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STUDIES OF CELL MOTILITY AND DIFFERENTIATION IN COMPLEX 3D IN VITRO MICROENVIRONMENT

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Abstract

Cell behaviors, such as motility and differentiation, which are highly regulated by the complex 3D in vivo microenvironment, have been extensively studied in the past decades to better understand the mechanisms of development, disease, and healing. Due to the highly complex nature of the in vivo environment and limited resources and access to study cell behaviors in vivo, great efforts have been made to develop in vitro systems to mimic the in vivo microenvironment for a better understanding of cell-microenvironment interactions. However, most of the commonly used in vitro systems are static, which cannot mimic the dynamics of the microenvironment in vivo. In this dissertation, our goal was to create a 3D complex in vitro microenvironment that can dynamically change its internal architecture and to employ such in vitro dynamic complex microenvironment to study cell motility and differentiation behaviors for the application of tissue engineering, regenerative medicine, and cancer metastasis.

In this work, 3D dynamic shape memory scaffolds were developed and employed to investigate cell motility and differentiation behaviors when the cellular microenvironment dynamically changes in vitro. The scaffolds have fibrous structure, which can potentially mimic the collagen matrix fibrous structure in vivo. And, more importantly, the scaffolds can dynamically change internal architecture on command, which can potentially mimic the dynamic ECM architectural change in vivo during tissue development and cancer metastasis.

To achieve this goal, the first part of this dissertation (Chapter 2 – 3) developed a programmable 3D shape memory electrospun scaffold that can dynamically change fiber alignment upon triggering under cytocompatible conditions. In these chapters, the programmable
dynamic 3D scaffold was employed to study stem cell motility, cancer cell polarization, and cancer cell motility, for ultimate application in stem cell homing and cancer metastasis studies. Stem cell motility, cancer cell motility, and cancer cell morphology were found to be directed by shape-memory-actuated changes in scaffold internal architecture.

In Chapter 4, the objective was to investigate stem cell differentiation when cells undergo dynamic scaffold internal architectural change for the application of bone tissue engineering and critical-sized bone defect treatment. The shape memory electrospun scaffold investigated in Chapter 2 and Chapter 3, as well as a shape memory foam scaffold, were employed to examine the human adipose-derived mesenchymal stem cells osteogenic differentiation capacity. We found that the dynamic change of the scaffold internal architecture would not hinder the stem cell osteogenic differentiation.

In Chapter 5, we utilized a scaffold-free 3D culture system and investigated the effect of a non-scaffold-related factor—low oxygen tension—on mature chondrocytes dedifferentiation behavior for the application of cartilage tissue engineering. Low oxygen has been frequently implicated as a limitation associated with synthetic 3D scaffolds when the scaffolds have small pore size or poor interconnectivity, or both, due to the fact that low oxygen and limited nutrient diffusion can cause cell death. However, low oxygen tension during culture could be beneficial for cartilage tissue engineering, as cartilage is an avascular tissue and low oxygen is present in vivo during chondrogenesis and in adult articular cartilage.

Finally, in Chapter 6, conclusions and future directions are discussed and summarized.
STUDIES OF CELL MOTILITY AND DIFFERENTIATION IN COMPLEX 3D IN VITRO MICROENVIRONMENT

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B.S., Beijing University of Chemical Technology, 2009

DISSEPTION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Bioengineering.

Syracuse University
December 2016
To my dearest family

This journey would not have been possible without the endless support and love from you all.
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Chapter 1: Introduction

1.1 Cell behaviors in complex microenvironments – bridging the gap between

*in vivo* and *in vitro* studies

Cell behaviors, such as proliferation, differentiation, and migration, have been extensively studied in the past decades to better understand the mechanisms of development, disease, and healing. Due to the highly complex nature of the *in vivo* environment and limited resources and access to study cell behavior *in vivo*, great efforts have been made to develop *in vitro* systems to mimic the *in vivo* microenvironment for a better understanding of cell-microenvironment interactions. With the help of advanced engineered biomimetic *in vitro* microenvironment, the ultimate goal is to develop engineered tissues that possess similar biochemical and mechanical functionality as native tissue, and the capability of regeneration, and to decipher the mechanisms of diseases then to come up with more sufficient diagnoses and treatments.

