seeded with EPCs but cultured in osteogenic medium for 9 days and in MCD131 medium for 27 days in parallel was indicated as O+M. The co-culture system was established as shown in **Fig. 3.1a** and the detailed timeline for various *in vitro* culture conditions applied to different samples was described in **Fig. 3.1b**.

Fig. 3.1 (a) Schematic illustration of the establishment of endochondral ossification model in vitro using LhCG constructs and EPCs. (b) Timeline showing different in vitro culture conditions applied to different samples and EPCs seeding on different days. (c) Timeline showing samples first cultured in vitro and then subcutaneously implanted in vivo on different days.

3.2.5 Seeding of green fluorescent protein (GFP) labeled EPCs

In order to evaluate the efficiency of cell seeding onto the LhCG construct, GFP labeled EPCs were fabricated. Adenovirus vector that contained GFP gene was constructed using Adeno-X ViraTrak ZsGreen1Expression System 2 (Clontech Laboratories, Mountain View, CA, USA). 24 h prior to cell seeding, EPCs were transfected at a multiplicity of infection of 100 in serum-free EPC growth medium for 4 hours and then changed back into normal EPC growth medium. GFP labeled EPCs were employed only for evaluating EPC attachment to the LhCG construct. Non-transfected EPCs were used in all other experiments and analysis of the samples. Four representative images of GFP-labeled EPCs were obtained for each time point and analyzed with ImageJ software. The GFP positive area was calculated as the sum of positive areas presented in one field, and expressed as a fraction of the total area in one field.

3.2.6 Gene expression analysis

Gene expression analysis was performed by using quantitative real-time polymerase chain reaction (qPCR). LhCG constructs were dissolved in TRIzol[®] for extraction of RNA, which was then converted into cDNA via reverse transcription prior to gene expression evaluation through qPCR. All the reverse transcription reagents used were obtained from Promega (Madison, MI, USA). Subsequently, qPCR was done using SsoAdvanced[™] Universal SYBR[®] Green Supermix (Bio-Rad) and the iQTM

qPCR system (Bio-Rad). For analysis, gene expression values relative to the housekeeping gene, porcine ribosomal protein L4 (RPL4), were calculated using the comparative $2^{-\Delta\Delta C_T}$ method. Briefly, the C_T values of all target genes in each group was first normalized to that of RPL4. Then, for each target gene, the ΔC_T values of all other groups were normalized to ΔC_T value of group CC on Day 1, followed by final expression ratio calculation. **Table 3.1** listed primers (AIT Biotech, Singapore) used in this experiment.

Table 3.1 qPCR primer sequences for porcine gene markers: forward (F) and reverse (R).

Gene	Accession Number/ reference	Primer sequence (both 5'-3')	Annealing temperature (°C)	Product size (bp)
Porcine type I collagen (Col I)	[29]	F: CCTGCGTGTACCCCACTCA R: ACCAGACATGCCTCTTGTCCTT	58	84
Porcine alkaline phosphatase (ALP)	[29]	F: CCTGCGTGTACCCCACTCA R: ACCAGACATGCCTCTTGTCCTT	58	175
Porcine osteonectin (ON)	[29]	F: AGATAACTGTCTGCCCAAGGCTGT R: ATGGCTCTGGGCTCTGAGAAGTTT	58	143
Porcine osteocalcin (OCN)	[29]	F: TATGGCATAGCCTAGACCTCGCA R: TTTGGAGCAGCTGGGATGATGG	58	143
Porcine Cbfa-1	[29]	F: TACCTCTACTATGGCACCTCGTCA R: TTTAATAGCGTGCTGCCGTTTCGAG	58	122
Porcine collagen type X (Col X)	[29]	F: CAGGTACCAGAGGTCCCATC R: CATTGAGGCCCTTAGTTGCT	58	117
Porcine matrix metalloproteinase 13 (MMP-13)	XM_003129808.2	F: ACCTGGACAAGTAGTTCCAAAC R: AGTGGTCAAGACCTAAGGAATGGC	58	82
Porcine type II Collagen (Col II)	[29]	F: GCTATGGAGATGACAACCTGGCTC R: CACTTACCGGTGTGTTTCGTGCAG	58	256
Porcine aggrecan (pAgg)	[29]	F: CGAGGAGCAGGAGTTTGTC AAC R: ATCATCACCACGCAGTCCTCTC	58	177
Porcine Sox9	[29]	F: GCTGGCGGATCAGTACCC R: CGCGGCTGGTACTTGTAA	58	165
Porcine ribosomal protein L4 (RPL4)	XM_005659862.1	F: CAAGAGTAACTACAACCTTC R: GAACTCTACGATGAATCTTC	58	122

3.2.7 Histology and immunohistochemistry staining

LhCG constructs harvested at each time point were fixed in 4% (w/v) paraformaldehyde for 24h before dehydration and paraffin embedment. Three samples for each group at each time point were collected for analysis. The embedded samples were then cut into sections of 10µm in thickness using a microtome. The sections from all groups were later stained with hematoxylin and eosin (H & E), Safranin-O and Alizarin Red S. For platelet endothelial cell adhesion molecule-1 (PECAM-1 / CD31) staining, prior to incubation with CD 31 primary antibody (2 µg/ml in 1 x PBS, goat polyclonal IgG, sc-1506, Santa Cruz Biotechnology), the sectioned specimens were first incubated with 10% horse serum (w/v, in PBS) for 20 min to prevent non-specific IgG binding. After that, sections were incubated with anti-IgG (5 µg/ml in 1 x PBS, AlexaFluor 488, A22078, Invitrogen) at room temperature for 1 h in the dark. For collagen type X staining, the sections were first blocked with 10% goat serum (w/v, in PBS), and then incubated with collagen type X primary antibody (2 µg/ml, mouse monoclonal IgG, ab49945, Abcam) at 4 °C overnight. Prior to PBS washing for 3 times, the sections were washed with 1 x PBS and incubated with Anti-IgG (5 µg/ml in 1 x PBS, Invitrogen Alexa Fluor 546, A11060, Invitrogen) at room temperature for 1 h in the dark. Nuclei were finally counterstained by 4', 6-diamidino-2-phenylindole (DAPI).

3.2.8 Animal study

All animal experiments were done under guidelines approved by the Institutional

Animal Care and Use Committees, SingHealth, Singapore. 9 days after cell seeding, four LhCG constructs from CC, Osteo, O+M and EPC respectively were subcutaneously implanted into nude mice on day 18. Each mouse received one LhCG constructs from CC, one LhCG constructs from Osteo, one LhCG constructs from O+M and one LhCG constructs from EPC. The mice were killed and constructs were harvested for analysis at designated time points (Day 27 and 36). Experiment used three male nude mice for each time point (6 in total) and three samples for each group at each time point were collected for analysis. The sections from all groups were later stained with hematoxylin and eosin (H & E), Safranin-O and Alizarin Red S. Immunofluorescent staining was also applied to sectioned specimens as previously mentioned. The detailed timeline for samples cultured *in vitro* and subcutaneously implanted *in vivo* was described in **Fig. 3.1c**.

3.2.9 Statistical analysis

All results are presented as mean $\pm$ SD with 3 samples in each group. One-way ANOVA was used for comparisons assuming equal variances followed by least significant difference (LSD) test, unless otherwise stated. $p < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Effect of osteogenic medium on endostatin production by LhCG

Cartilage ECM components, such as endostatin, are mainly responsible for anti-angiogenesis property of cartilage [197]. As an anti-angiogenic factor, endostatin inhibits endothelial cell proliferation and counteracts many VEGF induced effects [198]. Therefore, in this study, we regarded the level of endostatin as an indicator of the whole cartilage anti-angiogenic property. We tried to use osteogenic medium treatment to offset this property of cartilage and monitored the level of endostatin to verify the effect of such treatment and also determine optimal treatment time. We found that osteogenic medium treatment could significantly inhibit the synthesis of endostatin by LhCG (**Fig. 3.2**). During initial 5 days of osteogenic medium treatment, there was no obvious difference in endostatin level in medium between osteogenic medium treated group and CC medium group, showing a decrease trend in both groups. This might be resulted from alginate removal, which caused cell function be compromised temporarily. However, from Day 8 onwards, there was significant difference between these two groups. Significant decrease in endostatin concentration in osteogenic group was first observed on Day 8, with 69.53% decline compared with endostatin concentration on Day 2. After 8 days of osteogenic medium treatment, endostatin secretion remained in low level until the end of experiment. Because osteogenic medium significantly decreased endostatin synthesized by LhCG from Day 8 to Day 11, following

experiments would use 9 days of osteogenic medium treatment to induce chondrocyte hypertrophic change.

Fig. 3.2 ELISA determination of endostatin synthesis of LhCG construct treated with osteogenic medium. ** indicates $p < 0.01$, significantly different from LhCG constructs cultured in CC medium.

3.3.2 Co-culture of EPCs and LhCG constructs

The presence of GFP-labeled cells further demonstrated that EPCs were attached onto the LhCG construct (**Fig. 3.3a**). Thus, a direct co-culture system of chondrocytes and EPCs was established. On the first (Day 10) after cell seeding, EPCs showed rounded cell morphology on LhCG constructs, while they manifested stretched out cell morphology on Day 18, which indicated EPCs attached more tightly on LhCG surface

with the elongation of the culture time. However, fewer GFP-labeled cells could be observed on Day 18 and Day 27, compared with cells on Day 10 (**Fig. 3.3b**). The results indicated that EPCs decreased in their numbers over time during *in vitro* culture period. The reason for this might be that EPCs could only attach onto the surface of LhCG constructs but couldn't grow and function normally in the co-culture system established here, even though cells still maintained their stretched morphology.

Fig. 3.3 GFP-labeled EPCs seeded on osteogenic medium pre-treated LhCG constructs and cultured in MCD131 medium.

3.3.3 Gene expression studies

To investigate ossification of LhCG constructs and chondrocyte hypertrophy, gene

expression studies were performed via qPCR to evaluate the expression levels of four important osteogenic markers, Col I, ALP, ON, OCN and Cbfa-1, as well as two significant chondrocyte hypertrophic markers, Col X and matrix metalloproteinase (MMP)-13. For each of the genes tested, LhCG control group at Day 0 was designated a fold value of 1 and all groups were normalized to that of LhCG control group at Day 0. As shown in **Fig. 3.4a**, for LhCG constructs from Osteo group, there were a significant upregulation of osteogenic genes, with highest expression of Col I showing on Day 9 and highest expression of OCN showing on Day 18. Gene ON showed an increasing expression throughout the whole experiment period. The expression of gene ALP and gene Cbfa-1 was markedly higher than that of LhCG constructs from CC group on Day 27, even though the expression of gene ALP and gene Cbfa-1 showing a decreased trend. However, all osteogenic markers from O+M and EPC group were down-regulated and remained at a low level from Day 18 to Day 36. Despite the high expression of gene OCN in O+M group at Day 36, the result might be anomaly, because the rest osteogenic genes, like Col I, ALP, ON and Cbfa-1, were all low expressed in O+M group.

From gene expression results in **Fig. 3.4b**, Col X, a hypertrophic mark for chondrocytes, showed decreased expression throughout 36 days' culture period in Osteo group. However, compared with Col X expression in specimens from CC group, Col X expression in samples from Osteo group was significantly higher. In contrast, the expression of MMP-13, another hypertrophic mark for chondrocytes, first increased and then decreased, with highest expression level showing on Day 9. But no

significance difference was observed between CC group and Osteo group. In O+M and EPC group, Col X and MMP-13 showed decreased expression and remained at comparatively low level from Day 9 to Day 36, indicating chondrocytes hypertrophy in these two groups were attenuated.

From **Fig. 3.4c**, it was observed that chondrocytes in LhCG constructs from CC group expressed increasingly higher chondrocytic markers from Day 0 to Day 9, followed by a gradual drop in gene expression level. LhCG constructs from Osteo group showed similar trend, however, with approximately 1-3-fold higher than LhCG constructs from CC group. For O+M and EPC group, down-regulation of chondrocytic marker expression was observed and the expression remained at low level from Day18 to Day 36.

Fig. 3.4 Analyses of osteogenic (a), chondrocyte hypertrophy (b) and chondrocytic (c) markers expression of LhCG constructs in different groups. Based on the expression value of the particular gene in LhCG constructs from CC group at day 0, fold values for each gene were calculated. * indicates $p < 0.05$; significantly different from LhCG constructs in CC group at designated time points. # indicates $p < 0.05$; significantly different from LhCG constructs in CC group on day 0.

3.3.4 Histochemistry and immunohistochemistry staining of samples from *in vitro* experiments

A qualitative analysis of LhCG constructs status for ossification, vascularization and chondrocytic hypertrophy was performed via various histological stains (**Fig. 3.5**). Based on H & E staining results in **Fig. 3.5a**, chondrocytes showed typical round shape with lacunae clearly visible around some chondrocytes throughout specimens from different groups. There was no significant difference among four different groups, which indicated that the effects of EPCs seeding were minimal. In addition, at the edge of LhCG constructs from EPC group on Day 36, there were some spindle cells stained with pink color. It seemed that these cells which were distinctive from nearby chondrocytes were seeded EPCs. As shown in **Fig. 3.5b**, there was strong Alizarin Red S staining in Osteo group and negative Alizarin Red S staining in CC group from Day 18 to Day 36. The calcium deposition for the EPC group at Day 27 was very similar to that of osteogenic group. Previous study indicated that cartilage explants could mineralize with the addition of bone morphogenetic protein or by exposure to human vascular endothelial cell (HUVEC)-conditioned medium [183]. Therefore, the EPCs that attached to the LhCG constructs might facilitate the mineralization process of LhCG constructs, even though *in vitro* culture condition established in this study was not suitable for EPCs growth. However, EPCs gradually detach from LhCG constructs over time. As a result, the effects of EPCs were not as obvious as osteogenic medium treatment, since the intensity of calcium staining in EPCs group was similar to M+O group but significantly lower than that in Osteo group on Day 36. Safranin O staining

results in **Fig. 3.5c** showed similar orange color for samples from four different groups and there was no obvious difference could be observed among these different groups.

Fig. 3.5 Histochemistry staining of samples from in vitro experiments (a) H&E staining; (b) Alizarin Red S staining; and (c) Safranin O staining. Scale bar represented 100 μm and applied to according images.

Immunofluorescent staining for CD 31 was found to be negative in all groups, except for EPC group where EPCs were seeded onto LhCG constructs, as showed in **Fig. 3.6a**. However, only on the edge of LhCG constructs in EPC group, there were a few CD31-positive cells present, which indicated that co-culture system in this study was not well established. According to images in **Fig. 3.6b**, Col X was found to be expressed in Osteo group on Day 18 and highly expressed on Day 36. Col X remained insignificant in CC group from Day 18 to Day 36. Immunofluorescent staining for Col X in both EPC group and O+M group was slightly positive, which were much lower compared with Col X staining in Osteo group.

Fig. 3.6 Immunohistochemistry staining of samples from in vitro experiments (a) Immunofluorescent staining for CD 31; and (b) Immunofluorescent staining for Col X. Nuclei were stained blue (DAPI) in all immunohistochemistry images. Scale bar represented 200 μ m and applied to all images.

3.3.5 Animal studies

After 9 days of osteogenic induction in osteogenic medium and 9 days of co-culture in MCDB131 medium, samples in EPC group were subcutaneously implanted into nude mice on Day 18, together with samples from CC group as negative control, Osteo group as positive control, and O+M as control without cell seeding. The samples were harvested on Day 27 and Day 36 for histochemical and immunohistochemistry staining, respectively. As shown in **Fig. 3.7a**, host tissues just encapsulated LhCG constructs and there was no blood vessel that invaded into interior of LhCG constructs in CC group. In contrast, host tissues stained with pink through H & E staining could be observed

between nodules of LhCG constructs in the other three groups. In addition, specimens from Osteo group, as well as O+M group and EPCs groups, all showed lumen-like structures in the center of sample on Day 18 and Day 36, as indicated by arrowheads. From images in **Fig. 3.7b**, Osteo group showed most bright red staining, suggesting higher ossification degree than other three groups. Samples from CC group were negatively stained for Alizarin Red S, indicating that there was no sign of ossification. The intensity of Alizarin Red S staining of sectioning from EPC group was nearly the same as that of sectioning from O+M group. As shown in **Fig. 3.7c**, Safranin O staining for cartilage tissues among different groups showed no obvious difference.

Fig. 3.7 Histochemistry staining of samples from in vivo experiments (a) H & E staining; (b) Alizarin Red S staining; and (c) Safranin O staining. Arrowheads denoted lumen-like structure present in H & E staining. Insets showed low-magnification images and scale bar represented 400 µm. Scale bar represented 100 µm and applied to all high-magnification images.

Immunofluorescent staining for CD 31 was found to be positive for Osteo group and almost negative for CC group on Day 36, as shown in **Fig. 3.8a**. O+M group was slightly stained positive for CD 31 on Day 36, while EPC group was intensively stained positive for CD 31 both on Day 27 and Day 36, indicating the presence of CD31-positive cells. Col X was also found to be highly expressed in Osteo group on Day 18 and Day 36 in animal experiment. Col X still remained insignificant in CC group from Day 18 to Day 36, as indicated in **Fig. 3.8b**. Similar staining for Col X in EPC group and in O+M group was observed, however, both of which was much lower compared with Osteo group.

Fig. 3.8 Immunohistochemistry staining of samples from in vivo experiments (a)

Immunofluorescent staining for CD 31; and (b) Immunofluorescent staining for Col X. Nuclei were stained blue (DAPI) in all immunohistochemistry images. Scale bar represented 200 μ m and applied to all images.

3.4 Discussion

Our study demonstrated that osteogenic medium treatment was able to enable LhCG constructs hypertrophic induction. Hypertrophic marker, Col X, was highly expressed in LhCG constructs when cultured in osteogenic medium. Obvious matrix mineralization was also achieved within LhCG construct upon osteogenic medium treatment, as well as highly expressed osteogenic genes, such as Col I, ON and OCN. When subcutaneously implanted after 18 days of *in vitro culture*, osteogenic medium treated LhCG constructs still expressed Col X and showed significant calcium deposition. In addition, when osteogenic medium treated LhCG construct developed under *in vivo* environment, obvious blood vessel invasion into LhCG constructs was observed. Hypertrophic cartilage can attract blood vessels by releasing vascular-endothelial growth factor (VEGF) [194]. Therefore, this result also suggested that cartilage hypertrophy could be initiated by osteogenic medium treatment.

In our previous studies, we found that when cultured in osteogenic medium for 42 days, LhCG construct, primarily consisting of chondrocytes, expressed osteogenic markers as highly as LhCG constructs seeded with MSCs [196]. In addition, increasingly *in vivo* evidences show that hypertrophic chondrocytes appearing during endochondral ossification will survive and commit into osteogenic phenotypes, being

an alternative source of osteoblasts during endochondral bone formation rather than die due to apoptosis [182, 199]. Taken together, it is highly possible that hypertrophic chondrocytes in LhCG constructs will further differentiate into osteogenic precursors, which will enable bone formation. However, in our study, even though osteogenic medium could induce LhCG constructs into a hypertrophic status, LhCG constructs didn't proceed into the bone formation stage even after 18 days of subcutaneous implantation. There is a possibility that chondrocytes that remained to be non-hypertrophic in the constructs prevented the bone formation. Therefore, unlike cartilage hypertrophy in endochondral ossification which will be followed bone formation, our model established in this study is more like cartilage hypertrophy happened in OA where hypertrophic chondrocytes will express Col X and MMP-13 and lead to the mineralization and neovascularization of the cartilage ECM but the healthy chondrocytes remained in cartilage will still resist the bone formation [187].

In this study, we also found that osteogenic medium treatment could reduce the anti-angiogenic property of cartilage. Cartilage is an avascular tissue and the avascular nature of cartilage is primarily due to its ECM components that can prevent vascular invasion, such as thrombospondin-1, chondromodulin-1 and endostatin [197]. Endostatin, a 20 kDa C-terminal fragment of collagen XVIII [200], is extensively expressed in many avascular tissues, including cartilage [198]. As an antiangiogenic factor, endostatin, specifically inhibits endothelial cell proliferation and counteracts many VEGF induced effects, such as inhibition of VEGF-induced endothelial cell migration neovascularization [198]. Hence, we regarded the level of endostatin as an

indicator of cartilage anti-angiogenic property. As shown in **Fig. 3.2**, the level of endostatin significantly decreased after around 9 days of osteogenic medium treatment. We thus hypothesized that anti-angiogenic property of cartilage was inhibited and EPCs could be seeded on LhCG construct without interruption from chondrocytes. The presence of GFP-labeling EPCs on LhCG construct with spread out and elongated shape in subsequent experiment suggested EPCs could attach to the surface of osteogenic medium-treated LhCG constructs. However, regardless of initial cell seeding densities, EPCs that could remain on the surface of LhCG constructs after 9 days of co-culture was similar. Moreover, EPCs that could attach onto LhCG constructs surface decreased over time (**Fig. 3.3**). Therefore, we believe exposure of LhCG constructs to osteogenic medium for 9 days helps to create a microenvironment in favor of cell attachment but the microenvironment created in this study cannot completely support the growth and function of EPCs. Co-culture system was not completely set up in this study.

In addition, medium used to co-culture EPCs and LhCG constructs seemed not to sustain hypertrophic change of LhCG constructs initiated by osteogenic medium according to gene expression studies, which showed considerably low expression of hypertrophic and osteogenic genes, as well as genes associated with chondrocytes (**Fig. 3.4**). Even though both endothelial cells and chondrocytes could survive in MCDB131 medium that is routinely used for culture endothelial cells [201], MCDB131 medium may not support normal function of co-cultured chondrocytes. The reason for this was probably due to some critical components, such as dexamethasone, necessary for

osteogenic differentiation was absent in MCDB131 medium [202]. Hence, culture conditions for both endothelial cells and chondrocytes needs to be further optimized in the future so as to facilitate angiogenesis and cartilage hypertrophy simultaneously in single culture system.

Under co-culture conditions *in vitro*, EPCs seeding didn't show any improvement in the LhCG construct hypertrophy and matrix mineralization in comparison with LhCG constructs without cell seeding. When co-culture system was transferred into animals for further development, similar results could be observed as well. However, LhCG construct in Osteo group also showed obvious blood vessels invasion even without any cell seeding. These results indicated that the role of EPCs was not as significant as osteogenic medium treatment for LhCG constructs hypertrophy. Exposure to osteogenic medium treatment for a longer period (18 days in Osteo group while only 9 days in EPC group) may be more favorable than EPC seeding for cartilage hypertrophy.

Blood vessel invasion, which can deliver osteoblast precursors to cartilaginous model for bone formation, is one of critical events that occur during endochondral ossification [180]. In order to mimic vascular invasion *in vitro*, EPCs were employed to co-culture with LhCG constructs in this study. EPCs are bone marrow-derived cells that are present in the systemic circulation and are able to differentiate into a variety of mature vascular endothelial cell types to participate new blood vessel formation [203]. Since its first discovery in 1997, increasing evidence demonstrates that EPCs have a high capacity to promote tissue regeneration and vascularization [204, 205].

Therapeutic applications of EPCs have been reported for bone regeneration [206, 207] and vascularization [208, 209], as well as osteochondral regeneration [210].

Despite minimal function of EPCs in this study, EPCs seems to be more suitable for endothelialization of hypertrophic cartilage compared with vessel-derived endothelial cells, such as human umbilical vein endothelial cells (HUVECs). EPCs have superior elongation, microvascular network formation and lumen formation capacity [211]; EPCs can be easily isolated from peripheral blood, thereby making this cell type ideal for tissue engineering applications [212]; Under serum-deprived conditions, EPCs showed lower level of apoptosis than HUVECs [212]. Besides, a new type of endothelial cells with morphological, molecular and functional properties different from those of endothelial cells in other organs was founded in skeletal system [213]. These endothelial cells can release angiocrine signals that are necessary for perivascular osteoprogenitors differentiation and for chondrocyte maturation and hypertrophy [214]. This finding hints that EPCs without undergoing terminal differentiation may be more suitable for endothelialization of hypertrophic cartilage, as EPCs can acquire desired functional properties in hypertrophic and mineralized cartilage.

3.5 Summary

In this study, we successfully imitated some critical aspects of cartilage hypertrophy using *in vitro* engineered living cartilage templates and osteogenic

treatment. As a primary factor, osteogenic treatment induced cartilage hypertrophy and vascular invasion in LhCG constructs. EPCs seeded onto LhCG constructs showed no improvement in hypertrophic change and vascular invasion. Hypertrophic transition of LhCG constructs using osteogenic treatment may contribute to the establishment of an *in vitro* cartilage hypertrophy model.