To bridge the gap between the *in vivo* and *in vitro* cell microenvironment, this thesis focuses on several specific challenges to create *in vitro* complex microenvironments for studies of cell behaviors. These challenges include: (1) most of the *in vitro* systems currently available are static, which cannot mimic the dynamic nature of the *in vivo* microenvironment; (2) most of the *in vitro* systems culture cells under ambient oxygen condition, which cannot mimic the low oxygen environment in some tissues such as articular cartilage *in vivo*. This present dissertation focuses on addressing these challenges by: (1) introducing 3D scaffolds with dynamically changing internal architecture that mimic the dynamics of the extracellular matrix (ECM) *in vivo*, to study
the effect of the dynamic microenvironment architectural change on cell alignment, cell polarization, cell motility and stem cell differentiation; and (2) by investigating control of oxygen tension during \textit{in vitro} cell culture, a microenvironmental factor critical to many \textit{in vitro} culture approaches, both those involving scaffolds and those that are scaffold-free, to study the effect of low oxygen on stabilizing cell phenotype particularly for the application of cartilage tissue engineering.

\textbf{1.2 ECM structure and chemistry – critical regulator of cells \textit{in vivo}}

To create \textit{in vitro} systems that mimic the \textit{in vivo} microenvironment for cell behavior studies and biomedical research, it is critical to understand how the \textit{in vivo} microenvironment functions, how it interacts with cells, and why such interaction is important for tissue development, disease progression, and healing.

\textbf{1.2.1 ECM}

ECM is the major component of the \textit{in vivo} cellular microenvironment. It forms a fibrous mesh network that provides physical support, tissue boundary and signaling molecules to regulate cell functions. ECM is highly \textit{dynamic} and it constantly remodels. Such dynamic remodeling directs cell behavior, and cells in return remodel the ECM as well. Modification of ECM physical properties, such as stiffness, elasticity, and architecture, or biochemical properties, such as binding affinity of certain molecules, ligands and receptors, would result altered cell behavior. The dynamics of ECM plays a critical role in various cellular events during tissue development, disease progression, and healing.
1.2.2 ECM in tissue development

ECM is actively involved in virtually every stage of tissue development. One of the most well-studied cases is neural crest cell migration guided by ECM [1]. When neural tube closes, neural crest precursor cells delaminate and migrate through the periphery and then differentiate into different cell types. Such guided cell migration is achieved by a gradient of ECM molecule concentration. Cells migrate from regions of low ECM concentration where adhesion is weak, to regions of higher ECM concentration where adhesion is stronger, but slow down if the adhesion is too great. At the same time, migration is also directed by proteases which can digest ECM components and form cleavage [2]. When neural crest precursors migrate out and further differentiation, the stiffness, elasticity, topography of ECM, together with growth factors within ECM network together would further direct them to differentiate into various cells at various locations.

Branching morphogenesis is another example showing multiple aspects of ECM involved in development. Previous study showed that local anisotropies of tension produced by fibrous components of ECM could determine where the branching happens. Hinck and colleagues altered the branching direction by asymmetrical induction of sulfated GAGs at the mammary gland terminal end [3]. Besides biomechanical effect on morphogenesis, biochemical binding of ECM to receptors on cell surface could also affect how branching occurs. Linton and colleagues found that ECM protein nephronectin is an essential ligand that binds α8β1 integrin to induce glial cell-line derived neurotrophic factor (GDNF) which is critical for bud formation and branching in kidney development [4].
1.2.3 ECM in disease progression