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Chapter 4: The Effects of Gelatin-Dopamine Coating on Polydimethylsiloxane Substrates on Pluripotency Maintenance and Myocardial Differentiation of Cultured Mouse Embryonic Stem Cells

4.1 Introduction

Myocardial differentiation of pluripotent stem cells, such as induced pluripotent stem cell (iPSCs) and embryonic stem cells (ESCs), in microfluidic devices has recently attracted much attention in cardiac tissue engineering [215-218], due to the capacity of microfluidic technologies to manipulate cells in a well-controlled and precise manner [219, 220]. Polydimethylsiloxane (PDMS) is a widely-used biomaterial in the fabrication of microfluidic devices, especially in the exploratory stages of research, due to their numerous advantages, including wide-range adjustable stiffness, ease of molding, optical transparency, gas permeability, nontoxicity and low manufacturing costs [221, 222].

However, the surface of PDMS is highly hydrophobic [223], which often discourage cell adhesion and long-term cell culture on unmodified PDMS surfaces [224, 225]. This leads to the extensive investigations that focus on the PDMS surface modification to render its surface less hydrophobic for the enhanced cell adhesion and

growth [221, 226]. For instance, plasma treatment is a simple and direct approach to reduce the hydrophobicity of the PDMS surface, but its effects are often short-lived, which results in the later PDMS surface hydrophobic recovery [227]. On the other hand, although coating of protein may enhance cell adhesion, cell detachment and poor cell proliferation still can be observed on the PDMS surface after prolonged culture [224, 226, 228-230]. Recent investigation also explores the use of (3-aminopropyl)triethoxy silane (APTES) and glutaraldehyde to functionalize the PDMS surface for the stable and covalent attachment of proteins. Such silanized PDMS surfaces have been shown to improve and stabilize the adhesion, proliferation and osteogenic differentiation of mesenchymal stem cells (MSCs) [226]. Though effective, this multi-step PDMS surface functionalization is complex and time consuming. Moreover, the possible chemical residuals on PDMS surfaces can cause potential toxicity to cells seeded. More recently, coating the PDMS substrates with a layer of polydopamine and another layer of adhesive protein has been shown to improve MSC adhesion proliferation and differentiation on their surfaces [221]. However, two steps of coating are still required for such method. Therefore, a simple and one-step coating method is desired to effectively reduce PDMS surface hydrophobicity and support prolonged cell culture.

Embryonic stem cells (ESCs) possess the outstanding capacity to be differentiated into cell types representing all three germ layers in vitro and thus are frequently used for lineage commitment studies [138, 231]. ESCs are widely-used model cells for myocardial differentiation and ESC-derived cardiomyocyte is also one

of promising cell sources for cardiac tissue engineering [139, 140, 232, 233]. For the monolayer differentiation of ESCs into cardiomyocytes, ESCs need to be attached to substrate surface first and then grow into a single layer of cells, followed by induction through small molecules or growth factors [234-237]. While for the differentiation of ESCs into cardiomyocytes via embryoid bodies (EBs, spherical aggregates of ESCs in suspension) formation [238, 239], the attachment of EBs onto substrate surfaces is critical in the establishment of EB bilateral symmetry, which facilitates the following EB mesoderm induction and cardiac commitment [240, 241]. Therefore, in order to differentiate ESCs into cardiomyocytes on the PDMS substrate, the adhesion of ESCs or EBs onto PDMS surface is important. However, the intrinsic properties of PDMS surfaces often hinder ESC adhesion and its myocardial differentiation on PDMS-based substrates.

In our previous study, we grafted dopamine onto the gelatin backbone to synthesize a dopamine-conjugated gelatin [242]. Gelatin is an inexpensive and denatured collagen, which contains appropriate sites for cell adhesion and thus is commonly used to improve cell-material interaction [243-245]. Dopamine is a typical catechol-containing molecule, which can directly generate strong non-covalent interactions to various surfaces [246-249]. Moreover, catechol group can be oxidized under oxidative or alkaline conditions to produce quinone, which can result in the formation of high strength covalent bonding to substrate surfaces [249-253]. In this study, we used our synthesized gelatin-dopamine to coat the PDMS surface and the adhesion of ESCs and ESC-derived EBs onto the PDMS surface was evaluated (**Fig.**

4.1). We demonstrated that gelatin-dopamine could effectively bind to the PDMS surface, which in turn reduced the hydrophobicity of PDMS surfaces and provided adhesion sites to ESCs for their attachment. As a result, ESC adhesion, proliferation, pluripotency maintenance and myocardial differentiation on the PDMS surface were greatly improved.

Fig. 4.1 Schematic illustration of coating PDMS substrate with gelatin-dopamine and seeding ESCs or EBs on the gelatin-dopamine coated PDMS surface.

4.2 Methods

4.2.1. Synthesis and characterization of gelatin-dopamine

Gelatin-dopamine was synthesized via ethyl-dimethyl-aminopropylcarbodiimide (EDAC) and N-hydroxy-succinimide (NHS) coupling chemistry. 100 mL of phosphate buffered saline (1×PBS, pH 7.4) was used to dissolve gelatin (2.0 g, type A from porcine skin, Sigma-Aldrich) at 60 °C by stirring, followed by the addition of EDAC (0.5 g, Sigma-Aldrich) and NHS (0.3 g, Sigma-Aldrich). The pH value of the solution was maintained at around 6.0. After 30 minutes, 2 mL of 50% dopamine

hydrochloride (w/v in deionized (DI) water, Sigma-Aldrich) was added to the mixture in dropwise fashion. The pH value of the reaction solution was maintained at 5.0 to 6.0 for 24 hours at 37 °C. The reaction solution was then dialyzed in DI water for 24 hours prior to lyophilization.

The grafting of dopamine onto gelatin was verified by proton nuclear magnetic resonance spectroscopy (^1H NMR). 0.15 g gelatin-dopamine dissolved in 1 ml of deuterium oxide (D_2O) was transferred into a 5 mm NMR tube and the signals were recorded using Bruker Avance-300 NMR spectrometer. The ^1H NMR spectrum of gelatin was collected in parallel for comparison.

4.2.2 PDMS substrate preparation and surface coating

PDMS substrates was prepared by mixing 20 parts of silicone elastomer base to 1 part of curing agent (SYLGARD, Dow Corning, USA), followed by casting PDMS onto a flat polystyrene dish or well plate. The PDMS was then degassed in a vacuum oven for 60 minutes to remove air bubbles and cured at 70 °C for 120 minutes. The cured PDMS substrates were divided into three groups. 0.1% G group: PDMS substrates were immersed in 0.1% gelatin solution (w/v in DI water) overnight at 37 °C. 0.1% G-D group: PDMS substrates were immersed in 0.1% gelatin-dopamine solution (w/v in DI water) with 0.05% sodium periodate (Sigma-Aldrich) overnight at 37 °C. 1% G-D group: PDMS substrates were immersed in 1% gelatin-dopamine solution (w/v in DI water) with 0.5% sodium periodate (Sigma-Aldrich) overnight at 37 °C. After that, the gelatin or gelatin-dopamine solution was removed, and the

samples were washed twice with DI water prior to any experimental analysis. For cell culture experiments, the PDMS substrates coated with gelatin or gelatin-dopamine were sterilized with 30 minutes ultraviolet (UV) light exposure. Tissue culture well plates (IWAKI, Japan) immersed in 0.1% gelatin solution overnight at 37 °C were used as positive control.

4.2.3 Surface characterization of PDMS substrates

The PDMS substrates coated with gelatin or gelatin-dopamine were characterized for their surface hydrophilicity, nanotopography and roughness. Surface hydrophilicity was determined by measuring surface water contact angles via FTA200 Dynamic Contact Angle Analyzer (First Ten Angstroms, VA, USA). Briefly, 5 μ L of DI water was dropped onto the PDMS substrate, and a high resolution camera was used to capture the shape of the water droplet. The contact angles of the water droplets on different coated PDMS substrates were measured with the static sessile drop tangent method in the Drop Shape Analysis software. The water contact angles of five different spots (n=5) on the PDMS substrate were measured for each group.

The surface roughness and nanotopography of the PDMS substrates were measured by MFP-3D Atomic Force Microscopy (AFM, Asylum Research, CA, USA) with a AFM probe in tapping mode. The AFM probe consisted of a silicon tip with radius of 28 ± 10 nm and spring constant range of 0.5-4.4 N/m. 20×20 μ m area was scanned at 0.8 Hz, a set point of 0.7 V and with resolution of 256 pixels. Five

random areas with the same size (n=5) on the PDMS substrates were tested for each group.

4.2.4 Surface protein quantitation

The amount of gelatin or gelatin-dopamine absorbed on the PDMS substrates was determined by a micro-BCA Protein Assay Kit (ThermoScientific, Singapore). After immersion in gelatin or gelatin-dopamine solution overnight, the PDMS surfaces were washed twice with DI water to remove loosely bound or non-adherent protein molecules and were immersed in working solution at 37 °C for 1 hour. Following this, the absorbance of the working solution at 562 nm was measured with Multiskan Spectrum Microplate Photometers (ThermoScientific, Finland). The protein absorbed on the PDMS substrates was then calculated from the absorbance value and normalized against the absorbed protein in positive control group. Six replicates (n=6) from each group were measured.

4.2.5 Embryonic stem cell (ESC) culture

Murine ESC (mESC, E14TG2a, CRL-1821) was purchased from American Type Culture Collection (ATCC). mESCs were seeded and maintained on 0.1% gelatin coated tissue culture flasks (Falcon) in feeder-free conditions in a humidified atmosphere of 95% air and 5% CO₂. The media used was Dulbecco's Modified

Eagle's Medium (DMEM, Gibco, ThermoFisher Scientific) consisting of 15% fetal bovine serum (FBS, PAA Laboratories), 0.1 mM nonessential amino acids (NEAA, Invitrogen, ThermoFisher Scientific), 0.1 mM 2-Mercaptoethanol (2-ME, Sigma-Aldrich) and 1000 units/mL leukemia inhibitory factor (LIF, ESG1107, ESGRO, Millipore). Upon reaching 80% confluence, the cells were trypsinized and used for the subsequent experiments. Only cells with the passage number of 6 to 8 were used for the experiments in this study.

4.2.6 ESC adhesion determination

For the analysis of cell adhesion on the gelatin or gelatin-dopamine coated PDMS surfaces, phase contrast images of adhered cells on the surface were captured and their number were evaluated. Cells that adhered on the surface were imaged with Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus). Cell numbers were assessed through DNA content quantification as previously described with modification [226, 254]. Briefly, mESCs were seeded at a density of 1.0×10^5 cells/well onto the gelatin or gelatin-dopamine coated PDMS surfaces in a 12-well plate and incubated overnight in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The wells were then rinsed twice with 1×PBS to remove the non-adherent cells and the adherent cells were frozen at -80 °C. The frozen cells were then thawed and lysed with 0.2% (v/v in DI water) Triton X-100 (USB, USA) for 15 min at room temperature. Following this, 100 µL of 0.2 µg/mL

bisBenzimide Hoechst 33258 (DNA Quantitation kit, Fluorescence Assay, Sigma-Aldrich) was mixed with the equal volume of the cell lysis and the fluorescence intensity were measured with microplate readers (Infinite 200, Tecan, Austria, 340 nm for excitation and 465 nm for emission). The readings of each group were then normalized against the fluorescence intensity of the positive control group and expressed in percentage. Six replicates (n=6) from each group were measured.

4.2.7 ESC viability and proliferation assay

Following attachment onto the coated PDMS surfaces, mESCs were cultured over a period of 7 days and tested for viability and proliferation on day 1, day 3 and day 7. LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, ThermoFisher Scientific) was used to determine cell viability. mESCs were seeded at a density of 5.0×10^4 cells/well onto gelatin or gelatin-dopamine coated PDMS surfaces in a 24-well plate and incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C with culture medium changed daily. At the designated time points, the culture medium was removed, and the cells were incubated with 1×PBS containing calcein acetomethoxy and ethidium homodimer-1 for 30 minutes at 37 °C in a humidified, 5% CO₂ atmosphere, followed by observation and imaging with an Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus).

Cell proliferation reagent WST-1 (Roche, Singapore) was used for the quantification of cell proliferation. At the designated time points, the culture medium

was removed, and the cells were incubated with 500 μ L culture medium supplemented with 50 μ L of WST-1 reagent for 3 hours in a humidified, 5% CO₂ atmosphere at 37 °C. The supernatant was collected and its absorbance at 450 nm and 620 nm was measured respectively with the Multiskan Spectrum Microplate Photometers (ThermoScientific, Finland). Three replicates (n=3) from each group were assessed.

4.2.8 EB formation and cardiac differentiation

Upon reaching 80% confluence, mESCs were dissociated into single cells with 0.25% trypsin (Gibco, ThermoScientific, Singapore). To initiate embryoid body (EB) formation, cell suspension droplets (20 μ L of differentiation medium containing 1000 cells) were added onto the lid of the petri dish. The differentiation medium was composed of Iscove's modified Dulbecco's medium (Gibco, ThermoScientific) supplemented with 15% FBS, 0.1 mM NEAA and 0.1 mM 2-ME. 48 hours later, the EBs were transferred into a non-adherent petri dish and cultured in suspension conditions for another 3 days. On day 5 days, EBs were seeded onto the gelatin or gelatin-dopamine coated PDMS surfaces in a 24-well plate and incubated in a humidified atmosphere of 95% air and 5% CO₂ at with the differentiation medium changed every other day. EBs were also seeded onto the 0.1% gelatin coated tissue culture 24-well plates as positive control. On day 7, day 10 and day 14, EBs were imaged with Inverted Fluorescence Microscope (IX71, Olympus) equipped with

DP71 camera (Olympus). EB spread-out area was calculated from the captured images with the ImageJ software. EB spread-out area was calculated as percentage of cell-covered areas against the whole imaged areas. Six images from each group (n=6) at the designated time points were analyzed.

4.2.9 Gene expression analysis

Gene expression analysis was performed with quantitative real-time polymerase chain reaction (qPCR). mESCs (on day 1, day3 and day 7) or EBs (on day 7, day 10 and day 14) were dissolved in TRIzol (Gibco, ThermoScientific) for extraction of RNA, which was then converted into cDNA via reverse transcription prior to gene expression evaluation through qPCR. All the reverse transcription reagents used were obtained from Promega (USA). Subsequently, qPCR was done with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Singapore) and CFX Connect Real-Time System (Bio-Rad, Singapore). **Table 4.1** listed the primers (AIT Biotech, Singapore) used in this experiment. Gene markers analyzed in this study include OCT4 and Nanog for ESC pluripotency maintenance; Brachyury (T) for EB mesoderm commitment; PDGFR α and Flk-1 for EB cardiac mesoderm induction; cardiac troponin T (cTnT) for EB cardiomyocyte differentiation. For analysis, expression of target gene markers was first normalized to housekeeping gene β -actin in the corresponding sample. Then the relative target gene expression was further normalized to its expression in positive control group on day 1 (for ESC pluripotency

analysis) or day 7 (for EB myocardial differentiation analysis). Three replicates of samples (n=3) at the designated time points in each group were analyzed for the expression of the target genes.

Table 4.1 qRT-PCR primer sequences for mouse gene markers: forward (F) and reverse (R)

Gene	Accession Number	Primer sequence (both 5'-3')	Annealing temperature (°C)	Product size (bp)
OCT4	NM_013633.3	F: AAAGCAACTCAGAGGGAACCTCCT R: TAGCTCCTTCTGCAGGGCTTTCAT	58	122
Nanog	AF507043.1	F: TGGTGTCTTGCTCTTTCTGTGGGA R: ACACTCATGTCAGTGTGATGGCGA	58	180
Brachyury (T)	NM_009309.2	F: TCTGGTCTGTGAGCAATGGTA R: CCGTGTCACTACTGGCTGTCA	58	210
PDGFR α	NM_011058.2	F: CCTCGTGCTTGGTCGGATT R: TAACTTCTTGTGCTCTCGTCTG	58	384
Flk-1	NM_010612.2	F: GCAGGACCAAGGCGACTAT R: CCGTAGGACAATGACAAGAAGG	58	460
Cardiac troponin T (cTnT)	NM_011619.3	F: GCGGAAGAGTGGGAAGAGACA R: CCACAGCTCCTTGGCCTTCT	58	127
β -actin	NM007393.5	F: TCATGAAGTGTGACGTTGACATCCGT R: CCTAGAAGCATTGCGGTGCACGATG	58	285

4.2.10 Immunohistochemistry staining

mESCs (on day 7) or EBs (on day 14) cultured on the coated PDMS surfaces were fixed using 4% paraformaldehyde (w/v in 1×PBS, Sigma-Aldrich) at room temperature for 30 minutes and washed with 1×PBS prior to immunostaining. For pluripotency evaluation, samples were incubated with Nanog primary antibody (rabbit polyclonal IgG, sc-33760, Santa Cruz Biotechnology) or OCT4 primary antibody (goat polyclonal IgG, sc-8629, Santa Cruz Biotechnology) overnight at 4 °C. After

that, samples were washed with 1×PBS and incubated with Goat anti-Rabbit IgG (H+L) Secondary Antibody (Alexa Fluor 546, A-11010, Invitrogen, ThermoScientific) or Rabbit anti-Goat IgG (H+L) Secondary Antibody (Alexa Fluor 546, A-21085, Invitrogen, ThermoScientific) at room temperature in the dark for 1 hour. For myocardial differentiation evaluation, samples were incubated with cTnT primary antibody (goat polyclonal IgG, sc-8121, Santa Cruz Biotechnology) overnight at 4 °C. After that, samples were washed with 1×PBS and incubated with Rabbit anti-Goat IgG (H+L) Secondary Antibody (Alexa Fluor 488, A11078, Invitrogen, ThermoScientific) at room temperature in the dark for 1 h. All antibodies were diluted at 1:200 in 10% horse serum (v/v in 1×PBS, Gibco, ThermoScientific) and 0.4% Triton X-100 (v/v in 1×PBS). Nuclei were counterstained by 0.1% 4', 6-diamidino-2-phenylindole (v/v in 1×PBS, DAPI, Sigma-Aldrich). Stained samples were imaged with Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus). Fluorescent intensity of one fluorescent image was quantified with ImageJ. For each group, at least 6 images were captured and analyzed.

4.2.11 Flow cytometry analysis

On day 14, EBs on the coated PDMS surfaces were dissociated into single cells using 0.25% trypsin and fixed in 4% paraformaldehyde (w/v in 1×PBS) at room temperature for 20 minutes. After washing with 1×PBS, 5.0×10^5 cells from each group were incubated with cTnT primary antibody overnight at 4 °C. Subsequently,

samples were incubated with Rabbit anti-Goat IgG (H+L) Secondary Antibody at room temperature in the dark for 30 minutes, following washing with 1×PBS. Antibodies were diluted at 1:200 in 0.5% bovine serum albumin (w/v in 1×PBS, BSA, Sigma-Aldrich) and 1% Triton X-100 (v/v in 1×PBS). Prior to flow cytometry analysis, cells were washed and re-suspended in 1×PBS. Flow cytometry was performed on LSRFortessa X-20 (BD Biosciences) and data were collected and analyzed with the BD FACSDiva (BD Biosciences) and FlowJo (Tree Star) software respectively.

4.2.12 Statistical Analysis

All results are presented as mean $\pm$ SD. One-way ANOVA was used for comparisons assuming equal variances followed by least significant difference (LSD) test, unless otherwise stated. $p < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 Gelatin-dopamine synthesis

Dopamine is a catechol-containing molecule with strong adhesive capacity [242, 250]. In order to enhance the adhesion property of gelatin, dopamine was grafted onto gelatin backbone via EDC/NHS chemistry (**Fig. 4.2a**). To verify the successful conjugation of dopamine onto gelatin, the ^1H NMR spectrum of gelatin-dopamine was

analyzed and compared with that of gelatin alone. According to **Fig. 4.2b**, a peak at δ 2.81 ppm was observed in the spectrum of gelatin-dopamine, which corresponded to the protons of methylene group proximal to the phenyl ring in dopamine. However, there was no such peak at the same position of the gelatin spectrum. This result thus confirmed the successful synthesis of gelatin-dopamine in this study.

Fig. 4.2 Synthesis and characterization of gelatin-dopamine. (a) Gelatin-dopamine synthesis. ¹H NMR spectrum of gelatin-dopamine (b) and gelatin (c).

4.3.2 Properties of gelatin-dopamine coated PDMS surfaces

To assess the effect of gelatin-dopamine coating on PDMS surface properties, 0.1% and 1% gelatin-dopamine solution were coated on the PDMS substrates and their surface were characterized. The wettability of PDMS surfaces was determined

by the measurement of water droplet contact angle (**Fig. 4.3a**). The native PDMS surface was hydrophobic with contact angle around $120.11 \pm 2.45^\circ$. Coating PDMS surfaces with 0.1% unmodified gelatin only slightly decreased the hydrophobicity, with a contact angle of $107.24 \pm 1.23^\circ$. In contrast, 0.1% gelatin-dopamine coating decreased the contact angle to $62.90 \pm 4.19^\circ$ and increasing the concentration of gelatin-dopamine to 1% further decreased the contact angle to $8.42 \pm 0.90^\circ$. These results demonstrated that dopamine-gelatin coating could effectively decrease the hydrophobicity of PDMS surfaces.

The roughness and nanotopography of gelatin-dopamine coated PDMS surfaces were also evaluated with AFM. According to **Fig. 4.3b**, gelatin coating alone resulted in the increase of PDMS surface roughness, with roughness 0.84 ± 0.05 nm on pure PDMS surface and 3.42 ± 0.09 nm on 0.1% gelatin coated surface. Meanwhile, PDMS surface coated with 0.1% (4.39 ± 0.36 nm) and 1% gelatin-dopamine (3.77 ± 0.23 nm) showed a rougher surface than 0.1% gelatin coating. **Fig. 4.3c-f** also showed different nanotopography on gelatin-dopamine coated PDMS surface as compared to 0.1% gelatin coated PDMS surface.

Fig. 4.3 Properties of the gelatin-dopamine coated PDMS surfaces. (a) Water contact angle on the gelatin or gelatin-dopamine coated PDMS surfaces. (b) Roughness of the gelatin or gelatin-dopamine coated PDMS surfaces. (c-f) Nanotopography on the gelatin or gelatin-dopamine coated PDMS surfaces. The data are presented as the mean $\pm$ standard deviation (n=6). * indicates $p < 0.05$; ** indicates $p < 0.01$.

4.3.3 ESC adhesion and protein adsorption on gelatin-dopamine coated PDMS surfaces

As tissue culture well plates coated with 0.1% gelatin were extensively used for feeder cell-independent ESC culture [255], we used such surface as positive control in this study. According to **Fig. 4.4a-d**, adhered ESCs on gelatin-dopamine coated PDMS surface was much higher than ESCs on gelatin coated PDMS surface and almost comparable to ESCs in positive control group. The attached ESCs in 0.1% G group was

only $10.85 \pm 0.61\%$ of control group, while there was 9-fold increase in ESC attachment on 0.1% gelatin-dopamine coated PDMS surface and 6-fold increase on 1% gelatin-dopamine coated PDMS surface (**Fig. 4.4e**).

Since cell adhesion was relied on the protein adsorbed on the PDMS surfaces [256], the protein adsorption on different PDMS surfaces was also determined (**Fig. 4.4f**). The result revealed that the protein adsorption in 0.1% G-D group was $105.17 \pm 7.45\%$ of control group and only $44.88 \pm 2.44\%$ of control group in 0.1% G group, indicating higher adsorption efficiency of gelatin-dopamine on the PDMS surface than gelatin. Interestingly, although 1% gelatin-dopamine coating resulted in much more increased protein adsorption on the PDMS surface ($328.89 \pm 7.82\%$ of control group), the cell adhesion on 1% gelatin-dopamine coated surface was not improved accordingly.

Fig. 4.4 ESC adhesion and protein adsorption on the gelatin-dopamine coated PDMS surfaces. (a-d) ESC adhesion on the gelatin or gelatin-dopamine coated PDMS surfaces. (e) Quantification of

ESC adhesion on the gelatin or gelatin-dopamine coated PDMS surfaces. (f) Protein adsorption on the gelatin or gelatin-dopamine coated PDMS surfaces. The data are presented as the mean $\pm$ standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

4.3.4 ESC proliferation on the gelatin-dopamine coated PDMS surfaces

ESC viability and proliferation on gelatin-dopamine coated PDMS surface were subsequently assessed by LIVE/DEAD Viability/Cytotoxicity Kit and WST-1 assay respectively. **Fig. 4.5a-l** showed high cell viability with minimal cell death on gelatin or gelatin-dopamine coated PDMS surfaces. In addition, positive control (**Fig. 4.5d, h and l**), 0.1% G-D (**Fig. 4.5b, f and j**) and 1% G-D (**Fig. 4.5c, g and k**) groups all showed homogenously-distributed and progressively-enlarged ESC colonies. However, ESC colonies on 0.1% gelatin coated PDMS surface was quite small on day 1 and day 3(**Fig. 4.5a and e**) and even detached from PDMS substrate to form clear boundaries (indicated by arrows, **Fig. 4.5i**) on day 7, indicating poor cell attachment on the 0.1% gelatin coated PDMS surface.