ECM dynamics actively supports tissue development as previously mentioned. However, abnormal ECM dynamics could lead to disease. Over-produced ECM or decreased ECM turnover is considered to be associated with tissue fibrosis [5]. Increased collagen density has been showed to be an indicator of breast cancer and it could promote mammary tumor initiation and progression [6]. Not only the ECM composition changes, the architecture of the ECM is also different than healthy mammary tissue. In healthy breast tissue, the collagen fibrils are relaxed and randomly oriented. However in breast cancer happens, collagen fibrils are highly organized, either tangentially aligned around the tumor to restrain invasion in case of ductal carcinoma in situ (DCIS) [7], or radially aligned to provide “tracks” for cancer cells to invade into the surrounding stroma in pregnancy-associated breast cancer (PABC) [8,9]. As ECM participates in many signaling pathways, any abnormal deregulation of these pathways could also contribute to carcinogenesis. For example, overproduced CD44 would facilitate growth factor signaling, and further induce cancer [10].

Abnormal ECM physical and biochemical dynamics clearly play an important role in disease, especially in cancer. However, it still remains largely unknown how exactly the cell-ECM interactions would initiate and facilitate cancer progression.

1.2.4 ECM in healing

Similarly, the dynamics of ECM and each of its components, such as collagen, fibronectin, glycosaminoglycan, play a significant role in every stage of the healing process in vivo. When a wound is created, the healing starts immediately by blood clotting and fibronectin deposition to
create a temporary “scaffold”. Fibroblasts are then activated and recruited by growth factors, such as platelet-derived growth factor (PDGF) and insulin-like growth-factor-1 (IGF-1) to make the fibroblasts migrate towards the wound site, proliferate, and secret more ECM components such as collagen and glycosaminoglycan [11]. The fibroblasts migration is mainly guided by growth factor gradient. The physical structure, elasticity and the ECM composition dynamically remodel as well from a temporary matrix formed mainly from fibronectin, to a framework that is “intertwined” by many capillaries and has a woven structure with high content of hyaluronic acid and fibronectin, and then further to a matrix that has decreased amount of hyaluronic acid and high content of collagen [11].

1.3 Engineered in vitro microenvironments

In the past decades, engineers and scientists have made great progress on creating in vitro systems that are biocompatible, biomimetic, biodegradable, and can be precisely controlled to have specific physical or chemical properties, to study cell mechanobiology, and to achieve the ultimate goal of facilitating in vitro tissue growth and repair in order to produce more biofunctional engineered tissue.

Such systems can be generally categorized by their origin as naturally derived matrix, synthetic 2D substrates, and synthetic 3D scaffolds, as described in details below.

1.3.1 Natural polymers

Natural polymers have been widely used as in vitro cell culture platforms to study cell-microenvironment interactions, due to their diverse functions in the native setting. They are
cytocompatible, biodegradable, anti-inflammatory, and could be further tailored or engineered into
different structure or have specific binding affinity to fit the needs for tissue engineering and
regenerative medicine.

Collagen, as an example of protein-origin natural polymers, is considered as one of the
ideal scaffolds or matrix for tissue engineering as it is the major component of the ECM. It provides
essential network and the ability to incorporate growth factors for cells to attach, proliferate,
differentiate, and migrate. Because collagen has high mechanical strength, great cytocompatibility,
and the ability to be crosslinked and further tailored for different mechanical and degradation
properties [12], collagen gel or matrix has been widely used to study various cell behavior in vitro.
For example, collagen gel has been used to encapsulate cells to study external mechanical
stimulation effect on cell [13], primary neural precursor cell expansion and differentiation [14],
growth factor effects on stem cell differentiation for the application of tissue engineering [15],
human neutrophil motility in 3D matrix [16], and breast cancer metastatic invasion enhanced by
adhesion receptors such as CD44 [17], etc. However, most collagen gel or matrix used in the
in vitro studies is isolated from animal tissues. It brings out concerns regarding to inconsistency from
batch to batch and potential viral contamination. Alternative collagen produced by recombinant
technique has been explored but the cost is still high, and the production process and the
degradability are hard to control [12].

Polysaccharides, which are constituted by sugar monomers, are another popular class of
natural polymers that has been widely employed as scaffolds for in vitro studies. Most commonly
used polysaccharidic polymers are alginate, agarose, chitosan, hyaluronan, chondroitin sulphate,
dextran, etc.
Alginate has been widely used in cartilage tissue engineering studies. It can be used to encapsulate chondrocytes to have chondrocytes have round morphology to better maintain their chondrogenic phenotype when cultured in vitro, as chondrocytes tend to lose their chondrogenic phenotype when cultured on in vitro 2D surface on which they show a spread-out morphology. The effects of incorporating different growth factors on better maintaining chondrogenic phenotype when the chondrocytes are encapsulated in alginate has been extensively investigated in many studies [18–22]. Hyaluronan, as another examples, has been frequently used for studies focused on cartilage, bone, and osteochondral applications, implantable drug delivery devices [12] as it has excellent matrix water regulation, structural support, lubrication, viscoelastic properties.

Generally, polysacchridic polymers are non-toxic, biodegradable, and have lower cost than protein-origin polymers. However, their limitations include variability from batch to batch, hard to process, limited range of mechanical properties, and limited capability to be tailored to meet specific requirements.

1.3.2 Synthetic 2D substrates

Synthetic biomaterials are developing rapidly to fulfill the needs for tissue engineering application and fundamental biological studies – to better understand the cell-microenvironment interactions to improve the current disease diagnose and treatment technology. Recent advances include various non-toxic, cytocompatible synthetic biomaterials that have be engineered to have different bulk chemical and mechanical properties, complex surface modification techniques, as well as creation of elaborate surface topography that can be precisely designed and controlled to study their effect on cell behavior. The ultimate goal for these synthetic biomaterials is to
recapitulate the *in vivo* cellular events and potentially further enhance the tissue regeneration and prevent disease progression *in vitro*.

Many synthetic two-dimensional substrates have been developed with diverse bulk or surface features. When cells cultured *in vitro* in plastic petri dishes or flasks, they behave dramatically different than when they were *in vivo*. One of the main reasons is that the stiffness of the substrates the cells are attached to are far different. Cell morphology and their functions can highly depend on substrate stiffness when chemical conditions are constant. Therefore, synthetic substrates with well controlled bulk stiffness have been carefully studies to examine their effect on cell behavior. Yeung and colleagues [23] tested cell morphology, cytoskeletal structure and adhesion of multiple cell lines on 2D protein-laminated polyacrylamide gel substrates with various stiffness, ranging from 2 to 55,000 Pa. They found the morphologies of different cell types change both quantitatively and qualitatively with substrate stiffness, and the varied substrate stiffness can initiate the altered adhesion receptors.

Besides bulk material stiffness, substrate surface properties can also play an important role in corresponding cell behavior. For example, surface treatment such as RGD peptides (R: arginine; G: glycine; D: aspartic acid) grafting [24,25], dopamine [26] and Laminin-5 [27] coating, have been developed and widely employed to enhance cell attachment on synthetic substrates. Surface chemical modification not only can be utilized to enhance cell attachment, but could also influence stem cell proliferation and differentiation as shown in Chen and colleagues’ work [28]. They developed a series of poly-caprolactone (PCL) films with distinct pendant small functional groups, such as hydroxyl (−OH), methyl (−CH$_3$), carboxyl (−COOH) and amino (−NH$_2$), and examined the surface wettability, protein adsorption, and human mesenchymal stem cells lineage
commitment influenced by these functional groups. They found PCL−NH$_2$ was favorable for osteogenesis, PCL and PCL−CH$_3$ films enhanced the adipogenesis, while the PCL−CH$_3$ was the most favorable for chondrogenesis. It supported the hypothesis that substrate surface chemistry would affect stem cell lineage commitment.