WST-1 assay over a period of 7 days revealed a higher ESC proliferation capacity on gelatin-dopamine coated PDMS surface as compared to gelatin coated PDMS surface, even though ESC proliferation in both 0.1% G-D and 1% G-D was lower than positive control group (**Fig. 4.5m**). Meanwhile, cell proliferation rate on the 0.1% gelatin-dopamine coated PDMS surface was higher than 1% gelatin-dopamine coated PDMS surface, suggesting that lower concentration of gelatin-

dopamine is more likely to support robust cell proliferation (**Fig. 4.5m**).

Fig. 4.5 ESC viability and proliferation on the gelatin-dopamine coated PDMS surfaces. (a-l) LIVE/DEAD staining of ESCs on the gelatin or gelatin-dopamine coated PDMS surfaces. Green color indicates live cells and red color indicates dead cells. Arrows indicate the detachment of ESCs from the gelatin coated PDMS surface. (m) WST-1 assay of ESCs on the gelatin or gelatin-

dopamine coated PDMS surface. The data are presented as the mean $\pm$ standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

4.3.5 ESC pluripotency maintenance on the gelatin-dopamine coated PDMS surfaces

Besides cell viability and proliferation, pluripotency maintenance is also critical to ESCs when they are cultured *in vitro*. Pluripotent markers Nanog and OCT4 were analyzed at both gene (qPCR) and protein (immunostaining) expression levels to evaluate ESC pluripotency maintenance on the gelatin-dopamine coated PDMS surfaces. Gene expression study revealed a significantly higher expression of Nanog and OCT4 on the gelatin-dopamine coated PDMS surfaces than gelatin coated PDMS surface from day 1 to day 7 (**Fig. 4.6a and b**). Although Nanog and OCT4 expression on the 1% gelatin-dopamine coated PDMS surface was lower than positive control group, their expression on 0.1% gelatin-dopamine coated PDMS surface was almost comparable to positive control group (**Fig. 4.6a and b**), suggesting that the pluripotency of ESCs is well preserved on the PDMS surface coated with lower concentration of gelatin-dopamine.

The expression of these two genes was further confirmed by the immunostaining of protein Nanog (**Fig. 4.6c-f**) and OCT4 (**Fig. 4.6g-j**). The gelatin-dopamine coated PDMS surfaces showed stronger staining of Nanog and OCT4 (red color) as compared to the gelatin coated PDMS surface. Moreover, **Fig. 4.6 k and l** indicated higher fluorescent intensity of Nanog and OCT4 in gelatin dopamine groups than

gelatin group and the fluorescent intensity in 0.1% G-D group was even higher than positive control group.

Fig. 4.6 ESC pluripotency maintenance on the gelatin-dopamine coated PDMS surfaces. Gene expression of pluripotent markers Nanog (a) and OCT4 (b) of the ESCs on the gelatin or gelatin-

dopamine coated PDMS surfaces from day 1 to day 7. Immunostaining of pluripotent markers Nanog (c-f) and OCT4 (g-j) of the ESCs on the gelatin or gelatin-dopamine coated PDMS surfaces on day 7. Fluorescent intensity of pluripotent markers Nanog (k) and OCT4 (l) of the ESCs on the gelatin or gelatin-dopamine coated PDMS surfaces on day 7. Red color indicates positive staining. The data are presented as the mean $\pm$ standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

4.3.6 EB adhesion on the gelatin-dopamine coated PDMS surfaces

EBs are cell aggregates that formed as a result of ESC proliferation and differentiation in suspension conditions. Following adhesion to a substrate, they will spontaneously differentiate into cardiomyocytes under serum stimulation [255]. When EBs were seeded on the gelatin-dopamine coated PDMS surfaces, they showed a spread-out morphology on day 7 (**Fig. 4.7b and c**), which was similar to the morphology of EBs in control group (**Fig. 4.7d**). In contrast, EBs still maintained a spheroid morphology on the gelatin coated PDMS surface (**Fig. 4.7a**). From day 10 to day 14, EBs in 0.1% G-D, 1% G-D and positive control group still well adhered to the PDMS surface, while EBs in 0.1% G group detached from the PDMS surface (**Fig. 4.7e-l**).

Further quantification of EB spread-out area revealed higher cell-covered areas on the gelatin-dopamine coated PDMS surfaces than the gelatin coated PDMS surface from day 7 to day 14 (**Fig. 4.7m**). In addition, EB spread-out area in 1% G-D group was higher than 0.1% G-D group from day 7 to day 14 and as high as positive control

group from day 10 to day 14 (**Fig. 4.7m**).

Fig. 4.7 EB adhesion on the gelatin-dopamine coated PDMS surfaces. (a-l) Morphology of EBs seeded on the gelatin or gelatin-dopamine coated PDMS surfaces. (m) Quantification of EB spread-out areas on the gelatin or gelatin-dopamine coated PDMS surfaces. The data are presented as the mean $\pm$ standard deviation (n=6). * indicates $p < 0.05$; ** indicates $p < 0.01$.

4.3.7 ESC myocardial differentiation on the gelatin-dopamine coated PDMS surfaces

To investigate the ESC myocardial differentiation on the gelatin-dopamine coated PDMS surfaces, cardiac biomarkers in EBs were evaluated through gene expression study, immunostaining and flow cytometry analysis. During myocardial differentiation, ESCs first commit into mesoderm followed by cardiac mesoderm induction and final myocardial differentiation. Therefore, mesoderm marker (T), cardiac mesoderm markers (PDGFR α and Flk-1), and cardiomyocytes marker (cTnT) were assessed accordingly. T, PDGFR α , Flk-1 and cTnT were highly expressed on the gelatin-dopamine coated PDMS surfaces as compared to the gelatin coated PDMS surface (**Fig. 4.8a-d**). In addition, **Fig. 4.8a-d** also revealed that a higher concentration of gelatin-dopamine coating resulted in the increased gene expression of T, PDGFR α , Flk-1 and cTnT as compared to a lower concentration coating. More interestingly, the 1% gelatin-dopamine coated PDMS surface also showed higher expression of T than positive control group on day 14 and the gene expression of PDGFR α , Flk-1 and cTnT in 1% G-D group was higher than positive control group on day 7 and day 10 as well. These results suggest possibly enhanced ESC myocardial differentiation on the 1% gelatin-dopamine coated PDMS surface.

Immunostaining and flow cytometry analysis of cTnT-positive cells in EBs at the end of differentiation were then performed to confirm the results of gene expression study. Due to the unavailability of samples in 1% G group as a result of the EB detachment on the gelatin coated PDMS surface, only samples in 0.1% G-D, 1% G-D

and positive control groups were analyzed. **Fig. 4.8e-g** showed the stronger staining of cTnT in 1% G-D group and positive control group as compared to 0.1% G-D group. Flow cytometry analysis showed 35.5%, 50.8% and 43.6% cTnT-positive cells in 0.1% G-D, 1% G-D and positive control groups respectively (**Fig. 4.8h-j**). These results indicate that ESC myocardial differentiation efficiency on the 1% gelatin-dopamine coated PDMS surface was improved as compared to positive control group.

Fig. 4.8 EB myocardial differentiation on the gelatin-dopamine coated PDMS surfaces. Gene expression of mesoderm marker T (a), cardiac mesoderm markers PDGFRα (b) and Flk-1 (c), and cardiomyocyte marker cTnT (d) of EBs on the gelatin or gelatin-dopamine coated PDMS surfaces from day 7 to day 14. (e-g) Immunostaining of cardiomyocytes marker cTnT of EBs on the gelatin-dopamine coated PDMS surfaces on day 14. (h-j) Flow cytometry analysis of cTnT-positive cells of EBs on the gelatin-dopamine coated PDMS surfaces on day 14. Green color

indicates positive staining. The data are presented as the mean $\pm$ standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

4.4 Discussion

In the present study, gelatin-dopamine was synthesized by grafting dopamine onto gelatin backbone (confirmed by ^1H NMR, **Fig. 4.2**). Coating the PDMS surface with gelatin-dopamine resulted in the dramatic decrease of PDMS surface hydrophobicity, as well as increased roughness and more complex topography (**Fig. 4.3**). Even though the decrease of PDMS surface hydrophobicity and the increased surface roughness could also be found when using gelatin for coating, the effect was only minimal. These results demonstrated gelatin-dopamine coating could reduce PDMS surface hydrophobicity and increase its roughness more effectively than the conventional gelatin coating. Coating using various extracellular matrix proteins is a widely-used technique to improve the hydrophobicity on PDMS surfaces [222, 257-259]. Even though it is a simple and straightforward methodology, the effects for hydrophobicity reduction on PDMS surfaces is always minimal. The adsorption of unmodified proteins (e.g. gelatin) on material surfaces largely depends on weak non-covalent bonding, including electrostatic, van der Waals and hydrophobic forces [260]. As a result, the strength of gelatin adsorption on the PDMS surface is quite low and the adsorbed gelatin easily detaches from PDMS surface, which leads to very limited surface hydrophobicity improvement on the PDMS surface. On the other hand, because the catechol group in dopamine can generate strong interactions with

substrate surfaces via both non-covalent and covalent bonding formation [250], gelatin-dopamine possesses superior adhesive capacity and is able to attach strongly on the PDMS surface, thereby increasing its adsorption on the PDMS surface and reducing PDMS surface hydrophobicity significantly, especially when coating the PDMS surface with the higher concentration of gelatin-dopamine (1% gelatin-dopamine, **Fig. 4.3**).

As coating material, gelatin dopamine proved to be not cytotoxic to cultured ESCs in this study. Live-dead staining showed minimal cell death on gelatin dopamine coated PDMS substrates (**Fig. 4.5a-l**). Dopamine has been reported to coat methacrylate-based material and show improved cell interactivities [261]. Dopamine coated on PDMS is also not toxic to cultured bone marrow stromal cells [221]. Meanwhile, in our previous studies, we have showed that gelatin dopamine, as one component of tissue adhesive, is biocompatible both in vitro and in vivo [242].

Compared with gelatin coating, gelatin-dopamine coating greatly improved the ESC adhesion and proliferation on the PDMS surface (**Fig. 4.4 and 4.5**). A previous study reported that cell adhesion and proliferation was mainly determined by surface wettability, with moderate wettability supporting cell adhesion and proliferation best [262]. As gelatin-dopamine coating decreased PDMS surface hydrophobicity (**Fig. 4.3a**) and increased protein adsorption (**Fig. 4.4f**), the ESC adhesion and proliferation on the PDMS surface were enhanced. Although 1% gelatin-dopamine coating resulted in the more hydrophilic surface and the higher protein adsorption on the PDMS surface as compared to 0.1% gelatin-dopamine and positive control group (**Fig. 4.3a**

and 4.4f), however, the higher concentration of gelatin-dopamine coating showed the lower ESC adhesion on the PDMS surface (**Fig. 4.4e**). Gelatin-dopamine is synthesized based on gelatin, which still retains natural cell binding motifs, such as Arg-Gly-Asp (RGD) [263, 264]. High RGD concentrations was reported to inhibit MSC attachment and eliminate MSC spreading [265]. As such, too much RGD in the higher concentration of gelatin-dopamine may decrease the ESC adhesion on the PDMS surface.

Meanwhile, due to different stiffness of the PDMS substrate and the tissue culture polystyrene, ESC proliferation on the gelatin-dopamine coated PDMS substrate were suboptimal as compared to positive control group. As cell behavior can be affected by substrate stiffness [35, 112, 266, 267], the stiffer substrate (e.g. tissue culture polystyrene) may be responsible for the higher cell proliferation. However, the stiffness of tissue culture polystyrene cannot be changed easily [255, 268]. On the contrary, the stiffness of PDMS substrate is adjustable and wide-range [112, 259]. Therefore, the optimal culture stiffness for ESCs can be further explored on the PDMS-based substrates.

In addition, the ESC pluripotency maintenance and myocardial differentiation on gelatin-dopamine coated PDMS were also greatly improved as compared to gelatin coated PDMS surface (**Fig. 4.6 and 4.8**). Interestingly, different concentrations of gelatin-dopamine coating enhanced ESC pluripotency maintenance and cardiac differentiation to different degrees, with the lower concentration coating more favorable for ESC pluripotent maintenance (**Fig. 4.6**) while the higher concentration

coating more likely to support ESC myocardial differentiation (**Fig. 4.8**). Previous study pointed out that binding to RGD was prerequisite for ESC differentiation, which could be inhibited by antibodies against RGD [269]. As ESCs on the PDMS surface coated with the higher concentration of gelatin-dopamine are exposed to more RGD and have higher opportunity to bind them, their pluripotency is decreased and accordingly myocardial differentiation potential is enhanced. More importantly, the expression of cardiac mesoderm and cardiomyocyte markers on the 1% gelatin-dopamine coated PDMS surface was even higher than positive control from day 7 to day 10 (**Fig. 4.8b, c and d**) as well as the percentage of cTnT-positive cells (**Fig. 4.8i and j**). These results are in accordance with previous study showing that differentiation of human ESCs was sensitive to substrate mechanics at early stages of cardiac differentiation [236]. Therefore, the PDMS substrate coated with 1% gelatin-dopamine may be a superior platform for ESC myocardial differentiation than gelatin coated tissue culture polystyrene.

4.5 Summary

In this study, gelatin-dopamine was synthesized for PDMS surface coating. Compared with gelatin, gelatin-dopamine coating reduced hydrophobicity and increased protein adsorption on the PDMS surface. In addition, the gelatin-dopamine coated PDMS surface greatly improved ESC adhesion, proliferation and pluripotency maintenance as compared to the gelatin coated surface. ESC-derived EBs also showed

increased adhesion and facilitated myocardial differentiation on the gelatin-dopamine coated PDMS surface. These results demonstrated that gelatin-dopamine coating could effectively improve PDMS surface properties for the long-term ESC culture and enhanced ESC myocardial differentiation.

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Chapter 5: Optimized PDMS Microfluidic Biochips for ESC Pluripotency Maintenance and Cardiac Differentiation

5.1 Introduction

Myocardocyte derived from pluripotent stem cells, such as induced pluripotent stem cell (iPSCs) and embryonic stem cells (ESCs), is a promising cell source for cardiac tissue engineering [162, 231]. Combined with microfluidic technologies, heart-on-a-chip is very likely to be realized and works as a drug screen platform in the future [216, 218, 220]. Polydimethylsiloxane (PDMS) silicone elastomer is a widely-used biomaterial for investigation of cell-substrate interaction and biochip fabrication [215, 222, 223, 229, 230, 257-259, 270-272], due to their adjustable stiffness and surface properties, ease of molding, optical transparency, gas permeability, nontoxicity and low manufacturing costs [273, 274]. However, the surface of PDMS is highly hydrophobic, which inhibits effective cell adhesion on the uncoated PDMS surface [223, 224].

Many researches thus focus on PDMS surface modification to reduce its hydrophobicity [223]. For example, plasma treatment can reduce PDMS surface hydrophobicity, but hydrophobicity will recover after treatment [275, 276]. Coating PDMS surface with extracellular matrix (ECM) protein is simple and commonly-

used, which promotes cell adhesion on the PDMS surface. However, the effect is transient and the cells will detach after initial adhesion due to the dissociation of protein from the PDMS surface [222, 259]. Chemical modification of PDMS to covalently bind protein onto the PDMS surface was also explored [226]. Although this method is very effective in improving mesenchymal stem cell (MSC) adhesion, proliferation and osteogenic differentiation, the multi-step modification procedure is time-consuming and may leave chemical residuals that are toxic to cells. Modification of protein to increase its adhesive capacity and thereby improve its adsorption on PDMS surfaces is another strategy to reduce hydrophobicity of the PDMS surface [277]. However, complex chemical synthesis and product purification are still needed prior to PDMS surface coating. Coating the PDMS surface first with polydopamine (PD) and then with ECM protein has been shown to enhance MSC behaviors on its surface [278]. In addition, as PD and protein are coated separately, this is a flexible and simple strategy with no involvement of chemical synthesis and product purification. Nevertheless, whether such PD together with ECM protein coating can support growth and cardiac differentiation of pluripotent stem cell on the PDMS surface remains unknown.

In addition to surface wettability, cell behaviors on the PDMS substrate can also be affected by other PDMS physical properties, such as substratum stiffness, surface roughness and surface nanotopography. For instance, when the stiffness of PDMS substrate increased, the expression of mesoderm associated gene markers by human ESCs was enhanced [258]. The increased rigidity of the PDMS substrate promoted not only the cardiac differentiation of ESCs but also the maturation of already

specified myocytes [259]. The increased stiffness of PDMS substrate also resulted in improved cell viability [279]. Moreover, cells can change their morphologies in response to different rigidities of PDMS [222, 279, 280]. Meanwhile, surface roughness of the PDMS substrate can influence cell adhesion [270] and cytoskeleton organization of cells can also be altered by nanotopography on the PDMS surface [281]. As different PDMS formulations result in different physical properties of PDMS, which can subsequently influence cell behaviors, PDMS formulations also need to be optimized prior to PDMS-based biochip fabrication.

ESCs have remarkable long-term self-renewal capacity and developmental potential to form derivatives that represent all the three embryonic germ layers even after prolonged culture in vitro [282]. Due to almost indefinite proliferation in vitro and cardiac differentiation ability, ESC-derived myocyte is an ideal cell type for cardiac tissue engineering [234, 283-285]. However, the optimal conditions for the ESC pluripotency maintenance following long-term expansion [286-288] and cardiac differentiation [236, 240, 266, 289] in vitro are still under investigation. Due to tunable stiffness and surface features, PDMS provide a platform where ESC culture and cardiac differentiation can be studied on substrates with various stiffness and surface properties.

In present study, the effect of PD in combination with ECM protein gelatin coating on ESC adhesion, pluripotency maintenance and cardiac differentiation was first investigated, followed by optimal PDMS formulation determination. As microfluidic devices can substantially scale down the in vitro culture system and precisely control

the culture microenvironments within the microfluidic chambers [215-218, 220], biochips based on the optimized PDMS substrates were finally fabricated for ESC pluripotency maintenance and cardiac differentiation (**Fig. 5.1**).

Fig. 5.1 Schematic illustration of (a) coating PDMS substrate with PD and gelatin (b) ESCs or EB seeding (c) PDMS formulation optimization and (d) PDMS-based biochip fabrication.

5.2 Methods

5.2.1 PDMS substrate preparation

PDMS substrates were prepared by mixing silicone elastomer base to curing agent (SYLGARD, Dow Corning, USA), followed by casting PDMS onto a flat polystyrene dish or well plate. The PDMS was then degassed in a vacuum oven for 60 minutes to remove air bubbles and cured at 70 °C for 120 minutes. Four different PDMS substrates were prepared in this study by mixing silicone elastomer base to curing agent in a weight ratio of 5:1, 10:1, 20:1 or 40:1.

5.2.2 PDMS surface modification

The prepared PDMS substrate was surface-modified by immersion in 0.01% (w/v in 1M Tris-HCl buffer, pH 8.5) dopamine solution (Sigma) for 1 or 24 hours. PDMS surface was then washed twice with deionized (DI) water and further immersed in 0.1% (w/v in DI water) gelatin solution (Sigma) overnight at 37 °C for gelatin coating. Following this, the gelatin solution was removed, and the surface of samples were washed twice with DI water. For unmodified PDMS group, the surface was neither treated with dopamine solution nor gelatin solution, which was used as a negative control. For PDMS+G group, the surface was treated with gelatin solution alone. For PD1h or PD 24h group, the surface was treated with dopamine solution for 1 hour or 24 hours but not with gelatin solution. For PD1h+G or PD 24h+G group, the surface was first treated with dopamine solution and then with gelatin solution. Prior to any cell culture experiment, different modified PDMS substrates were sterilized by exposure to ultraviolet (UV) for 30 minutes.

5.2.3 PDMS substrate characterization

Substratum stiffness and surface hydrophilicity, protein adhesion, roughness and nanotopography were characterized for all the modified/unmodified PDMS substrates. The stiffness of PDMS substrates was determined by Tensile Meter (Instron, Model 5543) equipped with a 100 N load cell. The tests were performed at a tensile rate of 0.5 mm/minute. The recorded stress-strain curves were used to calculate elastic

modulus of PDMS substrates. Five samples (n=5) of each group were tested.

Surface hydrophilicity was assessed by measuring surface water contact angles via FTA200 Dynamic Contact Angle Analyzer (First Ten Angstroms, VA, USA). Briefly, 5 μ L DI water was dropped onto the surface of PDMS substrates, and the shape of the water droplet was captured with a high-resolution camera. The contact angles of the water droplets on the PDMS surface were then measured with the Drop Shape Analysis software. Five water droplets on different spots (n=5) of the PDMS surface were tested for each group.

The amount of gelatin absorbed on the PDMS surface was determined by a micro-BCA Protein Assay Kit (ThermoScientific, Singapore). After immersion in gelatin solution overnight at 37 °C, the PDMS surface was washed twice with DI water to remove loosely bound or non-adherent protein molecules and then incubated in the Micro BCA working reagent at 37 °C for 2 hours. Following this, the absorbance of the working solution was measured at a wavelength of 562 nm with Multiskan Spectrum Microplate Photometers (ThermoScientific, Finland). The protein absorbed on the PDMS substrates was then calculated from the absorbance value and normalized against the absorbed protein on the 5:1 PDMS surface. Six samples (n=6) of each group were measured.

The roughness and nanotopography of the PDMS surface were measured by MFP-3D Atomic Force Microscopy (AFM, Asylum Research, CA, USA) in a tapping mode. The silicon AFM sensor (POINTPROBE, NCST-10, NANO WORLD) was 2.8 μ m in thickness, 150 μ m in length, and 27 μ m in width with 160 kHz resonance

frequency and 7.4 N/m spring constant. Five random areas ($20 \times 20 \mu\text{m}$ in size, $n=5$) on the PDMS surface were scanned for each sample.

5.2.4 Microfluidic chip fabrication

AUTOCAD 2012 was used to design photomasks of chips and the designs were printed on high-resolution and transparent films (CAD/Art Services Inc., Oregon, US), which were used to produce masters on a 3-inch silicon wafer (LATCH, Singapore) with photolithography techniques. Briefly, SU-8 2150 (MICROCHEM, USA) was spin-coated at 1000 rpm to create an evenly-spread film on silicon wafers, followed by soft bake at 95 °C for 2 hours. After that, the SU-8 film was covered with the photomasks and exposed to UV light at a dose of 100 mJ/cm², prior to post exposure bake at 95 °C for 30 minutes. Then, the UV-exposed SU-8 film was immersed in SU-8 Developer (MICROCHEM, USA) for 1 hour with mild agitation and dried with nitrogen gas. Silicone elastomer base and curing agent with optimized combination ratios (5:1 for ESC channel and 40:1 for embryoid body (EB) channel) were poured over the masters, degassed in a vacuum oven for 60 minutes and cured at 70 °C for 120 minutes. Then the solidified PDMS was peeled off from the masters. Following this, the PDMS microfluidic chips were plasma-treated and bonded with PDMS substrate, followed by surface modification first with dopamine solution (1-hour treatment) and then with gelatin solution as described earlier. Before ESC or EB seeding, the surface-modified chips were sterilized by exposure to UV for 30 minutes.

To characterize the channel structure of microfluidic chips, field emission scanning electron microscopy (FESEM, JSM-6700F, JEOL, USA) were performed. Cross-section of microfluidic chips were coated with platinum prior to scanning with FESEM.

5.2.5 Embryonic stem cell (ESC) culture

Murine ESC (mESC, E14TG2a, CRL-1821) was purchased from American Type Culture Collection (ATCC). mESCs were seeded and maintained on 0.1% gelatin coated tissue culture flasks (Falcon) in feeder-free conditions in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The medium used was Dulbecco's Modified Eagle's Medium (DMEM, Gibco, ThermoFisher Scientific) consisting of 15% fetal bovine serum (FBS, PAA Laboratories), 0.1 mM nonessential amino acids (NEAA, Invitrogen, ThermoFisher Scientific), 0.1 mM 2-Mercaptoethanol (2-ME, Sigma-Aldrich) and 1000 units/mL leukemia inhibitory factor (LIF, ESG1107, ESGRO, Millipore). Upon reaching 80% confluence, the cells were trypsinized and used for the subsequent experiments. Only cells with the passage number of 9 to 12 were used for the experiments in this study.

5.2.6 ESC adhesion determination

For the analysis of cell adhesion on PDMS surfaces, adhered cells were imaged

with Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus). Briefly, mESCs were seeded at a density of 1.0×10^5 cells/well onto the PDMS surfaces in a 12-well plate and incubated overnight in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The wells were then rinsed twice with 1×phosphate buffered saline (PBS) to remove the non-adherent cells. To visualize cell nuclei, the adhered cells were stained with 5 µg/ml (w/v in PBS) Hoechst 33342 (ThermoFisher Scientific) for 5 minutes, followed by imaging.

On the other hand, total adherent cells were estimated through modified DNA content quantification as previously described [226, 254]. After washing, the remaining adherent cells were frozen at -80 °C overnight. The frozen cells were then thawed and lysed with 0.2% (v/v in DI water) Triton X-100 (USB, USA) for 15 min at room temperature. Following this, 100 µL of 0.2 µg/mL bisBenzimide Hoechst 33258 (DNA Quantitation kit, Fluorescence Assay, Sigma-Aldrich) was mixed with the equal volume of the cell lysis and the fluorescence intensity were measured with microplate readers (Infinite 200, Tecan, Austria, 340 nm for excitation and 465 nm for emission). The readings of each group were then normalized against the fluorescence intensity of unmodified PDMS group or 5:1 group and were expressed in percentage. Six replicates (n=6) of each group were measured.

5.2.7 ESC viability and proliferation assay

Following attachment onto PDMS surfaces, mESCs were cultured over a period

of 7 days and tested for cellular viability and proliferation on day 1, day 3, day 5 and day 7. LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, ThermoFisher Scientific) was used to determine cellular viability. mESCs were seeded at a density of 5.0×10^4 cells/well onto the PDMS surfaces in a 24-well plate and incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C with culture medium changed daily. At the designated time points, the culture medium was removed, and the cells were incubated in 500 µL of 0.05% (v/v in 1×PBS) calcein acetomethoxy and 0.2% (v/v in 1×PBS) ethidium homodimer-1 for 30 minutes, followed by imaging with an Inverted Fluorescence Microscope equipped with DP71 camera.

Cell proliferation reagent PrestoBlue (Molecular Probes, Invitrogen, ThermoFisher Scientific) was used to evaluate cell proliferation. At the designated time points, cells were incubated with 400 µL of 10% (v/v in culture medium) PrestoBlue at 37 °C for 1 hour in the darkness. The fluorescence intensity at an emission wavelength of 612 nm (excited by 540nm wavelength) was measured with a microplate reader (Infinite F200, TECAN, Austria). For each group at the specific time point, six replicates (n=6) were tested and cell proliferation was expressed as fold change as compared to the fluorescence intensity of day 1.

5.2.8 Embryoid body (EB) formation and cardiac differentiation

Upon reaching 80% confluence, mESCs were dissociated into single cells with

0.25% trypsin (Gibco, ThermoFisher Scientific). To initiate embryoid body (EB) formation, cell suspension droplets (20 μ L of differentiation medium containing 1000 cells) were added onto the lid of the petri dish. The differentiation medium was composed of Iscove's modified Dulbecco's medium (Gibco, ThermoFisher Scientific) supplemented with 15% FBS, 0.1 mM NEAA and 0.1 mM 2-ME. After 2 days, the EBs were transferred into a non-adherent petri dish and cultured in suspension conditions for another 3 days. On day 5 of differentiation, EBs were seeded onto PDMS substrates in a 24-well plate and incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C with half of the differentiation medium changed every other day. On day 7, day 10 and day 14 of differentiation, EBs were imaged with Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus).

5.2.9 Gene expression analysis

Gene expression analysis was performed with quantitative polymerase chain reaction (qPCR). mESCs (on day 1, day3 and day 7) or EBs (on day 5, day 7, day 10 and day 14) were dissolved in TRIzol (Gibco, ThermoFisher Scientific) for extraction of RNA, which was then converted into cDNA via reverse transcription prior to gene expression quantification through qPCR. All the reverse transcription reagents used were obtained from Promega (USA). Subsequently, qPCR was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Singapore) and generated signals were detected with CFX Connect Real-Time System (Bio-Rad, Singapore).

Table 5.1 listed the primers (AIT Biotech, Singapore) used in this experiment. Gene markers analyzed in this study include OCT4 and Nanog for ESC pluripotency; Actinin alpha 2 (Actn 2), myosin heavy chain 7 (Myh 7), myosin light chain 2 (Myl 2) and cardiac troponin T (cTnT) for cardiac differentiation; Integrin subunits ($\alpha 5$, $\alpha 8$, $\beta 1$ and $\beta 8$) for mechanism under ESC-PDMS surface interactions. The gene expressions were calculated using the comparative $2^{-\Delta\Delta C_T}$ method. For analysis, the expression of target genes was first normalized to housekeeping gene β -actin in the corresponding sample. Then the relative target gene expression was further normalized to its expression in 5:1 group on day 1 (for ESC pluripotency analysis) or EBs on day 5 (for EB myocardial differentiation analysis). Three replicates of samples (n=3) at the specific time point in each group were analyzed for the expression of the target genes.

Table 5.1 qPCR primer sequences for mouse gene markers: forward (F) and reverse (R).

Gene	Accession Number	Primer sequence (both 5'-3')	Annealing temperature (°C)	Product size (bp)
OCT4	NM_013633.3	F: AAAGCAACTCAGAGGGAACCTCCT R: TAGCTCCTTCTGCAGGGCTTTCAT	58	122
Nanog	AF507043.1	F: TGGTGTCTTGCTCTTTCTGTGGGA R: ACACTCATGTCAGTGTGATGGCGA	58	180
Actn 2	NM_033268.4	F: GTCAAACTCCCAAACCCGA R: CTCCAACAGCTCACTCGCTA	58	183
Myh 7	NM_080728.2	F: TGCCAATGACGACCTGAAGGAGAA R: TCTTCTGGTTGATGAGGCTGGTGT	58	197
Myl 2	NM_010861.3	F: AGATGCTGACCACACAAGCAGAGA R: TCCGTGGGTAATGATGTGGACCAA	58	125
cTnT	NM_011619.3	F: GCGGAAGAGTGGGAAGAGACA R: CCACAGCTCCTTGGCCTTCT	58	127
Integrin α 5	NM_010577.4	F: CAACCCAGAGGAGCGCAGTT R: TAAGGGTGGCCTGCTTGGAA	58	194
Integrin α 8	NM_001001309.3	F: CGAGGTGCAGTTAGATTTCCTGAAG R: TGTAGTTCAGGCTGATGTTGATTGG	58	200
Integrin β 1	NM_010578.2	F: ATGTAGCAGGCGTGTTGC R: TGCTGTGAGCTTGGTGTTC	58	244
Integrin β 8	NM_177290.3	F: ACAGACCAGACATCACATCTTGCTC R: AATGAAACTGCTTTCCTTGAAGTGC	58	199
β -actin	NM007393.5	F: TCATGAAGTGTGACGTTGACATCCGT R: CCTAGAAGCATTGCGGTGCACGATG	58	285

5.2.10 Immunofluorescent staining

mESCs (on day 7) or EBs (on day 14) cultured on the PDMS surfaces were fixed in 4% (w/v in 1×PBS) paraformaldehyde (PFA, Sigma-Aldrich) for 30 minutes, followed by treatment with 0.1% (v/v in 1×PBS) Triton X-100 (USB, USA) for 10 minutes and subsequent block with 1% (v/v in 1×PBS) bovine serum albumin (BSA, Sigma) for 30 minutes. For ESC pluripotency evaluation, samples were incubated

with Nanog primary antibody (rabbit polyclonal IgG, sc-33760, Santa Cruz Biotechnology) or OCT4 primary antibody (goat polyclonal IgG, sc-8629, Santa Cruz Biotechnology) at room temperature for 1 hour. After that, samples were washed with 1×PBS and incubated with Goat anti-Rabbit IgG (H+L) Secondary Antibody (Alexa Fluor 546, A-11010, Invitrogen, ThermoFisher Scientific) or Rabbit anti-Goat IgG (H+L) Secondary Antibody (Alexa Fluor 546, A-21085, Invitrogen, ThermoFisher Scientific) at room temperature in the darkness for 30 minutes. For ESC cardiac differentiation investigation, samples were incubated with cTnT primary antibody (goat polyclonal IgG, sc-8121, Santa Cruz Biotechnology) or Myl 2 primary antibody (goat polyclonal IgG, sc-34490, Santa Cruz Biotechnology) at room temperature for 1 hour. After that, samples were washed with 1×PBS and incubated with Rabbit anti-Goat IgG (H+L) Secondary Antibody (Alexa Fluor 488, A11078, Invitrogen, ThermoFisher Scientific) at room temperature in the darkness for 30 minutes. For F-actin staining, samples were incubated with phalloidin (Alexa Fluor 546, A22283, Molecular Probes, Invitrogen, ThermoFisher Scientific) at room temperature in the darkness for 30 minutes. All antibodies were diluted at 1:100 in 1% BSA and nuclei were counterstained by 0.1% (v/v in 1×PBS) 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Stained samples were imaged with Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus). Fluorescent intensity or fluorescence positive area was quantified with the software ImageJ. At least 6 images (n=6) were analyzed for each group.

5.2.11 Statistical analysis

All results are presented as mean $\pm$ SD. One-way ANOVA was used for comparisons assuming equal variances followed by least significant difference (LSD) test, unless otherwise stated. $p < 0.05$ was considered statistically significant.

5.3 Results

5.3.1 Improved ESC adhesion and proliferation on PDMS surfaces coated with both PD and gelatin

PDMS is a widely-used biomaterial for microfluidic device fabrication. However, the highly-hydrophobic surface of unmodified PDMS greatly limits cell adhesion and growth on its surface. Coating PDMS surface with PD alone or in combination with collagen has been shown to support long-term and stable MSC culture on its surface [278]. Nonetheless, whether such coating strategy can improve ESC adhesion and proliferation on PDMS surfaces remains unknown. To address this issue, PD or PD in combination with gelatin was coated on PDMS surfaces and ESC adhesion and proliferation were evaluated. Gelatin coating alone showed the least effect with a 4-fold increase in cell adhesion as compared to the uncoated PDMS surface. On the other hand, PD coating for 1 hour or 24 hours resulted in a higher cell adhesion, which increased by 13-fold and 17-fold respectively as compared to the uncoated PDMS surface. Coating gelatin on top of PD coating further improved the efficiency

of cell adherence with an 18-fold increase in cell adhesion (**Fig. 5.2a and b**).

ESC viability and proliferation on the different coated PDMS surfaces were also evaluated in this study. ESCs on all PDMS surface were viable with minimal death from day 1 to day 7, which proves that PD coated on the PDMS surfaces is not cytotoxic to ESCs. However, the PDMS surfaces coated with either gelatin or PD alone had few ESCs. In contrast, ESCs on the PDMS surfaces coated with PD in combination with gelatin were well-attached and quite confluent (**Fig. 5.2c**). PrestoBlue test also showed increased cell population in both PD1h+G and PD24h+G groups, while there was a decrease of cell population in PDMS, PDMS+G, PD1h and PD24 groups (**Fig. 5.2d**).

Cell attachment on different coated PDMS surfaces after 7 days of in vitro culture was also evaluated via F-actin staining (**Fig. 5.2e**). Cell aggregation could be observed on the PDMS surface coated with gelatin alone, which indicates poor cell adhesion as well as high possibility for cell to dissociate from the surface. Even though cell was well-adhered on PDMS surfaces coated with PD for 1 hour or 24 hours, cells did not reach confluence after 7 days of in vitro culture. Well-attached and confluent ESC cell sheet was only observed on the PDMS surface with both gelatin and PD coating. Quantification of F-actin positive area also revealed obviously higher percentage in PD1h+G and PD24h+G groups than the other groups (**Fig. 5.2f**).

Fig. 5.2 ESC adhesion and proliferation on the PDMS surface coated with PD and gelatin. (a) ESC adhesion. Blue color indicates cell nuclei. (b) Quantification of ESC adhesion. (c) LIVE/DEAD staining of ESCs on day 7 of in vitro culture. Green color indicates live cells and red color indicates dead cells. (d) PrestoBlue assay for the proliferation of ESCs. (e) F-actin staining of ESCs on day 7 of in vitro culture. Red color indicates F-actin staining; blue color indicates cell nuclei. (f) Quantification of F-actin positive areas of ESCs on day 7 of in vitro culture. The data

are presented as the mean $\pm$ standard deviation (n=6). * indicates $p < 0.05$; ** indicates $p < 0.01$.

5.3.2 Enhanced EB adhesion on the PDMS surface with both PD and gelatin coating

Following ESC adhesion and proliferation determination, EB attachment were also assessed on different coated PDMS surfaces. EBs on both unmodified PDMS surface and PDMS surface coated with gelatin alone exhibited clustered morphologies (**Fig. 5.3a**), suggesting that EBs are not well attached on these surfaces. In contrast, the PD coated PDMS surfaces with or without gelatin coating all supported EB attachment, which showed spread morphologies.

F-actin staining of EBs on day 14 showed similar results (**Fig. 5.3b**). For instance, EBs on the uncoated PDMS surface was aggregated, which may cause them to detach from surface easily. EB on the PDMS surface coated with gelatin was also not well-adhered with clear detachment that could be observed (as indicated by arrow). However, EBs in both PD and PD+G groups showed homogenously-distributed F-actin staining, which suggests improved adhesion of EBs as compared to unmodified PDMS or PDMS coated with gelatin alone. Quantification of F-actin-positive areas showed that coating gelatin on unmodified PDMS or PD-coated PDMS could lead to larger positive areas as compare to PDMS surfaces without gelatin coating, which indicates that gelatin coating on PDMS surfaces may benefit EB attachment (**Fig. 5.3c**).

Fig. 5.3 EB attachment on the PDMS surface coated with PD and gelatin. (a) Phase contrast images of EBs from day 7 to day 14. (b) F-actin staining of EBs on day 7 of in vitro differentiation. Red color indicates F-actin staining; blue color indicates cell nuclei. Arrows indicate the detachment of EBs. (c) Quantification of F-actin positive areas of EBs on day 14 of in vitro differentiation. The data are presented as the mean $\pm$ standard deviation (n=6). * indicates $p < 0.05$; ** indicates $p < 0.01$.

5.3.3 ESC pluripotency and cardiac differentiation on the PDMS surface with both PD and gelatin coating

Next, the influence of PD together with gelatin coating on ESC pluripotency

maintenance and cardiac differentiation were investigated. Pluripotent markers Nanog and OCT4 were intensively stained on the PDMS surfaces coated with PD in combination with gelatin, as shown in immunofluorescent staining (**Fig. 5.4a and c**). Additionally, the fluorescent intensities on the PD and gelatin-coated PDMS surfaces were higher than the PDMS surfaces coated with PD alone (**Fig. 5.4b and d**). These results demonstrate that PD together with gelatin coating greatly enhances the preservation of ESC pluripotency.

Cardiac marker cTnT staining on the unmodified PDMS surface and the PDMS surface coated with gelatin alone was weak and the fluorescent intensity was also not high (**Fig. 5.4e and f**), which suggests that ESC cardiac differentiation was inhibited. On the other hand, EBs on the PDMS surfaces coated with PD and gelatin exhibited a stronger cTnT staining as well as a higher fluorescent intensity than EBs on the PDMS surface coated with PD alone (**Fig. 5.4e and f**). These results prove that a more favorable cardiac differentiation environment can be provided on the PDMS surface coated with PD and gelatin as compared to other PDMS surfaces.

Fig. 5.4 ESC pluripotency maintenance and cardiac differentiation on the PDMS surface coated with PD and gelatin. Immunostaining of pluripotent marker (a) Nanog and (b) its fluorescent intensity. Immunostaining of pluripotent marker (c) OCT4 and (d) its fluorescent intensity. Immunostaining of cardiac marker (e) cTnT and (f) its fluorescent intensity. Red color indicates positive pluripotent marker staining; green color indicates positive cardiac marker staining; blue color indicates cell nuclei. The data are presented as the mean $\pm$ standard deviation (n=6). * indicates $p < 0.05$; ** indicates $p < 0.01$.

5.3.4 Substratum properties of different PDMS substrates

In addition to surface coating, substratum properties such as stiffness, hydrophobicity, affinity to protein, roughness and nanotopography, can also potentially influence cell adhesion, proliferation and differentiation [290]. Substratum properties of PDMS with different base to curing agent ratios (5:1, 10:1, 20:1 and 40:1) were then characterized. Increasing PDMS base resulted in decreased PDMS stiffness, with 5:1 group being stiffest and 40:1 group being softest (**Fig. 5.5a**). Water contact angel on PDMS surfaces coated with PD (1 hour) and gelatin increased with the increase of PDMS base (**Fig. 5.5b**). In contrast, protein adsorption on PDMS surfaces decreased when PDMS base increased (**Fig. 5.5c**). Roughness determined by AFM showed that 10:1 and 20:1 PDMS substrates after coating had the roughest surfaces, while surface on the 5:1 PDMS substrate was smoothest (**Fig. 5.5d**). The nanotopography profiles on the coated surfaces of PDMS with different formulations were also different (**Fig. 5.5e**).

Fig. 5.5 Physical properties of different PDMS substrates. (a) Elastic modulus. (b) Water contact angle. (c) Protein adsorption. (d) Roughness. (e) Nanotopography. The data are presented as the mean $\pm$ standard deviation (n=5). * indicates $p < 0.05$; ** indicates $p < 0.01$.

5.3.5 The effects of different PDMS substrates on ESC/EB adhesion and proliferation

After property characterization of different PDMS substrates, their effects on ESC adhesion and proliferation were investigated. The 40:1 PDMS substrate supported a higher ESC adhesion than the other three PDMS substrates, with a 35% increase of cell adhesion on its surface as compared to 5:1 PDMS substrate (**Fig. 5.6a and b**). Besides, cell proliferation rate on the 40:1 PDMS substrate was also more rapid than the other PDMS substrates (**Fig. 5.6c**). The reduced cell proliferation on all different substrates on day 7 is very likely due to the over-confluence of ESCs. F-actin staining of ESCs after 7 days of in vitro culture showed well-adhered and confluent ESC cell sheet on all different PDMS substrates (**Fig. 5.6d**). F-actin-positive area quantification showed no significant difference between different PDMS substrates (**Fig. 5.6f**). On the other hand, EBs on the 40:1 PDMS substrate showed larger F-actin-positive areas as compared to EBs on the rest of PDMS substrates after 14 days of in vitro differentiation (**Fig. 5.6e and g**).

Fig. 5.6 The effects of different PDMS substrates on ESC/EB adhesion and proliferation. (a) ESC adhesion. Blue color indicates cell nuclei. (b) Quantification of ESC adhesion. (c) PrestoBlue assay for the proliferation of ESCs. F-actin staining of (d) ESCs on day 7 of in vitro culture and (e) EBs on day 14 of in vitro differentiation. Red color indicates F-actin staining; blue color

indicates cell nuclei. Quantification of F-actin-positive areas of (f) ESCs on day 7 of in vitro culture and (g) EBs on day 14 of in vitro differentiation. The data are presented as the mean $\pm$ standard deviation (n=6). * indicates $p < 0.05$; ** indicates $p < 0.01$.

5.3.6 The effects of different PDMS substrates on ESC pluripotency maintenance

To investigate the effects of different PDMS substrates on ESC pluripotency maintenance, pluripotency gene markers Nanog and OCT4 were analyzed. While expressions of Nanog and OCT4 increased with time on all different substrates, the expression on 5:1 PDMS substrate were significantly higher than the rest of PDMS substrates on day 7 (**Fig. 5.7a and b**). These results are further supported by immunostaining of Nanog and OCT4, with a stronger staining observed on the 5:1 PDMS substrate (**Fig. 5.7c and d**). Additionally, fluorescent intensity in 5:1 group was significantly higher than the rest of the groups (**Fig. 5.7e and f**). These results suggest that the 5:1 PDMS substrate strongly supports ESC pluripotency preservation on its surface.

Fig. 5.7 The effects of different PDMS substrates on ESC pluripotency maintenance. Gene expression of pluripotent markers (a) Nanog and (b) OCT4. Immunostaining of pluripotent markers (c) Nanog and (d) OCT4 on day 7 of in vitro culture. Red color indicates positive staining; blue color indicates cell nuclei. Fluorescent intensity of pluripotent markers (e) Nanog and (f) OCT4 on day 7 of in vitro culture. The data are presented as the mean $\pm$ standard deviation (n=6). * indicates $p < 0.05$; ** indicates $p < 0.01$.

5.3.7 The effects of different PDMS substrates on ESC cardiac differentiation

To evaluate ESC cardiac differentiation on the surfaces of different PDMS substrates, four different gene markers Actn 2, Myh 7, Myl 2 and cTnT were analyzed (**Fig. 5.8a-d**). Actn 2, Myh 7 and Myl 2 in all groups were first highly expressed and then expressed at a low level and the highest expressions of genes were observed on the 40:1 PDMS substrate on day 10 of in vitro differentiation. Meanwhile, cardiac marker cTnT showed an increasing trend over the whole culture period and its expression on the 40:1 PDMS substrates was significantly higher than the other PDMS substrates from day 7 to day 14. Immunostaining of both Myl 2 and cTnT further confirmed the result of gene expression study, with stronger staining observed on the 40:1 PDMS substrates (**Fig. 5.8e and f**). Meanwhile, the quantification of fluorescence intensity of both Myl 2 and cTnT showed significantly higher value in 40:1 group than the other three groups (**Fig. 5.8g and h**). These results suggest that cardiac specification of ESCs is strongly promoted on the 40:1 PDMS substrate as compared to the other PDMS substrates.

Fig. 5.8 The effects of different PDMS substrates on ESC cardiac differentiation. Gene expression of cardiac markers (a) Actn 2, (b) Myh 7, (c) Myl 2 and (d) cTnT. Immunostaining of cardiac markers (e) Myl 2 and (f) cTnT on day 14 of in vitro differentiation. Green color indicates positive staining; blue color indicates cell nuclei. Fluorescent intensity of cardiac markers (g) Myl

2 and (h) cTnT on day 14 of in vitro differentiation. The data are presented as the mean $\pm$ standard deviation (n=6). * indicates $p < 0.05$; ** indicates $p < 0.01$.

5.3.8 Mechanism of ESC behaviors on different PDMS substrates

Cell-substrate interaction through transmembrane receptor integrin has a huge influence on ESC fate decision, with inactivation of integrin signaling in favor of ESC self-renewal while over-expression of integrin promoting ESC differentiation [291]. To understand the underlying mechanism of ESC behaviors on different PDMS substrates, we further investigated the expression of integrin subunits through qPCR. When cultured in medium with LIF where ESCs can maintain their pluripotency, ESCs on 5:1 PDMS substrate continuously expressed a low level of integrin $\alpha 5$, $\alpha 8$ and $\beta 1$ as compared to ESCs on other PDMS substrates over the whole culture period (**Fig. 5.9a-c**). On the other hand, under differentiation conditions, EBs on 40:1 PDMS substrate demonstrated significantly higher expressions of integrin $\alpha 8$, $\beta 1$ and $\beta 8$ than EBs on the other PDMS substrates on day 14 of cardiac differentiation (**Fig. 5.9d-f**). These results suggest that the 5:1 PDMS substrate promotes preservation of ESC pluripotency by decreasing expressions of integrin genes, while improved cardiac differentiation of ESCs on the 40:1 PDMS substrate is due to increased expressions of integrin genes.

Fig. 5.9 Gene expressions of integrin subunits by ESCs and EBs cultured on different PDMS substrates. Gene expression of integrin (a) $\alpha 5$, integrin (b) $\alpha 8$ and (c) integrin $\beta 1$ by ESCs; Gene expression of integrin (d) $\alpha 8$, (e) integrin $\beta 1$ and (f) integrin $\beta 8$ by EBs. The data are presented as the mean $\pm$ standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

5.3.9 ESC pluripotency maintenance in ESC microfluidic chips

As pluripotency of ESCs can be well-maintained on 5:1 PDMS substrate, the 5:1 PDMS substrate coated with PD and gelatin was utilized for the development of ESC microfluidic chips. Multiple and single channels were designed respectively to examine their individual performance for ESC culture. Compared with single channels, multiple channels could provide more spacious areas and accommodate

more culture medium (**Fig. 5.10a and b**). ESC attachment and pluripotency maintenance in these two different channels were examined after 7 days of in vitro culture. Multiple channels showed larger F-actin-positive areas as compared to single channels (**Fig. 5.10c and d**). In addition, immunostaining of pluripotent markers Nanog and OCT4, as well as their fluorescent intensity quantification, demonstrated higher staining intensities in multiple channels than single channels (**Fig. 5.10c, e and f**). These results suggest ESC microfluidic chips with multiple channels are superior to single channels in ESC adhesion and pluripotency maintenance.