Another surface modification technique, micropatterning, can create much more complicated substrate surface features than simple coating or grafting the entire surface, to control cell attachment within highly confined regions. A previous study performed by Balaban and colleagues micropatterned small squares or small circles on polydimethylsiloxane (PDMS) substrates and studied the real-time local force applied by the cardiac myocytes to the substrates and the assembly of focal adhesions [29]. Besides fundamental mechanobiology studies benefited by using micropatterning technique, stem cell lineage commitment and differentiation was also extensively studied by utilizing such highly defined and controlled patterns. For example, by controlling pattern shape to confine cell morphology [30,31], or by controlling pattern size to manipulate cell colony and aggregate size [32], or by controlling pattern alignment to create highly aligned cells in one direction [33] have all shown to direct stem cells to differentiation into various lineages. Asymmetric micropatterns have also been employed to study cell polarization and migration behavior [34].

Synthetic substrates could also be engineered to have complex surface topography to study the substrate topography effect on directing cell behavior, as microenvironment topography plays an important role in guiding cell behavior in vivo as well. Studies have shown the surface topography not only can affect cell adhesion and proliferation [35], but it could also direct cell differentiation [36], and migration in various cell types [37,38].
Many advanced techniques have been developed and well characterized to create highly defined and well controlled synthetic 2D substrates to study the cell-substrate interaction, with the hope to provide in-depth understanding of such cell-substrate interactions during cellular events such as development, disease progression and healing. However, 2D substrates can never truly mimic the *in vivo* microenvironment as the cells are always embedded in a 3D environment *in vivo*. They provide the ease of fabrication, property manipulation, and straightforward cell attachment. But to further explore the mechanism of cell-microenvironment interplay, 3D biomimetic scaffolds are in great needs.

### 1.3.3 Synthetic 3D scaffolds

Diverse techniques have been emerged to fabricate synthetic 3D scaffolds, which are more biomimetic than 2D substrates since the cells are exposed to 3D microenvironment *in vivo*, to study the dynamics of the cells and their surrounding matrix. These 3D scaffolds are aimed to be biocompatible, biodegradable, and able to support cellular activities, and further enhance tissue regeneration. Such scaffolds are desired to possess comparable mechanical properties and architecture as native tissues, high porosity and interconnectivity to permit nutrient diffusion, capability to incorporate healthy attached cells and signaling molecules, and the potential to be implanted into human body to further enhance new tissue formation without causing inflammatory reactions. Several commonly used techniques include porogen leaching, electrospinning, freeze drying, 3D printing, etc.

Porogen leaching method is one of the popular techniques that have been widely used to generate porous scaffolds for the application of tissue engineering. Salt, wax and sugars are most
commonly used porogens. The scaffold pores, percentage of porosity can be controlled by varying the amount of porogen, the size and the shape of the porogen. Scaffold fabrication is easy to carry out. Huang and colleagues [39] created a highly porous nano-sized hydroxyapatite (HA) reinforced poly-2-hydroxyethylmethacrylate (PHEMA)/polycaprolactone (PCL) scaffolds using porogen leaching technique. They achieved porosity as high as 84% and approximately 300 – 400 µm pore size, and robust growth and proliferation of primary human osteoblasts. Such porous nanocomposite scaffolds can be potentially employed for bone repair.

Electrospinning uses electrostatic force to produce polymer fibers and eventually create a fibrous mat as a 3D microenvironment for cells. A jet of polymer solution is charged, and the fiber collector on the other side is charged of opposite polarity. Once the polymer solution is ejected, it will be charged and the resultant fibers will be collected on the collector and accumulated into a mat. The fiber morphology, fiber diameter, pore size and fiber orientation are controlled by varying electrospinning parameters such as polymer solution concentration, voltage of the electric field, the distance between the fiber ejector and the collector, fiber collector drum rotation speed, etc. One of the most significant advantages of electrospinning technique is that it can produce 3D fibrous structure that mimics the ECM architecture in vivo. This technique has been widely used for the biomedical applications such as fundamental cell mechanobiology studies [40], tumor cell migration [41], wound healing [42–44], artificial blood vessels [45], drug delivery [46,47], etc. However, with the favorable capability of creating fibrous scaffold architecture with tunable fiber feature, current electrospinning technique still often faces the limitations of insufficient cell infiltration, time consuming fabrication process, difficulties to scale up, and low drug loading efficiency.
1.4 Shape memory polymers