Fig. 5.10 Biochips for ESC pluripotency maintenance. (a) Macroscopic view. (b) SEM images of cross-section. (c) ESC adhesion and immunostaining of pluripotent markers Nanog and OCT4 on day 7 of in vitro culture. Red color indicates positive staining; blue color indicates cell nuclei. (d) Quantification of F-actin-positive areas. Fluorescent intensity of pluripotent markers (e) Nanog and (f) OCT4 on day 7 of in vitro culture. The data are presented as the mean $\pm$ standard deviation (n=6). * indicates $p < 0.05$; ** indicates $p < 0.01$.

5.3.10 ESC cardiac differentiation in EB microfluidic chips

Due to the improved cardiac differentiation of ESCs on the 40:1 PDMS substrate, EB microfluidic chips were fabricated with the 40:1 PDMS substrate and combinatory coating of PD and gelatin. Like ESC microfluidic chips, two different channels (multiple and single) were designed for EB microfluidic chips, with more spacious areas and culture medium in multiple channels than single channels (**Fig. 5.11a and b**). To assess EB attachment in multiple and single channels, F-actin were stained after 14 days of in vitro cardiac differentiation and the percentage of positive area was quantified, which manifested more confluent cell sheet and a significantly higher percentage of F-actin-positive areas in multiple channels as compared to single channels. Cardiac differentiation of ESCs in multiple channels was also promoted as compared to single channels, as evidenced by the stronger Myl 2 and cTnT staining and their higher fluorescent intensities in multiple channels as compared with single channels (**Fig. 5.11c, e and f**).

Fig. 5.11 Biochips for EB cardiac differentiation. (a) Macroscopic view. (b) SEM images of cross-section. (c) EB adhesion and immunostaining of cardiac markers Myl 2 and cTnT on day 14 of in vitro differentiation. Red color indicates positive F-actin staining; green color indicates positive cardiac marker staining; blue color indicates cell nuclei. (d) Quantification of F-actin-positive areas. Fluorescent intensity of cardiac markers (e) Myl 2 and (f) cTnT on day 14 of in vitro differentiation. The data are presented as the mean $\pm$ standard deviation (n=6). * indicates $p < 0.05$; ** indicates $p < 0.01$.

< 0.01.

5.4 Discussion

In this study, ESCs on PDMS surfaces coated with PD and gelatin showed improved adhesion, proliferation and pluripotency maintenance as compared to the PDMS surfaces coated with gelatin or PD alone (**Fig. 5.2 and Fig. 5.4a-d**). The improved ESC adhesion and proliferation is very likely due to the increased gelatin adsorption on the PDMS surface coated with PD and gelatin. The adhesion of cells on substratum is mediated by specialized transmembrane receptors, such as integrins, which can recognize and bind to specific amino sequences on cell adhesive proteins [26]. As cell adhesive proteins hardly adsorbed on hydrophobic surface [292, 293], high hydrophobicity on unmodified PDMS surface also resists the adsorption of cell adhesive proteins, which results in poor cell adhesion on the PDMS surface and cell dissociation after initial attachment [224, 225]. Gelatin is a commonly-used adhesive protein, which can improve cell adhesion and proliferation [294]. Low cell adhesion and reduced cell proliferation on PDMS surface coated with gelatin alone (**Fig. 5.2a and b**) is probably because gelatin adsorbed on PDMS surface after gelatin coating is too little. PD contains catechol groups, which can attach protein onto PDMS surfaces stably via the formation of non-covalent and covalent bonding [250]. As such, adhesive proteins in culture medium are more easily adsorbed on PD-coated PDMS due to the function of PD [295], which can explain the improved cell adhesion on PDMS substrates by PD coating alone as compared to gelatin coating (**Fig. 5.2b**).

Although PD coating alone increased the initial ESC adhesion on the PDMS surface (**Fig. 5.2a and b**), ESC attachment on the PDMS surface coated with PD alone after 7 days of in vitro culture was still poor (**Fig. 5.2 c-f**). These results indicate that PD coating alone cannot increase adsorption of adhesive proteins that come from culture medium only on PDMS surfaces to the extent that stable and confluent ESC cell sheet can be formed on PDMS surfaces. Additional gelatin coating following PD coating increase the amount of adhesive proteins that can be adsorbed on PDMS surfaces. On the other hand, coated PD help the binding of gelatin onto PDMS surfaces. Therefore, the adsorption of adhesive proteins on PDMS surfaces was greatly improved, which results in not only increased initial ESC adhesion on PDMS substrates but also the formation of stable and confluent ESC cell sheet on PDMS substrate. Rapid cell proliferation is required for ESC pluripotency maintenance [296]. Because of improved ESC adhesion and proliferation, ESCs maintained their pluripotency on the PDMS surfaces coated with PD and gelatin at a higher level than the other PDMS surfaces (**Fig. 5.4a-d**).

EBs are three-dimensional cell aggregates self-assembled by ESCs when they are cultured in suspension conditions [297]. ESCs are commonly differentiated into myocytes in vitro by EB formation and following attachment of the formed EBs onto a substrate [162, 239]. EBs were not well adhered on the unmodified PDMS surface and the PDMS surface coated with gelatin alone, but PD coating alone improved EB adhesion on PDMS surfaces over the whole in vitro culture period (**Fig. 5.3**). In addition to increased adsorption of adhesive proteins from culture medium, the

adhesion capacity of EBs is stronger than undifferentiated ESCs. With the progress of differentiation, EBs express by themselves ECM proteins such as collagen I, collagen IV, fibronectin, laminin and vitronectin [298], which can facilitate EB adhesion on substrates. Therefore, unlike undifferentiated ESCs, EBs could attach and spread well on the PDMS surfaces coated with PD alone. Besides, EB adhesion on substrates is required for bilateral establishment and mesoderm induction, which can facilitate subsequent cardiac differentiation of ESCs [240, 241, 266]. Due to enhanced attachment of EBs, the cardiac differentiation of ESCs on the PDMS surface coated with PD alone was also improved (**Fig. 5.4e and f**). On the other hand, PD-mediated binding significantly increased the adsorption of gelatin on the PDMS surface coated with PD and gelatin, which further improved the adhesion of EBs as well as ESC cardiac differentiation on PDMS surfaces (**Fig. 5.3 and Fig. 5.4e and f**).

In this study, we noticed that the effects of gelatin coating following PD coating for 1 hour on ESC adhesion, proliferation, pluripotency, and cardiac differentiation was nearly the same as gelatin coating following PD coating for 24 hours (**Fig. 5.2-5.4**), although a longer PD coating resulted in a higher gelatin adsorption on the PDMS surface. Considering that PD coating for 1 hour followed by gelatin coating is fast and effective enough in improving ESC behaviors on PDMS surfaces, this coating strategy was adopted in PDMS formation optimization and microfluidic biochip fabrication.

In addition to surface coating, different ratios of PDMS base to curing agents, which resulted in varying profiles of substratum stiffness, surface wettability, protein

adsorption amount, roughness and nantopography (**Fig. 5.5**), also influenced the behaviors of ESCs and EBs on PDMS surfaces (**Fig. 5.6-5.8**). Combinatory effect of these substratum properties has been shown to affect mesenchymal stem cell adhesion and multi-lineage differentiation on PDMS surfaces [274]. In this study, ESC adhesion and proliferation, as well as EB attachment, were improved on the 40:1 PDMS substrate as compared to the other PDMS substrates (**Fig. 5.6**). Cells rely on integrins to bind with ECM proteins for adhesion [256, 299, 300]. The increased expression of integrin subunits $\alpha 5$, $\alpha 8$, and $\beta 1$ in ESCs cultured on the 40:1 PDMS substrate (**Fig. 5.9a-c**) may be responsible for the improved ECS adhesion; meanwhile, integrin subunits $\alpha 8$, $\beta 1$, and $\beta 8$ in EBs were more highly expressed in EBs culture on the 40:1 PDMS (**Fig. 5.9d-f**), which probably results in the enhanced attachment of EBs. In addition, ESC pluripotency preservation was improved on the 5:1 PDMS substrate as compared to the other PDMS substrates (**Fig. 5.7**), while the 40:1 PDMS substrate supported ESC cardiac differentiation more than the other PDMS substrates (**Fig. 5.8**). Integrin also play a critical role in mediating ESC differentiation through extracellular-signal-related kinase (ERK) and phosphatidylinositol-3-OH kinase (PI3K) pathways [256, 301]. For example, blocking the interactions between ECM and integrin inhibits ESC differentiation, whereas the activation of integrin signaling promotes the differentiation of ESCs [291]. Therefore, the low expressions of integrin subunits by ESC on the 5:1 PDMS substrate and the highly expressed integrin subunits in EBs on the 40:1 PDMS substrate may explain the difference in ESC pluripotency maintenance and cardiac differentiation on

different PDMS substrates (**Fig. 5.9**). Previously, a study shows that a rigid PDMS substrate promotes ESC cardiac differentiation as compared to a soft PDMS substrate [259], which contradicts our results. This is probably due to ECM protein coating alone on the PDMS surface in that study cannot stabilize EB adhesion, an intermediate procedure that is critical to ESC cardiac differentiation.

Biochips for ESC pluripotency maintenance and cardiac differentiation are fabricated respectively based on the optimized coating procedure and PDMS formulation. For each biochip, two channels (multiple and single) were designed and evaluated in this study. In multiple channels of ESC biochips, ESCs were well-attached and well maintained their pluripotency. However, ESCs in single channel showed poor cell adhesion and low pluripotency (**Fig. 5.10**). Due to the rapid proliferation rate of ESCs in vitro [302], living space and nutrients are quite critical to the growth of ESCs [303-305]. Compared with single channels, multiple channels can provide spacious areas and accommodate more nutrients, which leads to improved ESC adhesion as well as higher pluripotency in multiple channels. As EBs are ESC aggregates which are much larger than single ESC [306], the channels of EB biochips (**Fig. 5.11a and b**) are wider than the channels of ESC biochips (**Fig. 5.10a and b**). Since multiple channels can supply EBs with higher volume of culture medium than single channels, EB adhesion and cardiac differentiation in multiple channels were much improved as compared to single channels (**Fig. 5.11**). These results highlight the importance of channel design to the performance of biochips [307].

5.5 Summary

In this study, we found PD and gelatin coating greatly improved ESC adhesion, proliferation and cardiac differentiation on PDMS surfaces. In addition, different PDMS substrates exhibited different profiles of substratum properties, which influenced the behaviors of ESCs and EBs on PDMS surfaces. ESC adhesion and proliferation, as well as EB attachment, were improved on the 40:1 PDMS substrate as compared to the other PDMS substrates. Meanwhile, compared with the other PDMS substrates, ESC pluripotency preservation was improved on the 5:1 PDMS substrate, while the 40:1 PDMS substrate encouraged ESC cardiac differentiation. Based on the optimized coating method and PDMS formulation, biochips with two different designs were fabricated and evaluated. Multiple channels could provide spacious areas and accommodate more nutrients, which resulted in improved ESC pluripotency maintenance and cardiac differentiation. The use of this simple surface modification strategy to stabilize the adhesion of ESCs and EB while enhance their pluripotency and differentiation can possibly contribute to the development of a real heart-on-a-chip for high throughput drug screening in the future.

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Chapter 6: Enhancing vascularization of gelatin-based micro-cavitary hydrogel by increasing the density of micro-cavities

6.1 Introduction

Vascularization of engineered vascular tissue constructs plays a key role in the successful tissue construct transplantation since it is essential to guarantee availability of oxygen and other nutrients to the cells in the constructs [308, 309]. Currently, the mainstream strategy to achieve rapid and complete vascularization of engineered tissue constructs is scaffold-mediated pre-vascularization in vitro [283, 308, 310-312].

Hydrogels have exhibited great potential as candidate scaffolds for vascularization [313-316]. For example, matrigel and other natural protein-based hydrogels, such as fibrin hydrogels and collagen hydrogels, are commonly used scaffolds for in vitro vascularization models [317, 318]. These hydrogels can provide permissive environments for endothelial cells (ECs) or endothelial progenitor cells (EPCs) to form vascular networks both in vivo and in vitro [318-320]. In order to improve mechanical property and stability of these natural biomaterials, they are further modified [321, 322]. Methacrylate was used to modify gelatin and such gelatin-methacrylate (Gel-MA) hydrogel showed flexible mechanical properties and supported vascular network formation [263]. In addition, hydrogel can also be micro-patterned to create specific

geometrical shape, which can guide endothelial cells to form cord structures within the hydrogel [323] or improve cardiomyocyte alignment and functions on the hydrogel-based substrate [324]. Recently, a novel hydrogel that can respond to stimulation from environment was developed. Such hydrogel can be formed under hypoxia conditions and has been shown to induce vasculogenesis in vitro and rapid vascularization in vivo [325].

However, vascularization of hydrogel is still constrained by its submicron- or nano-scale polymer networks [326, 327]. When cells are entrapped in hydrogel bulks, they cannot easily free themselves from frameworks of hydrogels to stretch out and migrate, which greatly restrain their viability, proliferation, differentiation and self-organization into functional structures [326]. The effect of macro-pore size on vascularization has been investigated in poly(ethylene glycol) hydrogel, with larger pore size (50-150 μm in diameter) supporting in vitro vascular cell invasion and in vivo vascular tissue invasion more than smaller pore size (25-50 in diameter) [136]. However, the invaded vascular cells or tissues come from the outside of the hydrogel and the influence of macro-pore size on cells encapsulated in gel bulks is still unknown. Macro-porous spongy scaffolds have interconnected macro-pores and can overcome space limitation of hydrogels and thus are widely used for tissue vascularization [328-331]. Nonetheless, unlike hydrogels where encapsulated cells can homogeneously distribute within the whole gel bulks prior to gelation, spongy scaffolds are often pre-fabricated, followed by cell seeding. As such, there might be uneven cell distribution and non-uniform vascularization in the spongy scaffolds [328, 331].

Previously, we have developed a gelatin-based micro-cavitary gel (referred to as Gel-MCG) construct for the delivery of anchorage dependent cells, by using Gel-MA and temperature-cured dissolvable gelatin microspheres (MS) as precursors and porogens respectively [327]. Our initial studies show that the separate micro-cavities in the Gel-MCG construct can enhance its permeability as well as create interfaces around micro-cavities. In particular, the endothelial progenitor outgrowth cells (EPOCs) encapsulated in the gel can grow, spread, and connect with each other along the interfaces within the construct.

The inspiring results achieved in the Gel-MCG construct further motivate us to optimize the density of the interior micro-cavities to improve the vascularization of Gel-MCG constructs. Here, two Gel-MCG constructs with higher cavitory density (1.0 and 2.0 gram MS per mL Gel-MA precursor solution respectively) than the previous fabricated Gel-MCG construct (0.3 gram MS gelatin per mL Gel-MA precursor solution) were prepared and characterized. The effect of cavitory density on Gel-MCG construct vascularization was investigated both in vitro (**Fig. 6.1A**) and in vivo (**Fig. 6.1B**). A porous gelatin spongy construct was also established in parallel as comparison.

Fig. 6.1 (A) Schematic illustration of establishing Gel-MCG constructs in vitro. Two different constructs were fabricated: MS:Gelatin (2:1) and MS:Gelatin (1:1) constructs. (B) Schematic illustration of the in vivo study. After 3 days of in vitro culture, constructs were subcutaneously implanted into the nude mice, and the implants were harvest on day 7 (n=3 for each construct) and day 14 (n=3 for each construct), respectively.

6.2 Methods and materials

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich, and all cell-culture related reagents from Gibco.

6.2.1 Synthesis of gelatin methacrylate (Gel-MA) precursor

Gel-MA was synthesized according to our previous study [327]. Briefly, 11 g of gelatin type A (from porcine skin) was dissolved in 150 mL water under stirring at 40 °C, followed by the addition of 50 mL of dimethylformamide (DMF). 5 g of triethylamine (TEA) and 50 g glycidyl methacrylate (GMA) was added before stirring for 3 days at 40 °C. Then, the Gel-MA precursor was obtained by lyophilizing from the solution that was concentrated for another 3 days using a rotary evaporator and dialyzed against deionized (DI) water. Proton nuclear magnetic resonance (^1H -NMR) spectroscopy (Bruker Avance-300 spectrometer) was used to characterize the degree of methacrylation of Gel-MA.

6.2.2 Fabrication of gelatin-based micro-cavitary gel (Gel-MCG) constructs

Gelatin microspheres (MS) were fabricated and sterilized according to previous protocol with modification [195, 196]. Briefly, double emulsion method (oil/water/oil) was employed to generate gelatin microspheres and these gelatin microspheres were further sieved to obtain spheres from 150 μm to 180 μm in diameter. Then 1000 units/mL penicillin and 1000mg/mL streptomycin solution was utilized to sterile microspheres via overnight immersion following storage in phosphate buffer saline (PBS) at 4 °C.

20% (w/v) Gelatin-MA and 0.1% (w/v) photo-initiator Irgacure 2959 was dissolved

in 1x phosphate-buffered saline (PBS) and employed as precursor solution. Two mixture suspensions were prepared by suspending 1.0 or 2.0 g of MS into 1.0 mL of Gel-MA precursor solution, and they were named as MS:Gel-MA (1.0g/1mL) and MS:Gel-MA (2.0g/1mL) suspensions respectively. After that, 60 μ L of the mixture suspensions was pipetted into each cylindrical mold (5.2 mm in diameter) and exposed to ultraviolet (UV) light (365 nm, 30mW/cm²) for 5 minutes for gelation. The fabricated hydrogels were transferred to PBS at 37 °C for the dissolution of MS to get Gel-MCG constructs. The Gel-MCG constructs fabricated from MS:Gelatin (1.0g/1.0mL) and MS:Gelatin (2.0g/1.0mL) suspensions were named as MS:Gelatin (1:1) and MS:Gelatin (2:1) constructs, respectively.

Gelatin spongy was used as control and was engineered from directly lyophilizing glutaraldehyde crosslinked gelatin. Briefly, 5.0 g of gelatin was dissolved in 100 ml water and was crosslinked using 0.1% (w/v) glutaraldehyde. A punch with diameter of 6 mm (Miltex, USA) was used to cut small pieces of gelatin hydrogel out from the hydrogel. The hydrogel was pre-frozen and lyophilized to obtain Gelatin spongy.

6.2.3 Characterization of Gel-MCG constructs

To evaluate the dissolution of encapsulated MS, 0.4 mg of fluorescein isothiocyanate isomer I (FITC) was dissolved in 20 mL of PBS and incubated with 0.5 g of MS to label gelatin microspheres for overnight at 4 °C. After dialysis in PBS for one week at 4 °C, the labeled MS were filed through 100 μ m strainer (FALCON, USA)

and store in PBS at 4 °C until use. Two different constructs (MS:Gelatin (1:1) and MS:Gelatin (2:1)) were engineered using FITC-labeled MS as previously described and were incubated in PBS at 37 °C for 5 days to track the dissolution of microspheres.

To characterize pore structure left behind after microsphere dissolution, scanning electron microscopy (SEM, JSM-6700F, JEOL USA) and hematoxylin and eosin (H&E) staining were performed. The constructs were incubated in PBS at 37 °C for 5 days with change of PBS every day and then were fixed in 4% (w/v) paraformaldehyde solution in PBS for 24 hours. For examination of the microstructure of constructs using SEM, samples were lyophilized and coated with platinum prior to scanning using SEM. For H & E staining, the samples were embedded in Tissue Freezing Medium (Leica) for cryosectioning, and then cut into sections of 8 µm in thickness using sectioning cryostat (CM1900, Leica®). The sections were later stained with H&E and observed under microscope. The living space, defined as free space in constructs, was also calculated. Six representative images of H&E staining were obtained and analyzed with ImageJ software. The area of living space was calculated as the sum of unstained area in one field and expressed as a fraction of the total area of that field.

6.2.4 Fabrication of cell-laden Gel-MCG constructs

6.2.4.1 Cell culture

Murine endothelial progenitor outgrowth cells (EPOCs) were purchased from BioChain Institute (USA) and maintained in EPOCs growth medium (MCDB131

(Sigma-Aldrich) supplemented with 2% (v/v) FBS, 0.2% (v/v) bovine brain extract (Hammond Cell Tech, Healdsburg, California, USA), 50 µg/mL L-ascorbic acid, 0.1 mg/mL heparin, 1 µg/mL hydrocortisone. Cells were then incubated in humidified air with 5% CO₂ at 37 °C. Cells of passages 17-20 were used in this study.

6.2.4.2 Engineering cell-laden Gel-MCG constructs

EPOCs were suspended (5×10^6 cells/mL) into the mixture suspensions containing 1.0 g or 2.0 g of MS per mL Gel-MA solution, respectively. 60 µL of the cell suspensions was transferred into each cylindrical mold (5.2 mm in diameter) and crosslinked via exposure to UV light (Figure 1A). The size of such fabricated cell-laden constructs was around 5.2 mm × 2.8 mm (diameter × depth). For cell seeding of spongy constructs, 60 µL of cell suspension (5×10^6 cells/mL) in EPOCs growth medium was added directly to the sterile gelatin spongy. The size of cell-laden gelatin spongy was 4.8 mm × 2.2 mm (diameter × depth). Gel-MCG constructs and gelatin spongy with the same size as above mentioned were used for all subsequent experiments in this study. All these constructs were then cultured for 14 days in angiogenic medium that is comprised MCD131 basal medium supplemented 2% (v/v) FBS, 0.2% (v/v) bovine brain extract (Hammond Cell Tech, Healdsburg, California, USA), 50 µg/mL L-ascorbic acid, 0.1 mg/mL heparin, 1 µg/mL hydrocortisone, and the following growth factors: 10 ng/mL murine epidermal growth factor (EGF, Bio Basic Inc.), 10 ng/mL murine vascular endothelial growth factor (VEGF, Bio Basic Inc.) and 20 ng/mL

murine fibroblast growth factor-basic (bFGF, Bio Basic Inc.).

6.2.5 Cell viability assay

6.2.5.1 Qualitative live/dead staining

Qualitative live/dead fluorescent staining was performed using the LIVE/DEAD[®] Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen) for samples on day 1, 7 and 14. Briefly, calcein acetomethoxy (0.5 μ L) and ethidium homodimer-1 (2 μ L) were added to 1.0 mL of PBS to create staining solution. After 30 min of incubation of each cell-laden constructs in 1.0 mL of staining solution at 37 $^{\circ}$ C, the medium was replaced with equal volume of PBS and the constructs were examined under fluorescence microscope (Olympus).

6.2.5.2 Quantitative Prestoblue Cell viability assay

Quantitative PrestoBlue cell viability assay was performed using PrestoBlue[™] Cell viability reagent (Molecular Probes, Invitrogen) for samples on day 1, 7 and 14. Briefly, each cell-laden construct was incubated in 500 μ L of culture medium containing 10% (v/v) PrestoBlue reagent at 37 $^{\circ}$ C for one hour in dark. The fluorescence intensity at 560 nm (for excitation) and 590 nm (for emission) was collected using Fluorescence MicroPlate Reader (Bio-tek Synergy MX, USA). For each group, readings at different time points were normalized against the reading of day 1. Three

samples (n=3) from each group were assessed at designated time point.

6.2.6 Gene expression analysis

Gene expression analysis was performed by using quantitative real-time polymerase chain reaction (qPCR) for samples on day 3, 7 and 14. Cell-laden constructs were dissolved in TRIzol® for extraction of RNA, which was then converted into cDNA via reverse transcription prior to gene expression evaluation through qPCR. All the reverse transcription reagents used were obtained from Promega (Madison, MI, USA). Subsequently, qPCR was done using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and the iQTM qPCR system (Bio-Rad). For analysis, gene expression values relative to the housekeeping gene (β -actin) were calculated using the comparative $2^{-\Delta\Delta C_T}$ method. **Table 6.1** listed primers (AIT Biotech, Singapore) used in this experiment. Three samples from each group (n=3) were used to determine each target gene expression at designated time point.

Table 6.1 qRT-PCR primer sequences for murine gen markers: forward (F) and reverse (R).

Gene	Accession Number/ reference	Primer sequence (both 5'-3')	Annealing temperature (°C)	Product size (bp)
CD31	AK037551.1	F: CACCCATCACTTACCACCTTATG R: TGTCTCTGGTGGGCTTATCT	58	102
CD105	XM_006497664.1	F: TACCTCTGGATACCGGATAAGG R: GATGACAAACAGCAGGGTAATG	58	99
β -actin	NM007393.3	F: TCATGAAGTGTGACGTTGACATCCGT R: CCTAGAAGCATTTGCGGTGCACGATG	58	285

6.2.7 Histology and immunohistochemistry staining

Cell-laden constructs harvested on day 3, 7 and 14 were fixed in 4% (w/v) paraformaldehyde in PBS for 24 hours before embedding in Tissue Freezing Medium (Leica) for cryosectioning. Three samples for each group at each time point were collected for analysis. The processed samples were then cut into sections of 8 μ m in thickness using sectioning cryostat (Leica® CM1900). The sections from all groups were later stained with hematoxylin and eosin (H&E). For CD 31 staining, prior to incubation with CD 31 primary antibody (2 μ g/mL in PBS, goat polyclonal IgG, Santa Cruz Biotechnology), the sectioned specimens were first incubated with 10% horse serum (w/v, in PBS) for 20 minutes to prevent non-specific IgG binding. After that, sections were incubated with anti-IgG (5 μ g/mL in PBS, AlexaFluor 488, Invitrogen) at room temperature for 1 hour in the dark. Nuclei were finally counterstained by 4', 6-diamidino-2-phenylindole (DAPI).

6.2.8 CD31-positive staining area analysis

CD31-positive area of three different samples on day 3, 7 and 14 was calculated according to images of immunohistochemistry staining of CD31. Three representative images per construct and a total of nine images per group were obtained and analysed with the ImageJ software. The area of CD31-positive was calculated as the sum of CD31 positively-stained area in one field and expressed as a fraction of the total area of that field.

6.2.9 Animal Study

All animal experiments were done under guidelines approved by the Institutional Animal Care and Use Committees, SingHealth, Singapore. Four constructs were subcutaneously implanted into 5-week old male nude mice following 3 days of in vitro culture. Each mouse received one cellular Gel-MCG construct (MS:Gelatin (2:1)), one acellular Gel-MCG (MS:Gelatin (2:1)), one cellular gelatin spongy construct, and one acellular gelatin spongy. The mice were killed and constructs were harvested for analysis at designated time points (day 7 and 14). Three samples for each group at each time point were collected for analysis. The sections from all groups were later stained with H&E as previously described. Immunofluorescent staining of CD 31 was also applied to sectioned specimens as previously mentioned. The detailed timeline for samples cultured in vitro and subcutaneously implanted in vivo was described in **Fig. 1B**.

6.2.10 Statistical analysis

All results are presented as mean $\pm$ SD with 3 samples in each group. One-way ANOVA was used for comparisons assuming equal variances, unless otherwise stated. $p < 0.05$ was considered statistically significant.

6.3 Results

6.3.1 Synthesis and characterization of Gel-MA

The synthesis of Gel-MA and its ^1H -NMR spectrum was showed in **Fig. 6.2**. The peaks at 5.69 and 6.10 ppm could be assigned to protons in methacrylate groups, which demonstrates the successful methacrylation of gelatin. The degree of methacrylation of gelatin-MA was calculated using integral intensities of methylene protons (δ 5.69 and 6.10 ppm, as indicated by dash line, **Fig. 2Bb**) and aromatic groups (δ 7.23, **Fig. 2Ba**) as previously described [332], which was about 91.5%.

Fig. 6.2 (A) The synthesis of Gel-MA. (B) ^1H NMR spectrum of gelatin and Gel-MA precursor.

6.3.2 Dissolution of encapsulated MS

To track the dissolution of MS encapsulated in gel constructs, fluorescent dye FITC was used to label MS. As showed in **Fig. 6.3**, the decrease in florescent signals over time was observed in both two Gel-MCG constructs, indicating the dissolution of MS. For the constructs fabricated from MS:Gelatin (2.0g/1.0mL), green florescence disappeared after 24 hours of culture (**Fig. 6.3d**) and well-delineated micro-cavities could be clearly seen within the resultant Gel-MCG at hour 72 (**Fig. 6.3e**). Meanwhile, for the constructs prepared from MS:Gelatin (1.0g/1.0mL), there was still green florescence in the background at hour 72 (**Fig. 6.3j**) and even on day 5 (data not shown), which was due to the FITC-labelled MS residuals or gelatin molecules that still remained within the constructs. These results suggest more rapid dissolution of MS in MS:Gelatin (2:1) constructs than in MS:Gelatin (1:1) constructs.

Fig. 6.3 Dissolution of FITC-labeled MS encapsulated in MS:Gelatin (2:1) and MS:Gelatin (1:1) constructs in PBS at 37 °C over time.

6.3.3 Structures of Gel-MCG constructs

In order to characterize pore structures formed due to MS dissolution, SEM and H&E staining of samples were performed (**Fig. 6.4**). SEM images revealed that pore structures surrounded by gelatin walls in Gel-MCG constructs, which was different from sheet-like structures with wide gaps in gelatin spongy (**Fig. 6.4A**). H & E staining showed evenly distributed cavities in both Gel-MCG constructs and higher cavitory density in MS:Gelatin (2:1) constructs than MS:Gelatin (1:1) constructs (**Fig. 6.4Ba**). Interestingly, slightly pink staining could be observed within some cavities of MS:Gelatin (1:1) constructs, suggesting gelatin MS do not completely dissolve or diffuse into PBS even after 5 days of incubation. This result agrees with previous gelatin tracking experiment, which might be resulted from the low permeability of MS:Gelatin (1:1) constructs. To show free living spaces in the constructs available for cells growth, area of living space of each construct was presented (**Fig. 4Bd**). The living space in MS:Gelatin (2:1) constructs was $48.98 \pm 2.20\%$, which was significantly higher than that in MS:Gelatin (1:1) constructs ($32.82 \pm 2.54\%$). Gelatin spongy had the highest percentage of living space ($58.86 \pm 2.40\%$). This result illustrates that the increase in ratio of MS can enlarge the living space of Gel-MCG and even make it close to that of gelatin spongy.

Fig. 6.4 Structure characterization of Gel-MCG constructs. (A) SEM images of MS:Gelatin (2:1), MS:Gelatin (1:1), and gelatin spongy constructs. (B) H & E staining of MS:Gelatin (2:1), MS:Gelatin (1:1) and gelatin spongy constructs, and area of living space in Gel-MCG and gelatin spongy. The data were presented as the mean $\pm$ standard deviation (n=6). ** indicates $p < 0.01$.

6.3.4 Cell viability assays

After structure characterization of Gel-MCG, cell-laden Gel-MCG constructs were fabricated and cell viability in the constructs were investigated. According to **Fig. 6.5A**, most of cells in each group were stained green with very few red cells from day 1 to day 14. Besides, green signals on day 14 (**Fig. 6.5Ag, h and i**) were more widespread than day 1 (**Fig. 6.5Aa, b and c**). These results indicate high cell viability and a gradual increase in cell numbers in these constructs over time. In particular, well-defined round pores (indicated by asterisks) could be identified in both Gel-MCG constructs on day 14, which suggests that cells form networks along the boundaries created by gelatin MS. Quantitative PrestoBlue cell viability assay revealed an increase trend of fluorescence

intensity, indicating the active cell proliferation in these three constructs (**Fig. 6.5B**). In addition, MS:Gelatin (2:1) constructs showed 1.2-fold and 2.4-fold increase in fluorescence intensity on day 7 and day 14 respectively, which was obviously higher than MS:Gelatin (1:1) constructs (0.1-fold increase on day 7 and 1.9-fold increase on day 14). This result indicates that cells in MS:Gelatin (2:1) constructs grow in a more rapid way than MS:Gelatin (1:1) constructs. For gelatin spongy, even though there was 0.6-fold increase in fluorescence intensity on day 7, only 0.7-fold increase in fluorescence intensity was observed on day 14. The fold increase in gelatin spongy was significantly lower than both two Gel-MCG constructs on day 14.

Fig. 6.5. Live/dead staining (A) and proliferation (B) of cell-laden MS:Gelatin (2:1), MS:Gelatin (1:1), and gelatin spongy constructs. The data were presented as the mean $\pm$ standard deviation ($n=6$). * indicates $p < 0.05$; ** indicates $p < 0.01$.

6.3.5 Gene expression study

To further explore cell differentiation within constructs, expressions of endothelial markers, CD31 and CD105, were evaluated via qPCR (**Fig. 6.6**). CD31, an endothelial

differentiation marker, is highly expressed on differentiated endothelial cells (ECs) [203]. CD31 expression of MS:Gelatin (2:1) constructs was consistently higher than that of MS:Gelatin (1:1) constructs from day 7 to day 14 (**Fig. 6.6A**), indicating superior differentiation-inducing microenvironments in MS:Gelatin (2:1) constructs than in MS:Gelatin (1:1) constructs.

CD105 is also an endothelial marker, which was a proliferation-associated protein, and highly expressed in angiogenic ECs [333, 334]. Expression of CD105 in MS:Gelatin (2:1) constructs was significantly higher than that in MS:Gelatin (1:1) constructs on day 14 (**Fig. 6.6B**). This result demonstrate that MS:Gelatin (2:1) constructs can provide a more permissive microenvironment for neovascularization than MS:Gelatin (1:1) constructs. However, compared with gelatin spongy constructs, both of these Gel-MCG constructs showed lower expression of CD105 on day 7 and day 14 (**Fig. 6.6B**).

Fig. 6.6 Gene expression of CD31 (A) and CD105 (B) in MS:Gelatin (2:1), MS:Gelatin (1:1), and gelatin spongy constructs. Fold values were normalized against gene expressions in gelatin spongy constructs on day 3. The data were presented as the mean $\pm$ standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

6.3.6 In vitro vascularization of Gel-MCG constructs

In order to evaluate vascularization of cell-laden Gel-MCG constructs during the in vitro culture period, H&E staining and immunostaining of CD31 were performed.

According to the result showed in **Fig. 6.7A**, EPOCs distributed homogenously within

both MS:Gelatin (1:1) and MS:Gelatin (2:1) constructs, respectively, on day 3 (**Fig. 6.7Aa and b**). While some cells existed within the cavities created by dissolved gelatin MS, they still remained to be isolated. On day 7, cell connection within the micro-cavities were observed, indicating the micro-cavity-mediated vascularization (**Fig. 6.7Ad and e**). Cell further increased in their numbers in both of Gel-MCG constructs from day 7 to 14 (**Fig. 6.7Ag and h**). Excitingly, the neighboring micro-cavities were observed to be fused in the MS:Gelatin (2:1) constructs and cells in adjacent cavities interconnected to form cellular networks (shown by arrowheads in **Fig. 6.7Ag**). This phenomenon might be attributed to the metalloproteinase (MMP)-dependent proteolysis of gelatin hydrogel [335, 336]. However, numerous cells accumulated in MS:Gelatin (1:1) constructs (**Fig. 6.7Ah**), suggesting that thick gelatin gel wall inhibited the formation of interconnected cellular networks. For cell-laden gelatin spongy, a few cells were unevenly distributed throughout the whole structures (**Fig. 6.7Ac, f and i**).

To evaluate the distribution of differentiated EPCs within the constructs, Immunostaining of CD31 was performed. CD31 was positively stained in all three groups over 14 days (**Fig. 6.7B**). In both Gel-MCG constructs on day 7, cells that grew along the boundaries of micro-cavities were more positively stained than the ones entrapped in gel bulk, enabling cavity structure to be clearly delineated (as evidenced by asterisks in **Fig. 6.7Bd and e**). On day 14, as shown by arrowheads in **Fig. 6.7Bg**, positively-stained EPOCs grew along with the interconnected cavities in MS:Gelatin (2:1) constructs, while separate cavities surrounded by positively stained cells still

existed in MS:Gelatin (1:1) constructs. In gelatin spongy, cells unevenly located and several cellular colonies were formed (**Fig. 6.7Bc, f and i**). Quantification of CD31 positive area in the constructs showed that the positive area of both Gel-MCG constructs were significantly higher than that of gelatin spongy constructs from day 3 to day 14. Moreover, compared with MS:Gelatin (1:1) constructs, MS:Gelatin (2:1) constructs had higher percentage of positive area on day 3 and day 7 (**Fig. 6.7Bj**). Collectively, these results indicate that gel-cavity interfaces facilitate the proliferation and differentiation of EPOCs. More importantly, MS: Gelatin (2:1) constructs allow cells in adjacent cavities to interconnect with each other so as to form cellular networks.

Fig. 6.7 (A) H&E staining of MS:Gelatin (2:1), MS:Gelatin (1:1), and gelatin spongy constructs. (B) CD31 immunostaining of MS:Gelatin (2:1), MS:Gelatin (1:1), and gelatin spongy constructs, and CD31 positive area in the constructs. The data were presented as the mean $\pm$ standard deviation (n=6). * indicates $p < 0.05$; ** indicates $p < 0.01$.

6.3.7 In vivo vascularization of Gel-MCG constructs

Finally, vascularization of cellular and acellular MS:Gelatin (2:1) constructs was evaluated in vivo. As results shown in **Fig. 6.8A**, compared with pale appearance of acellular constructs, both cellular constructs were more red in color, indicating more

abundant vascular invasion into these two cellular constructs.

H&E staining showed more cells existed in cellular MS:Gelatin (2:1) constructs (**Fig. 6.8Ba and e**) than the acellular counterparts (**Fig. 6.8Bb and f**) both on day 7 and day 14. Significantly, red blood cells could be observed within the cellular constructs on day 14 as indicated by arrowheads in **Fig. 6.8Ba and e**, demonstrating vascular networks within constructs are connected with the host vasculature. However, this phenomenon was absent in acellular MS:Gelatin (2:1) constructs. This difference indicates that pre-seeded EPOCs in the MS:Gelatin (2:1) constructs play a critical role in host vasculature invasion. For gelatin spongy constructs, similar results could also be found, with more vascular invasion in cellular constructs (indicated by arrowhead in **Fig. 6.8Bg**) than acellular constructs (**Fig. 6.8Bd and h**).

Immunostaining of CD31 was also carried out to display the vascularization of constructs in vivo (**Fig. 6.8C**). Positive staining of CD31 only appeared in the cellular constructs. Meanwhile, lumen-like structures consisting of CD31 positive cells were obvious in cellular MS:Gelatin (2:1) constructs on day 14 (arrowheads in **Fig. 6.8Ce**). Gelatin spongy also showed the similar tubular structures (arrowheads in **Fig. 6.8Cg**) on day 14. Furthermore, quantification of CD31 positive-staining area in the constructs showed that positive area in cellular MS:Gelatin (2:1) constructs were significantly higher than that in cellular gelatin spongy constructs on day 7 and day 14, respectively (**Fig. 6.8Ci**). However, CD31-positive areas for both acellular constructs were nearly undetectable over the whole in vivo culture period. In combination with the results of H&E staining, EPOCs seeding in advance facilitate the invasion of host vasculature

into Gel-MCG constructs.

Fig. 6.8 (A) Gross views of cellular and acellular constructs on day 7 and on day 14, respectively. (B) H&E staining of cellular and acellular constructs over a period of 14 days. (C) CD31 immunostaining of cellular and acellular constructs, and CD31 positive areas in the constructs. The data were presented as the mean $\pm$ standard deviation ($n=6$). * indicates $p < 0.05$; ** indicates $p < 0.01$.

6.4 Discussions

Herein, two types of Gel-MCG constructs with increased cavity density, namely MS:Gelatin (1:1) and MS:Gelatin (2:1), were developed by elevating the ratio of microspherical porogens (namely gelatin MS) based on our previous model study [332]. Due to methacrylate groups, Gel-MA precursors in solution can be crosslinked to form stable polymer gel networks via UV exposure [337], while gelatin MS quickly dissolve in the hydrogels at 37 °C. As a result, numerous micro-cavities are created within the hydrogel bulks. However, the difference between these two types of Gel-MCG constructs is

obvious. More rapid MS dissolution and higher cavitory density could be observed in MS:Gelatin (2:1) constructs than MS:Gelatin (1:1) constructs (Figure 3 and 4). Compared to MS:Gelatin (1:1) constructs, MS:Gelatin (2:1) constructs have more MS and fewer gelatin gel, which leads to more thinner gelatin wall formation within MS:Gelatin (2:1) constructs. As a result, when MS in MS:Gelatin (2:1) constructs melt, gelatin molecules can more quickly diffuse through thinner gelatin wall into surrounding culture mediums. Meanwhile, for MS:Gelatin (1:1) constructs, higher gelatin gel content results in thicker gelatin wall formation in the constructs. These thick gelatin walls may delay the diffusion of gelatin molecules out from the constructs and thus slow down the dissolution of MS. These results agree with previous studies which show cavities can result in high hydrogel permeability and consequential more rapid diffusion of solute (such as proteins) across hydrogel [305, 338].

After establishment of the improved Gel-MCG, cell-laden Gel-MCG constructs were fabricated to explore the vascularization of constructs. The proliferation rate of EPOCs in MS:Gelatin (2:1) constructs is higher than that in MS:Gelatin (1:1) constructs (**Fig. 6.5B**). This results are due to the presence of more living spaces/interfaces in MS:Gelatin (2:1), which can enhance cell attachment, growth, and proliferation [305]. Interestingly, the proliferation of EPOCs in MS:Gelatin (2:1) constructs is higher than that in the gelatin spongy constructs which are commonly used scaffolds for vascularization and have been shown to support neo-vascularization well [339, 340]. This result might be due to more homogenous cell distribution in MS:Gelatin (2:1) constructs as compared to spongy constructs and suggests MS:Gelatin (2:1) constructs

are more superior than gelatin spongy for active cell proliferation. More importantly, we found that gel walls between cavities degraded over cell culture period (**Fig. 6.7Ag**), which facilitate the formation of interconnected cavities and cellular networks in MS:Gelatin (2:1) constructs.

Higher cavitory density in the MS:Gelatin (2:1) constructs also provide more living spaces/gel-cavity interfaces for the enhanced EPOC differentiation as evidenced by the higher CD31 and CD105 gene expression in MS:Gelatin (2:1) constructs than MS:Gelatin (1:1) constructs (**Fig. 6.6**). The gene expression of CD31 in MS: Gelatin (2:1) constructs was also comparable to that in gelatin spongy (Figure 6A). However, CD105 expression in MS: Gelatin (2:1) constructs was lower than that in gelatin spongy (**Fig. 6.6B**), which might be due to the aggregated cell colony formation in gelatin spongy. Besides as markers of angiogenic ECs [333, 334], CD105 will also be up-regulated in hypoxia conditions [341, 342]. In gelatin spongy, cell crowded in very limited spaces instead of homogenous and wide distribution, which might easily cause oxygen over-consumption and subsequent low oxygen pressure. As a result, CD 105 is highly expressed in gelatin spongy.

As MS:Gelatin (2:1) constructs showed superior vascularization capacity than MS:Gelatin (1:1) constructs in vitro, the MS:Gelatin (2:1) constructs were evaluated in following animal study. Gelatin spongy were also subcutaneously implanted as control. As evidenced by the presence of red blood cells within the cellular constructs (**Fig. 6.8B**), the cellular constructs, including cellular MS:Gelatin (2:1) and cellular spongy constructs, can provide permissive environments for host vasculature invasion.

Immunostaining of CD31 in both cellular constructs further verified this observation (**Fig. 6.8C**). These results agree with previous studies showing that pre-vascularized constructs induced host tissues and vessels to grow into these constructs [263, 343]. Pre-seeded EPOCs can release angiopoietin-2 (ANG-2), fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF) and other chemokines to recruit cells from host tissues [336]. Compared with gelatin spongy, more extensive vascular invasion was observed in the MS:Gelatin (2:1) constructs, which is probably due to more homogenous cells encapsulation in Gel-MCG constructs (**Fig. 6.8B**). These results demonstrate that cell seeding or pre-vascularization in vitro is critical for host vasculature invasion and consequential functional vascular network formation in constructs [311, 344, 345].

Although Gel-MCG scaffold has been created as delivery vehicle of anchorage dependent cells in our recent study [332], this work shows for the first time that the Gel-MCG can support the formation of neo-vascular networks across Gel-MCG constructs by fine tuning the internal cavitory density.

6.5 Summary

In this study, the vascularization of Gel-MCG scaffold was optimized by adjusting its cavitory density. The increase of cavitory density greatly enhanced the proliferation and endothelial differentiation of encapsulated EPOCs in Gel-MCG constructs. In particular, compared to MS:Gelatin (1:1) counterpart, MS:Gelatin (2:1) constructs with

higher cavitory density allows the interconnected vascular network formation within the whole constructs upon the degradation of thinner gel walls among neighboring cavities. Extensive vascular invasion could also be observed within the MS:Gelatin (2:1) constructs when implanted in vivo. These findings demonstrate the vascularization of Gel-MCG constructs can be improved by increasing cavitory density, and Gel-MCG constructs may contribute to the establishment of tissue constructs with homogenous vascularization.

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Chapter 7: Improvement of EPOC-Mediated Vascularization in Gelatin-Based Hydrogels through Pore Size Manipulation

7.1 Introduction

Vascularization of biomaterials is essential for successful clinical applications of engineered tissues, as implanted cells in tissue constructs need blood vessels for supply of oxygen and nutrients as well as removal of waste products to survive and maintain their normal functions [346-349]. Various strategies have been explored to develop vascular networks within engineered tissues, including angiogenic molecule delivery to promote ingrowth of host vessels into biomaterials [331, 350, 351], encapsulation of endothelial cells with or without perivascular cells within scaffold [31, 344, 352, 353], and in vivo pre-vascularization of tissue constructs prior to their therapeutic application [354]. However, regardless of approaches for scaffold vascularization, physical properties of scaffold need to be optimized at the same time, as these parameters not only affect behaviors of seeded endothelial cells but also ingrowth of host vascular tissues [136].

Pore structure is one of critical physical properties of scaffolds, which can regulate scaffold vascularization in vivo [355]. For example, pore structures can directly result in neovascularization at the interface between synthetic membranes and host tissues

[356]. An increased porosity in polyesterurethane scaffolds supports enhanced vascularization in vivo [357, 358]. In addition, pore size also plays an important role in scaffold vascularization in vivo. Chitosan scaffolds with 90 μm pore size exhibit vascular infiltration throughout the whole scaffolds, whereas there is no neovascularization in chitosan scaffolds with 35 μm pore size [359]. Polyurethane foams with 30 μm micro-pores induce more fibrovascular tissue ingrowth than foams with 10-15 μm micro-pores [357]. Diameters of invaded blood vessels in β -tricalcium phosphate scaffolds increase with pore sizes [54]. Although scaffold vascularization in vivo can be improved by larger pores, there is an upper limit of pore size for vascularization. A pore size larger than 400 μm in β -tricalcium phosphate scaffolds cannot promote scaffold vascularization in vivo further [360].

Besides synthetic membranes, polymer sponges and calcium phosphate scaffolds, the effects of pore structures and pore sizes on vascularization has been investigated in hydrogels as well. The induction of pore structures can induce endothelial progenitor cells (EPCs) to form vascular networks along pore-gel boundaries [361]. Increasing hydrogel porosity can improve hydrogel vascularization both in vitro and in vivo [362]. Poly(ethylene glycol) (PEG) hydrogels with 25-50 μm pores limit vascular invasion, while complete vascularization of the whole constructs in vivo can be observed in PEG hydrogels with pores from 50-150 μm [136]. However, the influence of pore sizes on vascular network formation by endothelial cells encapsulated in hydrogels remains unknown.

In our previous studies, we have developed a gelatin-based micro-cavitary

hydrogel by crosslinking gelatin-methacrylate containing dissolvable gelatin microspheres (MS) [361]. Since gelatin-methacrylate is synthesized by modification of gelatin with methacrylate groups, gelatin-methacrylate hydrogels possess advantages of both natural and synthetic biomaterials [363]. Due to gelatin backbone, gelatin-methacrylate has low immunogenicity, excellent degradability and cell adhesive sites [364-367]. Modulation of gelatin methacrylation degree and gelatin-methacrylate crosslinking density can adjust mechanical and chemical properties of the hydrogels [365, 368, 369]. Additionally, this hydrogel has been shown to support robust vascular network formation by encapsulated endothelial colony-forming cells (ECFCs) and mesenchymal stem cells (MSCs) [370]. Meanwhile, MS fabricated from unmodified gelatin spontaneously dissolve in water at 37 °C, which can create pore structures in hydrogels [254, 371, 372]. The pore sizes in hydrogels can be simply controlled by the size of dissolvable MS. Therefore, micro-cavitary hydrogels based on dissolvable MS and gelatin-methacrylate provide us a unique platform to study the effect of pore size on vascularization by endothelial cells encapsulated in hydrogels.