The aforementioned in vitro models, including naturally occurring polymers as well as synthetic 2D substrates and 3D scaffolds, have all made tremendous progress on providing various approaches to study cell-microenvironment interactions and potential applications in biomedical research, tissue engineering and regenerative medicine. However, one of the most significant limitations is the static nature of these systems, which cannot mimic the dynamic nature of the in vivo environment. As ECM dynamics plays a huge role in regulating cell function in vivo as mentioned in section 1.2, in vitro systems that can have dynamic changes of properties, such as stiffness, topography, surface feature, 3D architecture, are in great need to better mimic the in vivo microenvironment. To fulfill this need, here we introduce a category of “smart” materials, called shape memory polymers (SMPs).

Shape memory polymers (SMPs) are a class of smart synthetic biomaterials, that can undergo programmed changes in shape from a temporarily fixed shape to a permanently memorized shape when exposed to an external stimulus [48], such as direct heat [49], UV irradiation [50], electrical current [51], or magnetic field [52]. As they can dynamically change their certain properties upon trigger, this active process could be further designed and tailored to mimic the dynamic change of the microenvironment in vivo. Many research results have shown great promise for the application of biomedical research, tissue engineering and regenerative medicine by using such smart programmable polymers.
1.4.1 Mechanism of shape memory polymers

Direct heating is currently the most commonly used triggering event. Such SMP smart materials can memorize their permanent shape by chemical or physical crosslinking. When heated above their transition temperature – glass transition temperature ($T_g$, for amorphous SMPs) or melting transition temperature ($T_m$, for semi-crystalline SMPs), they can be manipulated into a temporary shape and then fixed at this temporary state by cooling below their transition temperature. When heated again above the transition temperature, SMPs can recover back to their original, permanent shape. How well they can be fixed to the temporary shape and further recover back to their permanent shape can be defined and quantified as fixing ratio ($R_f$) and recover ratio ($R_r$).

\[
R_f \% = \frac{(L_u - L_i)}{(L_t - L_i)} \times 100 \% \tag{Eq 1-1}
\]

\[
R_r \% = \frac{(L_u - L_f)}{(L_t - L_i)} \times 100 \% \tag{Eq 1-2}
\]

Here $L_i$, $L_t$, $L_u$ and $L_f$ are initial length, temporary length, fixed length, and final length, respectively.

The process of SMP deforming, fixing, and recovering can also be quantified by a one-way shape memory cycle (Figure 1-1). First, the temperature increases to be above the SMP’s transition temperature ($T_g$ or $T_m$). And a force is applied to the SMP to deform it into a temporary shape. Second, while the applied force remains constant, the temperature is decreased below its transition temperature, fixing the temporary shape by immobilizing the polymer chains. Third, the applied deformation force is removed while the SMP remains cooled and fixed. Forth, the
temperature is increased again above the SMP’s transition temperature, and the SMP recovers freely back to its original permanent shape. During this four-step process, the temperature, the applied force (stress), and the strain are recorded and further analyzed for calculating the fixing ratio and recover ratio to assess the shape memory functionality of a SMP.

### 1.4.2 Applications of SMPs in biomedical research

As SMPs can dynamically change their surface topography, bulk shape, and internal architecture, they immediately drew researchers’ attention and interests to engineer them into cytocompatible platforms to be used for biomedical research – with the goal to mimic the dynamics of the *in vivo* microenvironment. Tremendous progress has been made so far from SMP substrates that can change surface topography to study cell attachment and morphology, to SMP scaffolds that can be implanted into animals to serve as medical devices.