In the present study, pore structures with different sizes were created in gelatin-methacrylate hydrogels with dissolvable gelatin MS varying in sizes. The influence of pore sizes on vascular network formation by endothelial progenitor outgrowth cells (EPOCs) encapsulated in hydrogels was investigated both in vitro and in vivo (**Fig. 7.1**). Vascularization in vitro by EPOCs is improved in hydrogels with middle pores as compared to hydrogel with large or small pores, while large pores supported hydrogel vascularization in vivo better than middle or small pores. These findings can provide

useful information on how pore sizes in hydrogels influence vascularization both in vitro and in vivo.

Fig. 7.1 (a) Schematic illustration of pore structures created by dissolvable gelatin microspheres in gelatin-based hydrogels, (b) pore-mediated vascularization in micro-cavitary hydrogels, (c) micro-cavitary hydrogel degradation and vascularization in vivo. After 3 days of incubation in PBS or culture in vitro, non-cell-laden hydrogels or cell-laden hydrogels were subcutaneously implanted into nude mice and the implants were harvested for examination on day 7 and day 14.

7.2 Methods

7.2.1 Fabrication of gelatin microspheres (MS) with different sizes

Gelatin microspheres (MS) were fabricated with double emulsion (oil/water/oil) method as previously described [373, 374]. The fabricated MS were then filtered with a set of sieves. Based on their sizes, MS were divided into different groups. 80-100 group: MS that couldn't pass through a sieve with mesh number 80-100; 100-200 group: MS that could pass through a sieve with a mesh number 80-100 but not a sieve with mesh number 100-200; 200-300 group: microspheres that could pass through a sieve with a mesh number 100-200 but not a sieve with mesh number 200-300. The sorted MS were immersed in 1000 units/mL penicillin and 1000mg/mL streptomycin solution overnight for sterilization and then stored in phosphate-buffered saline (PBS) at 4 °C until use. To determine the average size of MS in each group, MS were first dispersed in 4 °C PBS and then observed with a microscope (Olympus). For each group, diameters of at least 160 MS were measured with the ImageJ software.

7.2.2 Synthesis of gelatin-methacrylate

Gelatin-methacrylate was synthesized as previously described [361, 362]. Briefly, 11 g of gelatin (type A, from porcine skin, Sigma) was dissolved in 150 mL deionized (DI) water under stirring at 40 °C, followed by addition of 50 mL of dimethylformamide

(DMF, BDH), 5 g of triethylamine (TEA, Sigma-Aldrich) and 50 g of glycidyl methacrylate (GMA, Aldrich), which were then stirred at 40 °C for 3 days. After that, the reaction solution was dialyzed with dialysis sacks (MWCO: 12000, YouLab Scientific, Singapore) for 5 days and freeze-dried prior to use. Proton nuclear magnetic resonance (^1H -NMR) spectrometer (Bruker Avance II, 300 MH) was used to determine the percentage of gelatin modified with methacrylate groups.

7.2.3 Fabrication of gelatin-based micro-cavitary hydrogels

1 mL of 20% (w/v in PBS) gelatin-methacrylate containing 0.1% (w/v) Irgacure 2959 (Ciba) were mixed with 2 g of MS from 80-100, 100-200 or 200-300 group. 80 μL of the mixture was then pipetted into a cylindrical mold (5.2 mm in diameter and 2.8 mm in height) and exposed to ultraviolet (UV) light (365 nm, 30mW/cm²) for 5 minutes. After crosslinking, hydrogels were incubated in PBS at 37 °C for 3 days to dissolve the encapsulated MS. The micro-cavitary hydrogels fabricated with MS in 80-100 group was named as 80-100; hydrogels with MS in 100-200 group was named as 100-200; hydrogels with MS in 200-300 group was named as 200-300. Hydrogels without MS were named as Gel, which were used as a control in this study.

7.2.4 Characterization of gelatin-based micro-cavitory hydrogels

Porosity of hydrogels was estimated based on total volume, wet and dry weight of hydrogels. Briefly, the total volume of samples was calculated according to diameter and height of samples. After the wet weight of samples was recorded, samples were frozen and lyophilized to obtain the dry weight. Pore volume was determined based on the weight loss of samples and the density of water (1 g/mL). The porosity was then calculated as a percentage of the pore volume to the total volume of samples. For each group, four samples (n=4) were tested.

Compressive modulus of hydrogels was measured with Tensile Meter (Model 5543, Instron) equipped with a 10 N load cell. Recorded stress-strain curves were used to calculate compressive modulus of hydrogels. Three samples (n=3) from each group were tested.

Degradation properties of hydrogels were determined with collagenase A. Samples were incubated in 4mL of 200 µg/mL (w/v in PBS) collagenase A (Roche, Germany) at 37 °C. After incubation for 0, 15, and 30 minutes, the samples were weighed individually. The degradation of hydrogels was calculated as a percentage of weight of samples at the designated time points to their original weight. For each group, three samples (n=3) were tested.

Pore structures created by dissolvable MS in hydrogels were observed with a scanning electron microscope (SEM, JSM-6700F, JEOL USA) or a confocal microscope (LSM710, Zeiss). For SEM scanning, samples were frozen at -80 °C for 12

hours and then lyophilized and coated with platinum. For confocal imaging, samples were labeled with 0.05 mg/mL (w/v in PBS) fluorescein isothiocyanate (FITC, Sigma) at 4 °C overnight and then washed with PBS.

Cross-section structures of hydrogels were observed with a microscope (Olympus) following hematoxylin and eosin (H&E) staining. After incubation in PBS at 37 °C for 3 days and fixation in 4% PFA at 4 °C for 24 hours, samples were embedded in Tissue Freezing Medium (Leica) and then cut into 10- μ m-thick sections with sectioning cryostat (CM1900, Leica). The sectioned samples were stained with Harris hematoxylin solution (Sigma-Aldrich) and eosin Y solution (Sigma-Aldrich) respectively, which were then observed with a microscope. Total interface and free space in hydrogels were measured. The total interface was defined as total length of pore-gel boundaries in hydrogels, which was calculated as the length of pore-gel boundaries in one image and expressed as mm per mm². The free space was defined as total area of pores in hydrogels, which was calculated as pore area in one image and expressed a percentage of whole area in one image. 6 representative images (n=6) from each group were taken and analyzed with the ImageJ software.

7.2.5 Fabrication of cell-laden micro-cavitary hydrogels

7.2.5.1 Cell culture

Murine endothelial progenitor outgrowth cells (EPOCs, BioChain Institute, USA) were cultured in MCDB131 medium (Sigma-Aldrich) supplemented with 2% (v/v) fetal

bovine serum (FBS, PAA Laboratories), 0.2% (v/v) bovine brain extract (BBE, Hammond Cell Tech, Healdsburg, California, USA), 50 $\mu\text{g/mL}$ (w/v) L-ascorbic acid (Sigma), 0.1 mg/mL (w/v) heparin (Sigma-Aldrich), 1 $\mu\text{g/mL}$ (w/v) hydrocortisone (Sigma). Cells were maintained in humidified air with 5% CO_2 at 37 °C. Cells with passage number 18 to 20 were used in this study.

7.2.5.2 Engineering cell-laden micro-cavitary hydrogels

5×10^6 EPOCs were suspended in 1 mL of 20% gelatin-methacrylate containing 0.1% Irgacure 2959, which were then mixed with 2 g of MS from 80-100, 100-200, or 200-300 group. 80 μL of the mixture was transferred into cylindrical molds (5.2 mm in diameter and 2.8 mm in height) and crosslinked via UV light exposure. After that, the fabricated cell-laden constructs were cultured in MCDB131 medium supplemented with 2% FBS, 0.2% BBE, 50 $\mu\text{g/mL}$ L-ascorbic acid, 0.1 mg/mL heparin, 1 $\mu\text{g/mL}$ hydrocortisone, 10 ng/mL murine epidermal growth factor (EGF, Bio Basic Inc.), 10 ng/mL murine vascular endothelial growth factor (VEGF, Bio Basic Inc.) and 20 ng/mL murine fibroblast growth factor-basic (bFGF, Bio Basic Inc.) for 14 days.

7.2.6 Confocal imaging

1×10^6 EPOCs were suspended in 1mL of 0.5% (v/v in serum-free MCDB 131 medium) DiO Cell-Labeling Solution (Vybrant, ThermoFish Scientific) and incubated

at 37 °C for 5 minutes. The labeled cells were then washed with PBS and cultured in normal medium overnight for cell recovery. After that, cells were used for cell-laden construct fabrication as aforementioned. On day 7 and day 14, constructs were stained with 5 µg/mL (w/v in PBS) Hoechst 33342 (ThermoFisher Scientific), followed by observation with a confocal microscope (LSM710, Zeiss). Confocal images of constructs at different planes were taken and 3-dimensional (3D) images were reconstructed based on these confocal images.

7.2.7 Cell viability and proliferation in micro-cavitary hydrogels

7.2.7.1 Live/dead staining

Cell viability was evaluated with the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen). Briefly, on day 1, 7 and 14, 0.5 µL of calcein acetomethoxy and 2 µL of ethidium homodimer-1 were added into 1 mL of PBS to create staining solution. Cell-laden constructs were incubated in the prepared staining solution at 37 °C for 30 minutes in the darkness. After that, the constructs were washed with PBS and observed with a fluorescent microscope (Olympus).

7.2.7.2 WST-1 assay

Quantitative cell viability was measured via WST-1 assay. Briefly, on day 1, 7 and 14, each cell-laden construct was incubated in 500 µL of cell culture medium

supplemented with 50 μ L of cell proliferation reagent WST-1 (Roche) at 37 °C for two hours in the darkness. The supernatant was then collected and absorbance at wavelength of 450 nm and 620 nm were measured respectively with Multiskan Spectrum Microplate Photometers (ThermoScientific, Finland). The absorbance was expressed as OD value. Three replicates (n=3) from each group were tested.

7.2.8 Gene expression study

Gene expressions were analyzed with quantitative polymerase chain reaction (qPCR) on day 3, 7 and 14. At the designated time points, cell-laden constructs were dissolved in TRIzol for RNA extraction and the extracted RNA was converted into cDNA via reverse transcription. All the reverse transcription reagents used were obtained from Promega (Madison, MI, USA). qPCR was then performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and synthesized cDNA. Generated signals after each round of reaction were detected with CFX Connect Real-Time System (Bio-Rad, Singapore). Gene expressions were calculated with the comparative $2^{-\Delta\Delta C_T}$ method. Endothelial marker CD31 [375, 376], CD105 [377, 378], and vascular endothelial (VE)-Cadherin [379, 380] were analyzed with β -actin as a housekeeping gene. **Table 7.1** listed the primers (IDT, Singapore) used in this experiment. Three samples from each group (n=3) were used to determine the expressions of the target genes at the designated time points.

Table 7.1 qPCR primer sequences for vascularization markers: forward (F) and reverse (R).

Gene	Accession Number	Primer sequence (both 5'-3')	Annealing temperature (°C)	Product size (bp)
CD31	AK037551.1	F: CACCCATCACTTACCACCTTATG R: TGTCTCTGGTGGGCTTATCT	58	102
CD105	XM_006497664.1	F: TACCTCTGGATACCGGATAAGG R: GATGACAAACAGCAGGGTAATG	58	99
VE-Cadherin	NM_009868.4	F: TACCTCTGGATACCGGATAAGG R: GATGACAAACAGCAGGGTAATG	58	109
β -actin	NM007393.3	F: TCATGAAGTGTGACGTTGACATCCGT R: CCTAGAAGCATTGCGGTGCACGATG	58	285

7.2.9 Histology and immunofluorescent staining

Cell-laden constructs were harvested on day 7 and day 14 and fixed in 4% PFA for 24 hours. After that, samples were embedded in Tissue Freezing Medium (Leica) and cut into 10- μ m-thick sections with sectioning cryostat (CM1900, Leica). The sections were stained with H&E, CD31 and VE-Cadherin respectively. H&E were stained as aforementioned. For CD 31 and VE-Cadherin staining, the sectioned samples were incubated with 2 μ g/mL CD 31 primary antibody (goat polyclonal IgG, Santa Cruz Biotechnology) or 2 μ g/mL VE-Cadherin primary antibody (rabbit polyclonal IgG, Santa Cruz Biotechnology), following block with 1% (w/v in PBS) bovine serum albumin (BSA, Sigma) for 20 minutes at room temperature. After that, sections were incubated with 10 μ g/mL Rabbit anti-Goat IgG (Alexa Fluor 488, ThermoFish Scientific) or Goat anti-Rabbit IgG (Alexa Fluor 568, ThermoFish Scientific) at room temperature for 1 hour in the darkness. All the antibodies were diluted with 1% BSA. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). The stained

sections were then observed with a fluorescent microscope (Olympus).

7.2.10 In vivo degradation of micro-cavitary hydrogels

All animal experiments were performed under guidelines approved by the Institutional Animal Care and Use Committees, SingHealth, Singapore. After incubation in PBS for 3 days, micro-cavitary hydrogels were implanted into subcutaneous pockets on back of 5-week-old male nude mice (**Fig. 7.1c**). Each mouse received one 80-100 hydrogel construct, one 100-200 hydrogel construct, one 200-300 hydrogel construct and one hydrogel construct without pore structures (served as a control). Total 6 mice were implanted with hydrogel constructs and sacrificed on day 7 and day 14 for sample harvest (3 mice at each time point). The harvested hydrogel constructs were weighed, sectioned and stained with H&E, followed by observation with a microscope. The degradation of hydrogel constructs was calculated as a percentage of weight of samples at the designated time points to their original weight. The cellular infiltration area was calculated as cellular area in hydrogel constructs in one image and expressed as a percentage of whole area in one image. 9 representative images per construct were taken and analyzed with the ImageJ software.

7.2.11 In vivo vascularization of cell-laden micro-cavitary hydrogels

After culture in MCDB131 medium for 3 days, cell-laden micro-cavitary hydrogels were implanted into subcutaneous pockets on back of 5-week-old male nude mice (**Fig. 7.1c**). Each mouse received one cell-laden 80-100 hydrogel construct, one cell-laden 100-200 hydrogel construct, one cell-laden 200-300 hydrogel construct and one cell-laden hydrogel construct without pore structures (served as a control). Total 6 mice were implanted with cell-laden hydrogel constructs and sacrificed on day 7 and day 14 for sample harvest (3 mice at each time point). The harvested samples were sectioned and stained with H&E, CD31 and VE-Cadherin respectively for in vivo vascularization evaluation. The vessel density of each group was quantified based on 9 representative images of H&E staining per construct. The vessels were identified as luminal structures containing red blood cells. The vessel density was calculated as number of vessels in hydrogel constructs in one image and expressed as vessels per mm². The CD31-positive or VE-Cadherin-positive area was calculated as expressed positively-stained area in hydrogel constructs in one image and expressed as a percentage of whole area in one image. 9 representative images per construct were obtained and analyzed with the ImageJ software.

7.2.12 Statistical analysis

All results are presented as mean $\pm$ SD. One-way ANOVA was used for

comparisons assuming equal variances followed by least significant difference (LSD) test, unless otherwise stated. $p < 0.05$ was considered statistically significant.

7.3 Results

7.3.1 Gelatin microspheres (MS) with different sizes

Through size screening with a set of sieves, the fabricated MS were divided into different groups. Increased mesh number of sieves resulted in smaller pores. The size of MS in 80-100 group was $287.99 \pm 74.58 \mu\text{m}$; in 100-200 group was $205.22 \pm 55.58 \mu\text{m}$; and in 200-300 group was $146.45 \pm 33.15 \mu\text{m}$ (**Fig. 7.2**). As MS are engineered from unmodified gelatin that can dissolve in water at 37°C , pore structures with the same size as MS can be created in hydrogels after MS dissolution. Pore sizes in hydrogels thus can be controlled by the size of MS. According to the average pore size of MS in different groups, the micro-cavitary hydrogels fabricated with MS in 80-100 group was named as $300 \mu\text{m}$; hydrogels with MS in 100-200 group was named as $200 \mu\text{m}$; hydrogels with MS in 200-300 group was named as $150 \mu\text{m}$.

Fig. 7.2 Size distribution of gelatin microspheres in different groups. Insets show representative images of gelatin microspheres in different groups.

7.3.2 Characterization of micro-cavitory hydrogels

^1H -NMR spectroscopy confirmed the successful methacrylation of gelatin and the degree of methacrylation was 83.95%. With MS varying in sizes, the synthesized gelatin-methacrylate was then used for fabrication of micro-cavitory hydrogels and their physical properties were characterized.

All micro-cavitory hydrogels had a significantly higher porosity than pure hydrogels (**Fig. 7.3a**) and only hydrogels with 200 μm pore size ($92.69 \pm 0.26\%$) showed a slightly lower porosity as compared to hydrogels with 300 μm pore size

(93.34 ± 0.02 %). Compressive modulus of micro-cavitary hydrogels increased with the decrease of pore sizes. Hydrogels with large pores had a lower compressive modulus (5.26 ± 0.04 KPa), while compressive modulus in hydrogels with smaller pores was higher (11.02 ± 0.36 KPa in hydrogels with 200 μm pore size and 9.38 ± 0.17 KPa in hydrogels with 150 μm pore size, **Fig. 7.3b**). Hydrogels without pore structures was the most elastic with compressive modulus of 237.73 ± 0.52 KPa (**Fig. 7.3b**). Hydrogels with larger pores were degraded more rapidly as compared to hydrogels with smaller pores (**Fig. 7.3c**). Only 10% of hydrogels with 300 μm pore size were left after collagenase treatment for 15 minutes, whereas hydrogel constructs with 150 μm pore size still had 30% of their original weight. However, the degradation of pure hydrogels was much slower than micro-cavitary hydrogels. After 30-minute collagenase treatment, pure hydrogels showed only 20% weight loss, while all micro-cavitary hydrogels were completely degraded (**Fig. 7.3c**). Both SEM and confocal images (**Fig. 7.3f and g**) revealed densely-stacked pore structures in micro-cavitary hydrogels, with large pores in hydrogels with 300 μm pore size, middle pores in hydrogels with 200 μm pore size, and small pores in hydrogels with 150 μm pore size. Pore structures in pure hydrogels, however, were quite small, randomly-distributed and irregular in shape. Confocal images also showed that pore structures in hydrogels in a wet status were not interconnected (**Fig. 7.3f**). H&E staining further confirmed these results, with larger pores in hydrogels with 300 μm pore size and smaller pores in hydrogels with 200 μm pore size or 150 μm pore size. Pore structures in pure hydrogels, however, was much smaller than pores in micro-cavitary hydrogels (**Fig. 7.3h**). The total interface increased

with the decrease of pore sizes in micro-cavitary hydrogels (**Fig. 7.3d**). Free space in hydrogels with 300 μm pore size ($70.17 \pm 7.76 \%$) was higher than hydrogels with 200 μm ($63.70 \pm 6.28 \%$) or 150 μm pore size ($65.75 \pm 5.10 \%$), even though there was no significant difference between different micro-cavitary hydrogels (**Fig. 7.3e**). The total interface of pure hydrogels was comparable to hydrogels with 200 μm pore size, but free space of pure hydrogels was much lower than all micro-cavitary hydrogels (**Fig. 7.3d and e**). The physical parameters of different micro-cavitary hydrogels were summarized in Table 2.

Fig. 7.3 Characterization of micro-cavitory hydrogels. (a) Porosity; (b) Compressive modulus; (c) Degradation in vitro; (d) Total interface; (e) Free space; (f) SEM images; (g) Confocal images; (h) H&E staining. The data are presented as the mean $\pm$ standard deviation. * indicates $p < 0.05$; ** indicates $p < 0.01$.

Table 2 Physical parameters of different micro-cavitary hydrogels

Group	Pore size (μm)	Porosity (%)	Compressive modulus (KPa)	Total interface (mm/mm^2)	Free space (%)
300 μm	287.99 ± 74.58	93.34 ± 0.02	5.26 ± 0.04	18.56 ± 1.01	70.17 ± 7.76
200 μm	205.22 ± 55.58	$92.69 \pm 0.26^*$	$11.02 \pm 0.36^{**}$	$27.52 \pm 1.38^{**}$	63.70 ± 6.28
150 μm	146.45 ± 33.15	93.01 ± 0.36	$9.38 \pm 0.17^{**}$	$33.01 \pm 2.87^{**}$	65.75 ± 5.10
Gel	N.A.	60.86 ± 1.50	237.73 ± 0.52	25.91 ± 2.63	16.04 ± 2.22

The data are presented as the mean $\pm$ standard deviation. * indicates $p < 0.05$; ** indicates $p < 0.01$, compared with hydrogels with 300 μm pore size. N.A. indicates not applicable.

7.3.3 Pore-mediated vascularization in micro-cavitary hydrogels

To investigate effects of pore structures on vascularization by EPOCs encapsulated in hydrogels, EPOCs were labelled with DiO, a green fluorescence cell tracker, and tracked in real time for 14 days. In micro-cavitary hydrogels, cells aligned, spread and connected with each other along pore-gel boundaries, which resulted in vascular network formation around pores (**Fig. 7.4**). In contrast, cells in pure hydrogels showed round morphologies and connections between cells close to each other couldn't be established due to inhibited cell spreading (**Fig. 7.4**). These results indicate that cells can respond to pore structures in hydrogels and the induction of pore structures in hydrogels induce vascularization surrounding pores by EPOCs.

Fig. 7.4 Pore-mediated vascularization in micro-cavitary hydrogels. Representative confocal images of cell-laden micro-cavitary hydrogels with different pore sizes on 7 day and on 14 day. Green color indicates DiO-labelled cells; blue color indicates cell nuclei.

7.3.4 Viability, proliferation and differentiation of EPOCs in micro-cavitary hydrogels

After showing pore-mediated vascularization, the effects of pore sizes on viability, proliferation and differentiation of EPOCs encapsulated in hydrogels were evaluated. Cells in micro-cavitary hydrogels showed a high viability over the period of in vitro

culture. Even though cells in pure hydrogels were viable, their number decreased with time (**Fig. 7.5a**). More importantly, cells spontaneously assembled along the pore-gel boundaries and form vascular networks that surrounded pores. In contrast, cells in hydrogels without pore structures exhibited a round morphology continuously (**Fig. 7.5a**).

Cell proliferation increased in micro-cavitary hydrogels, while cells in pure hydrogels exhibited a reduced proliferation (**Fig. 7.5b**). Moreover, cell proliferated more rapidly in 80-100 group than cells in 100-200 and 200-300 groups on day 7 and day 14, which indicates that larger pores encourage cell proliferation in hydrogels as compared to smaller pores.

Differentiation of EPOCs in micro-cavitary hydrogels was investigated through gene expression analysis of endothelial markers CD31, CD105 and VE-Cadherin. The increased expressions of the endothelial markers in micro-cavitary hydrogels showed the improved differentiation of EPOCs in micro-cavitary hydrogels (**Fig. 7.5c-d**). Additionally, the endothelial markers were expressed at a higher level in 100-200 group than in 80-100 group or 200-300 group (**Fig. 7.5c-d**), which indicates that middle pores support promoted EPOC differentiation in hydrogels as compared to larger or smaller pores.

Fig. 7.5 Viability, proliferation and differentiation of EPOCs in micro-cavitory hydrogels. (a) Live/dead staining; (b) WST-1 assay. Gene expression of (c) CD105, (d) CD31, and (e) VE-Cadherin. Green colour indicates live cells; red colour indicates dead cells. The data are presented as the mean $\pm$ standard deviation ($n=3$). * indicates $p < 0.05$; ** indicates $p < 0.01$.

7.3.5 In vitro vascularization of micro-cavitory hydrogels

The influence of pore sizes on in vitro vascularization of hydrogels by EPOCs were further investigated through H&E staining. In micro-cavitory hydrogels, cells that located at inner side of pores organized themselves along the pore-gel boundaries with spreading morphologies and continuous connections between cells. However, in pure hydrogels, scattered cells were separated from each other by gel bulks and exhibited round morphologies (**Fig. 7.6a**). Moreover, vascularization in hydrogels with middle pores was improved as compared to hydrogels with larger or smaller pores. Cells that

aligned and spread along pore structures were more in 100-200 and 200-300 groups than in 80-100 group on day 7. On day 14, cellular boundaries along pore structures in 80-100 group was still incomplete, while pore structures in 100-200 group were well-vascularized. However, due to the small pores in 200-300 group, cells aggregated with each other, which resulted in the formation of cell colonies within some pores.