For example, 2D shape memory surface topography was previously created in our lab to study the effect of active topography change on cell body and nuclear alignment [49,53]. In these two studies, SMP 2D substrates had temporary paralleled micro-grooves, or temporary flat surface, respectively, while the cells were attached. Upon heating to body temperature, the micro-grooves became flat or the flat surface formed nano-sized wrinkles. During this dynamic change of the surface topography, the cells reoriented themselves from highly aligned along the grooves to randomly oriented, or from randomly oriented to highly aligned along the wrinkles, respectively. These two studies are one of the very first SMP studies that the recovery can be triggered at body temperature 37 °C while cells were attached. They opened the doors to discovering cell behavior, cell mechanobiology on dynamically changing substrates under cytocompatible condition.
Besides SMP 2D substrates, great attention has also been focused on creating SMP 3D scaffolds that could be used as 3D cell culture platform for the applications ranging from fundamental mechanobiology studies, tissue engineering, to deployable medical device. For instance, a 3D SMP electrospun scaffold was developed in our lab that can dynamically change fiber alignment upon triggering at body temperature [54]. Human adipose derived mesenchymal stem cells were cultured on this scaffold at 30 °C when the scaffold had temporary highly aligned architecture. Once the construct was heated to 37 °C, the scaffold recovered from highly aligned architecture to randomly oriented architecture. And such dynamic fiber alignment changing event caused cell body re-orientation from highly aligned along the fibers to randomly distributed without any preferential alignment.

Several other studies have further demonstrated the ability to have SMPs as potential biomedical devices, for instance, to treat bone defect. Some previous in vitro studies have shown robust osteoblast adhesion, proliferation and osteogenic gene expression in SMP scaffolds [55,56]. Liu and colleagues developed a SMP poly-caprolactone (PCL) scaffold containing hydroxyapatite that was used as a cell-free synthetic bone graft in rabbit mandibular critical size defect. They found that the SMP scaffold promoted greater bone repair than sham controls [57]. Our lab has recently reported the use of SMP foam and electrospoon scaffolds to fill and further stabilize the mice femur critical sized defect [58]. Collectively, these studies demonstrated the potential for employing programmable dynamic SMP scaffolds in treatment of bone defects. However, none of these examined if the dynamic change of the scaffold would have any effect on cell phenotype or stem cell differentiation capacity. The extent to which stem cell differentiation capacity is preserved following programmed shape change has not yet been determined.
As SMP scaffolds have recently merged as new tools for active in vitro cell culture platforms, a lot still remains unknown regarding to how cell would respond to the dynamically changing scaffold. Especially the cell migration and cell differentiation in such in vitro dynamic systems are barely explored yet. In part of this thesis, we will be focusing on developing 3D SMP scaffolds to study guided cell migration and differentiation in such in vitro dynamic microenvironment. These two topics will be discussed in details in the following sections.

1.5 In vitro guided cell migration

Microenvironment architecture plays an important role in guiding cell migration in tissue development, healing, and disease progression, as previously discussed in section 1.2. ECM fibers become highly aligned to guide cells to migrate when tissue branching occurs. Collagen fibers change from randomly oriented to highly aligned, and such change from unaligned to aligned ECM architecture is considered to facilitate tumor cell invasion. In-depth studies of how such dynamic ECM architectural change would affect or direct cell migration in both healthy and disease conditions would greatly benefit the diagnosis and treatment of disease such as cancer, as well as cell homing for the application of tissue engineering and regenerative medicine.

1.5.1 Guided stem cell migration for the application of cell homing

Guided stem cell migration has been extensively studied, with the goal to deliver stem cells to a specific location to promote local tissue regeneration by inducing stem cells to differentiation to desired lineages. Such guided stem cell migration can be referred to as cell homing, a technique that attracts stem cells to sites of injury. Such technique could not only direct stem cells to traffic
towards the desired site, but potentially also at the same time deliver molecules as chemical cues to induce differentiation, or deliver drugs for specific treatments [59–61].