Immunostaining of CD31 and VE-Cadherin showed positively-stained ring-structures (indicated by asterisks) in 100-200 group on day 14, whereas these structures were not obvious in 80-100 and 200-300 groups (**Fig. 7.6b and c**). These results also demonstrate that middle pores promote hydrogel vascularization as compared to larger or smaller pores. On the other hand, staining of CD31 and VE-Cadherin in pure hydrogels was quite weak, which suggests an undesirable microenvironment for EPOC-mediated vascularization in hydrogels without pore structures.

Fig. 7.6 In vitro vascularization of the micro-cavitary hydrogels. Representative images of (a) H&E staining, (b) CD31 immunostaining, and (c) VE-Cadherin immunostaining. Green color indicates CD31staining; red color VE-Cadherin CD31staining; blue color indicates cell nuclei.

7.3.6 In vivo degradation of micro-cavitary hydrogels

Degradation of micro-cavitary hydrogels in vivo were assessed in nude mice. A macroscopic view of explanted hydrogels revealed a more obvious size decrease in micro-cavitary hydrogels than pure hydrogels (**Fig. 7.7a**). The degradation rate of

hydrogels with larger pores was more rapid than hydrogels with smaller pores (**Fig. 7.7b**). Hydrogels in 80-100 showed 50% weight loss on day 7 and 56% weight loss on day 14. The degradation of hydrogels in 200-300 group was slower than hydrogels in 80-100, with 46% weight loss on day 7 and 50% weight loss on day 14. Hydrogels in 100-200 degraded even more slowly, with 34% weight loss on day 7 and 39% weight loss on day 14. In contrast, pure hydrogels first increased their weight and then slowly degraded (7% increase of weight on day 7 and only 19% weight loss on day 14, **Fig. 7.7b**), showing a different degradation profile from micro-cavitary hydrogels.

To explore possible reasons for improved degradation in hydrogel with large pores as compared to hydrogels with smaller pores, host response to micro-cavitary hydrogels were determined. Macroscopic views of hydrogels at site of implantation showed reduced sizes and improved integration of micro-cavitary hydrogels with the subcutaneous tissues as compared to pure hydrogels (**Fig. 7.7c**, insets). Hydrogels with large pores induced more host tissue invasion than hydrogels with smaller pores (**Fig. 7.7c**). Quantification of cellular infiltration area in 80-100 group showed $5.25 \pm 0.47\%$ on day 7 and $4.04 \pm 0.51\%$ on day 14, which were higher than cellular infiltration area in 100-200 ($0.61 \pm 0.21\%$ on day 7; $1.60 \pm 0.30\%$ on day 14) and 200-300 groups $2.02 \pm 0.32\%$ on day 7; $1.60 \pm 0.25\%$ on day 14, **Fig. 7.7d**). However, no host tissues invaded into hydrogels without pore structures (**Fig. 7.7c**).

Fig. 7.7 In vivo degradation of the micro-cavitary hydrogels. (a) Macroscopic views of different micro-cavitary hydrogels. (b) In vivo degradation of micro-cavitary hydrogels. (c) Representative images of H&E staining of micro-cavitary hydrogels. Insets show representative images of micro-cavitary hydrogels at sites of implantation. (d) Quantification of cellular infiltration area in micro-cavitary hydrogels. The data are presented as the mean $\pm$ standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

7.3.7 In vivo vascularization of micro-cavitary hydrogels

Vascularization of micro-cavitary hydrogels in vivo was investigated through subcutaneous implantation of cell-laden micro-cavitary hydrogels into nude mice. Compared with pure hydrogels, micro-cavitary hydrogels was smaller in sizes and surrounded by more blood vessels from subcutaneous tissues (**Fig. 7.8a**, insets). Furthermore, micro-cavitary hydrogels showed red blood cell-containing vessels (indicated by yellow arrow heads), while there were no such structures in pure hydrogels (**Fig. 7.8a**). These results demonstrate functional connections between

EPOCs in micro-cavitary hydrogels and host vasculature were established. On the other hand, hydrogel with larger pores induced enhanced vascular invasion as compared to hydrogel with smaller pore structures (**Fig. 7.8a**). Quantification of vessel density (**Fig. 7.8b**) showed high vessel density in 80-100 group, with 8.20 ± 3.17 vessels/mm² on day 7 and 8.84 ± 3.18 vessels/mm² on day 14. Vessel density in 100-200 group was 2.51 ± 2.37 vessels/mm² on day 7 and 2.40 ± 2.25 vessels/mm² on day 14, which was fewer than vessel density in 80-100 group. Few invaded vessels were observed in 200-300 group, with no vascular invasion on day 7 and vessel density of 1.75 ± 1.90 vessels/mm² on day 14.

Distribution of endothelial cells in micro-cavitary hydrogels was determined through immunostaining of CD31 and VE-Cadherin. CD31 and VE-Cadherin were extensively-stained in micro-cavitary hydrogels, whereas only a few round cells were positively stained in pure hydrogels. In addition, hydrogels with larger pores showed larger positively-stained areas than hydrogels with smaller pores (**Fig. 7.8b and c**). Both CD31-positive and VE-Cadherin-positive areas in 80-100 group were significantly higher than 100-200 and 200-300 groups on day 7. The positive areas in 80-100 and 100-200 groups were similar while higher than 200-300 group on day 14 (**Fig. 7.8e and f**). These results indicate that large pores support improved hydrogel vascularization in vivo as compared to smaller pores.

Fig. 7.8 In vivo vascularization of cell-laden micro-cavitory hydrogels. Representative images of (a) H&E staining, (b) CD31 immunostaining, and (c) VE-Cadherin immunostaining. Insets show representative images of micro-cavitory hydrogels at sites of implantation; arrow heads indicate red blood cell-containing vessels; green color indicates CD31 staining; red color indicates VE-Cadherin

staining; blue color indicates cell nuclei. Quantification of (d) blood vessel densities, (e) CD31-positive area, and (f) VE-Cadherin-positive area in micro-cavitary hydrogels. The data are presented as the mean $\pm$ standard deviation (n=3). * indicates $p < 0.05$; ** indicates $p < 0.01$.

7.4 Discussions

In this study, vasculogenesis around pore structures was observed in micro-cavitary hydrogels. However, cells in pure hydrogels were entrapped in polymer networks of hydrogels and showed round morphologies (**Fig. 7.4**). Vasculogenesis is de novo development of tube-like structures from individual endothelial cell precursors [381, 382]. Pore structure in hydrogels may provide important physical cues to encapsulated cells. In response to these physical signals, cells migrate, align, and adhere along pore-gel boundaries [361, 362]. In addition, because cells located at inner side of pores are free from constraints of polymer networks, cells can spread and connect with each other to form tube-like structures as they do in 2-dimensional (2D) conditions [326]. Therefore, pore structures are important in inducing vascular network formation in hydrogels.

The influence of pore sizes on cell viability and proliferation were then investigated and we found large pores improved cell proliferation as compared to smaller pores in hydrogels (**Fig. 7.5b**). The increased cell proliferation in hydrogels with large pores is probably due to improved nutrient diffusion within the hydrogel constructs [383]. Porosity in hydrogels with 300 μm pore size was higher than hydrogels with 200 μm pore size (**Fig. 7.3a**). Compressive modulus in hydrogels with 300 μm pore size was

significantly lower than hydrogels with 200 μm or 150 μm pore size (**Fig. 7.3b**). Even though there was no significant difference in free space between different micro-cavitary hydrogels, the free space in hydrogels with 300 μm pore size was also higher than hydrogels with 200 μm or 150 μm pore size (**Fig. 7.3e**). These results indicate that hydrogel constructs with 300 μm pore size are more porous than hydrogel constructs with 200 μm or 150 μm pore size, which leads to improved diffusion of water, ions, and small molecules in the hydrogel constructs [383-385]. As such, cells proliferate more rapidly in micro-cavitary hydrogels with large pores.

On the other hand, both differentiation of EPOCs and vascularization was improved in hydrogels with middle pores as compared to hydrogels with larger or smaller pores (**Fig. 7.5c-e and Fig. 7.6**). Previous studies show that pore structures can improve muscle cell differentiation and osteogenic differentiation of MSCs [386, 387]. Pore-gel boundaries may also influence differentiation of EPOCs. As pore-gel boundaries increased with the decrease of pore sizes (**Fig. 7.3d**), hydrogels with the middle pores can provide more physical cues from pore-gel boundaries to encapsulated EPOCs than hydrogels with large pores, which leads to the improved EPOC differentiation and more rapid hydrogel vascularization. As a result, the expression of CD105, a marker of angiogenic endothelial cells [377, 378], in hydrogels with middle pores was higher than hydrogels with larger pores on day 14. In hydrogels with smaller pores, even though total pore-gel boundaries were longer (**Fig. 7.3d**), cell aggregates were formed in some smaller pores (**Fig. 7.6**), which has been shown to inhibit efficient vascularization [362]. The ineffective vascularization may result in the decreased

CD105 expression on day 14. On the other hand, CD31 and VE-Cadherin mediate adhesion between endothelial cells [376, 380]. As pore-gel boundaries in hydrogel constructs with larger pores were fewer (**Fig. 7.3d**), cell-cell connections along pores are less, which leads to a lower expression of CD31 and VE-Cadherin in hydrogel constructs with larger pores than hydrogel constructs with middle pores. In hydrogels with smaller pores, the formation of cell aggregates reduced cell-cell connections (**Fig. 7.6**), thereby resulting in the decreased gene expressions of CD31 and VE-Cadherin as compared to hydrogel constructs with middle pores.

After in vivo implantation, hydrogel with 300 μm pore size showed significantly more host tissue invasion than hydrogel with 200 μm or 150 μm pore size (**Fig. 7.7c and d**). Because gelatin can be degraded by enzymes secreted by cells [388, 389], degradation of hydrogels with larger pores in vivo was more rapid than hydrogels with smaller pores accordingly (**Fig. 7.7a and b**). Even though hydrogels with 200 μm pore size had larger pore sizes than hydrogels with 150 μm pore size, hydrogels with 200 μm pore size showed fewer host tissue invasion than hydrogels with 150 μm pore size on day 7 (**Fig. 7.7c and d**). Substrate stiffness can affect cell movement [39, 40] and speed of cell migration decreases with the increase of hydrogel stiffness [390]. A higher stiffness in hydrogels with 200 μm pore size than hydrogels with 150 μm pore size (**Fig. 7.3a**) may delay host tissue ingrowth. However, the difference in host tissue invasion between hydrogels with 200 μm pore size and hydrogels with 150 μm pore size was not obvious with the time of in vivo implantation. Both two groups showed similar host tissue invasion on day 14 (**Fig. 7.7c and d**). It is highly possible that the effect of pore

size smaller than 300 μm on inducing host tissue invasion into hydrogel constructs is not obvious. Previous studies also show that pore sizes smaller than 400 μm in β -tricalcium phosphate scaffolds limit ingrowth of host tissues [54, 360]. In addition, we found cell infiltration area in hydrogels with 300 μm pore size decreased from day 7 to day 14 (**Fig. 7.7d**). In hydrogels with 300 μm pore size, the invaded cells accumulated in pore structures in hydrogels on day 7 and then infiltrated into gel phase on day 14 (**Fig. 7.7c**). The re-distribution of invades cells from pore structures to gel bulk may be responsible for the decreased infiltration area. Previous studies show that cellular infiltration into porous PEG hydrogels or β -tricalcium phosphate scaffolds increases with the time of in vivo implantation [54, 136]. Because the invaded cells cannot immigrate into material phase of PEG hydrogels or β -tricalcium phosphate scaffolds, cells accumulate in pore structures of constructs with time. However, cells can infiltrate into gelatin based hydrogels [370]. Due to cell migration, the infiltration area on day 14 decreased as compared to day 7. Although hydrogels with 150 μm pore size had fewer host tissue invasion than hydrogels with 300 μm pore size on day 7, cell infiltration into gel phase could also be observed on day 14 (**Fig. 7.7c**). Thus, cell infiltration area in this group showed a similar trend as hydrogels with 300 μm pore size (**Fig. 7.7d**). In contrast, cell infiltration into pore structures in hydrogels with 200 μm pore size was quite limited on day 7 and no infiltration of cells into gel phase could be observed on day 14 (**Fig. 7.7c**). As a result, cell infiltration area in this group increased from day 7 to day 14 (**Fig. 7.7d**).

When implantation of cell-laden micro-cavitary hydrogels in vivo, red blood cell-

containing vessels were observed in hydrogels (**Fig. 7.8a**), which indicates functional connections between EPOCs encapsulated in hydrogels and host vasculature were established. However, invaded tissues in micro-cavitary hydrogels without EPOC encapsulation showed no such red blood cell-containing vessels (**Fig. 7.7c**), which suggests that EPOCs play an important role in inducing host vascular invasion into hydrogels. Other studies also show that endothelial cells or endothelial progenitor cells are essential in neovascularisation within scaffolds in vivo [343, 370]. Angiopoietin-2 (ANG-2), fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF) and other chemokines released by EPOCs may be responsible the recruitment of vascular cells from host tissues [336]. Meanwhile, large pores promoted in vivo hydrogel vascularization as compared to smaller pores (**Fig. 7.8**). Hydrogel vascularization in vivo is mainly completed by the invaded vascular tissues [135, 136, 360, 370]. In our studies, we also observed that most of the vascular tissues in hydrogels were host-derived (**Fig. 7.8a**). As larger pore sizes in hydrogels could induce more host vascular invasion as compared to small pores (**Fig. 7.8**), hydrogel vascularization in hydrogels with 300 μm pore size was improved best in vivo. In addition, previous studies show that vessel densities of porous scaffolds first increase with the time of in vivo implantation and then keep constant [360]. Besides, scaffolds with small pores take a longer time to reach the maximal vessel densities as compared to scaffolds with large pores [54, 135]. Our studies show the similar results. In hydrogels with 300 μm or 200 μm pore size, vessel densities on day 7 were comparable to vessel densities on day 14 (**Fig. 7.8d**), which indicates that maximal vessel densities have been reached by

day 7 in these two groups. However, vessel densities in hydrogels with 150 μm pore size increased from day 7 to day 14 (**Fig. 7.8d**). It is likely that vascular invasion still occurs in this group on day 14 and vessel densities haven't reached the peak yet.

In the future studies, we will further explore the use of micro-cavitary gelatin-hydrogels with an optimized pore size to treat ischemia diseases, such as myocardial infarction [391] and critical limb ischemia [392]. Due to the proangiogenic property of the micro-cavitary gelatin-hydrogel, it may be an effective treatment to these diseases.

7.5 Summary

In this study, the effects of pore sizes on hydrogel vascularization by EPOCs were evaluated both in vitro and in vivo. Vascular networks were formed around pore structures in micro-cavitary hydrogels. The middle pore size supported best differentiation of EPOCs and thus best hydrogel vascularization in vitro. When implantation in vivo, functional connections between EPOCs encapsulated in micro-cavitary hydrogels and host vasculature were established. Vascularization in vivo was promoted best in hydrogels with the large pore size due to the increased vascular tissue invasion. These results highlight the difference between in vitro and in vivo culture conditions and indicate that pore sizes shall be designed for in vitro and in vivo hydrogel vascularization respectively. Pore sizes for hydrogel vascularization in vitro shall be middle ones and pore sizes for hydrogel vascularization in vivo shall be large ones.

Chapter 8: Conclusion and future work

8.1 Conclusion

In this thesis, we promote vascularization of tissue constructs and cardiac differentiation of ESCs through manipulation of cell-induced or biomaterial-derived physical signals. We verified the feasibility of using PLGA nanoparticles to label and track ESC status during EB development. The unique cell viability monitoring function of the nanoparticles allowed clear presentation of live cell distribution within EBs, which has high potential to be a powerful tool to screen EBs with comparable sizes.

In addition, we successfully imitate some critical aspects of cartilage hypertrophy using *in vitro* engineered living cartilage templates and osteogenic treatment. Osteogenic treatment induces cartilage hypertrophy and creates channels for vascular invasion in LhCG constructs when implantation *in vivo*. Hypertrophic transition of LhCG constructs using osteogenic treatment may contribute to the establishment of an *in vitro* cartilage hypertrophy model that mimic initial stage of endochondral ossification.

Besides manipulation of cell behavior-origin physical signals, stiffness and topography of biomaterials are also controlled. Gelatin-dopamine was synthesized for PDMS surface coating. Compared with gelatin, gelatin-dopamine coating reduced hydrophobicity and increased protein adsorption on the PDMS surface. In addition, the gelatin-dopamine coated PDMS surface greatly improved ESC adhesion, proliferation

and pluripotency maintenance as compared to gelatin-coated surfaces. ESC-derived EBs also showed increased adhesion and facilitated myocardial differentiation on the gelatin-dopamine coated PDMS surface. These results demonstrated that gelatin-dopamine coating could effectively improve PDMS surface properties for the long-term ESC culture and enhanced ESC myocardial differentiation. Following the establishment of coating strategy, the influence of PDMS substrate stiffness on ESC pluripotency maintenance and cardiac differentiation is further investigated. Different PDMS substrates exhibit different profiles of substratum properties, which affect the behaviors of ESCs and EBs on PDMS surfaces. ESC adhesion and proliferation, as well as EB attachment, were improved on the 40:1 PDMS substrate as compared to the other PDMS substrates. Meanwhile, compared with the other PDMS substrates, ESC pluripotency preservation was improved on the 5:1 PDMS substrate, while the 40:1 PDMS substrate encouraged ESC cardiac differentiation. These results are successfully applied in fabrication of biochips for ESC pluripotency maintenance and cardiac differentiation. The use of this simple surface modification strategy to stabilize the adhesion of ESCs and EB while enhance their pluripotency and differentiation, as well as substrate stiffness optimization, can possibly contribute to the development of a real heart-on-a-chip for high throughput drug screening in the future.

Finally, the effects of pore density and pore sizes on hydrogel vascularization is studied both in vitro and in vivo. An increase of pore density greatly enhances the proliferation and endothelial differentiation of encapsulated EPOCs in Gel-MCG constructs. In particular, compared to MS:Gelatin (1:1) counterpart, MS:Gelatin (2:1)

constructs with higher pore density allows the interconnected vascular network formation within the whole constructs upon the degradation of thinner gel walls among neighboring pores. Extensive vascular invasion is observed within the MS:Gelatin (2:1) constructs when implanted in vivo. These findings demonstrate the vascularization of Gel-MCG constructs can be improved by increasing cavitory density. The effects of pore sizes on hydrogel vascularization by EPOCs is also evaluated in vitro and in vivo. Differentiation of EPOCs and hydrogel vascularization are promoted by middle pores as compared to large or small pores. When implantation in vivo, hydrogels with large pores induce more red blood cell-containing vessels and vascular tissues than hydrogels with small or middle pores. These results indicate that middle pores promote hydrogel vascularization in vitro, while hydrogel vascularization in vivo is improved by large pores. These study reveals the importance of pore densities and pore sizes in hydrogel vascularization both in vitro and in vivo and these findings can help design an optimal pore density and size for hydrogel constructs to enhance their vascularization.

In summary, the studies in this thesis prove that physical signals can be utilized to improve performances of tissue engineered constructs. By utilization of physical signals from cells, we develop a new tool to monitor ESC viability during EB development to screen EBs with homogenous sizes and vascularize chondrocyte-derived cartilage templates with hypertrophic induction. Through manipulation of stiffness and topographic cues pf biomaterials, we improve efficiency of ESC cardiac differentiation and hydrogel vascularization.

8.2 Future work

Though effective in screening EB with homogenous sizes, monitoring cell viability in EBs is still low in throughput. We will explore the control of EB sizes in a more straightforward while scalable way in the future. Due to resistance of alginate hydrogels to protein adsorption, ESCs can aggregate in pore structures of alginate hydrogels to form EBs. Pore structures can be created by dissolvable gelatin microspheres and pore sizes can be controlled by the sizes of gelatin microspheres. EBs with homogenous sizes can thus form in pore structures with comparable sizes. Additionally, alginate hydrogels provide a 3D environments for EB formation and the generated EBs can free from alginate hydrogels by simply sodium citrate treatment to unlink alginate polymer networks. Since EBs are generated in a 3D rather than 2D setting, the efficiency of EB formation can be enhanced dramatically.

Stiffness and topography of ECM have combinatorial effects on cell behaviours. However, few studies have investigated ESC responses to substrate stiffness in combination with geometric cues. In the future, we will use photolithography to fabricate micropatterned PDMS substrates, which can be used to study the influence of topographic cues on ESC pluripotency preservation and cardiac differentiation. Together with flexibly-adjusted PDMS substrates, numerous information regarding effects of substrate stiffness and topography on ESC self-renewal and specification can be obtained. In addition, the improved ESC pluripotency or cardiac differentiation potential can be further verified in 3D conditions or in vivo. Increased efficiency of EB formation in vitro and teratoma formation in vivo can confirm the enhanced ESC.

Facilitated maturation of ESC-derived myocytes in 3D scaffold in vitro and in vivo can support potentiated cardiac differentiation of ESCs.

The effects of pore density and pore sizes on hydrogel vascularization by EPOCs have been proved by our previous studies. However, whether hydrogel vascularization by co-culture of EPOCs and MSCs can be regulated by pore density and pore sizes haven't been evaluated yet. Paracrine function of MSCs can provide nutrients to cocultured endothelial cells. Meanwhile, MSCs can differentiate into perivascular cells to mechanically support endothelial cells. Vascular Networks formed by both MSCs and endothelial cells are more likely to mimic blood vessels in vivo. In the future, we will prove the hypothesis that hydrogel vascularization by co-culture of MSCs and endothelial cells can also be modulated by pore densities and pore sizes.

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List of Publications

Journal articles

1. **J. Fu**, C. Wiraja, R. Chong, C. Xu and D.A. Wang, Real-time and non-invasive monitoring of embryonic stem cell survival during the development of embryoid bodies with smart nanosensor. *Acta Biomater*, 2017. **49**: p. 358-367.
2. **J. Fu**, S. Lim, P. He, C. Fan and D.A. Wang, Osteogenic Treatment Initiating a Tissue-Engineered Cartilage Template Hypertrophic Transition. *Ann Biomed Eng*, 2016. **44**(10): p. 2957-70.
3. **J. Fu**, K. Quek, Y. Chuah, C. Lim, C. Fan and D.A. Wang, The effects of gelatin–dopamine coating on polydimethylsiloxane substrates on pluripotency maintenance and myocardial differentiation of cultured mouse embryonic stem cells. *Journal of Materials Chemistry B: Materials for biology and medicine*, 2016. **4**(48): p. 7961-7973.
4. **J. Fu**, C. Fan, W. Lai and D.A. Wang, Enhancing vascularization of a gelatin-based micro-cavitary hydrogel by increasing the density of the micro-cavities. *Biomed Mater*, 2016. **11**(5): p. 055012.
5. C. Fan, **J. Fu**, W. Zhu and D.A. Wang, A mussel-inspired double-crosslinked tissue adhesive intended for internal medical use. *Acta Biomater*, 2016. **33**: p. 51-63.
6. P. He, **J. Fu**, and D.A. Wang, Murine pluripotent stem cells derived scaffold-free cartilage grafts from a micro-cavitary hydrogel platform. *Acta Biomater*, 2016. **35**: p. 87-97.

Book chapter

1. **J. Fu**, P. He, and D.A. Wang, Tissue Engineering for Artificial Organs: Regenerative Medicine, Smart Diagnostics and Personalized Medicine, A. Hasan, Editor. 2017, Wiley VCH. p. 243-296.

Manuscripts in process

1. **J. Fu**, Y. Chuah, W. Ang, N. Zheng, D.A. Wang, Optimized PDMS Microfluidic Biochips for ESC Pluripotency Maintenance and Cardiac Differentiation. (Accepted by Biomaterial Science)
2. **J. Fu**, H. Muhammad, C. Wiraja, C. Xu and D.A. Wang, Improvement of EPOC-mediated vascularization in gelatin-based hydrogels through pore size manipulation. (Accepted by Acta Biomaterialia)

Manuscripts in preparation

1. **J. Fu**, and Dong-an Wang, The effects of substratum stiffness on pluripotency maintenance and cardiac differentiation of iPSCs.
2. **J. Fu**, and Dong-an Wang, Vascularization in Engineered Tissues for Regenerative Medicine: Trends toward Remodeling Inspiration and Organ-Specificity.
3. **J. Fu**, and Dong-an Wang, Co-culture of MSCs and HUVECs in porous gelatin-based hydrogels for therapeutic application in vivo.