Although the in vivo cell homing mechanism and cascade events are not fully understood, various in vitro studies have investigated biomaterial scaffolds for cell homing for the application of tissue repair and tissue regeneration. Schantz and colleagues employed a 3D PCL scaffold that incorporated cytokines to guide site-specific cell homing [62]. They found mesenchymal stem cells preferentially migrated towards scaffold in response to cytokine SDF-1, and such growth factor deliverable system further showed promise of tissue formation due to guided cell homing. Kim and colleagues [63] used 3D printed PCL and hydroxyapatite scaffolds with interconnected micro-channels. With the implementation of Stromal-derived factor-1 (SDF1) and bone morphogenetic protein-7 (BMP7), significantly more endogenous cells were recruited within the scaffolds comparing to control scaffolds without the incorporation of growth factors. However, without implementation of growth factors, cell migration directed by scaffold architecture, or particularly the dynamic change of scaffold architecture, remains largely unknown.

1.5.2 Cancer cell polarization and migration

Cancer cell polarization and subsequent directional migration are essential for cancer metastasis. A cell needs to be polarized to migrate. Proteins such as Cdc42 (a master regulator of cell polarity), Par, aPKC are heavily involved at leaching edge to re-organize the microtubules of the migrating cell. In 2D systems, polarized cells often have Golgi apparatus in front of the nucleus. Once the cell is polarized, a protrusion must form and stabilize by attaching to the ECM or surrounding cells. Integrin, as one of the most important components during cell migration events,
acts as the “feet” that links the actin filaments of the migrating cell to its surrounding environment. It anchors the migrating cell to the ECM or its neighbor cells so the cell can pull forward. It also serves as mechanosensors to initiate corresponding signaling cascade. When the cell migrates forward, the adhesion disassembles at the leading edge, then form new adhesions. At the same time, at the rear end of a migrating cell, adhesions must also disassemble as well and retract [64].

Rapid progress has been made in the past years to understand the molecular mechanism of cancer cell migration, and key regulatory molecules have been identified. In the work that Deakin and colleagues published in 2011 [65], MDA-MB-231 breast cancer cells were cultured in Collagen I gel matrix. The cell morphology, invasion and metastasis were compared among control cells, with cells depleted of paxillin, and cells depleted of Hic-5. Paxillin and Hic-5 are both identified as key mediators of cancer cell migration. They found paxillin-depleted cells had highly polarized and elongated mesenchymal morphology, whereas Hic-5-depleted cells had non-polarized amoeboid morphology. They also analyzed paxillin effect on microtubule acetylation, Golgi structure, and polarized migration using multiple cancer cell lines to further understand the molecular mechanism on cell polarity in relation to cancer cell migration [66]. The results showed that paxillin regulates both Golgi integrity and cell polarized migration in both 2D and 3D microenvironment. Similarly, some other studies have also identified molecules that regulate the Golgi organization, cell polarity and motility [67,68]. Collectively, it is apparent that cell polarity plays an important role in guiding directional cancer cell migration and metastasis.

However, there are still many questions remained unclear about how cells migrate in microenvironment which constantly remodels, how cells establish their polarity and how cell polarity exactly associates with directional migration, and how the dynamics of microenvironment
in return regulate the cell motility and polarity. Most of the previously published studies used static 2D substrates or 3D scaffolds to examine the cell motility and polarity. But such static systems cannot mimic the dynamic nature of the *in vivo* microenvironment. In this dissertation, we developed a programmable fibrous 3D SMP scaffold as a dynamic *in vitro* microenvironment and explored the stem cell motility and cancer cell polarization and migration behavior when the internal architecture of the scaffold dynamically changes. The findings would provide valuable information about how cell motility and polarity would respond to *dynamic* change of their surrounding microenvironment *in vitro*.

### 1.6 In vitro microenvironments for controlled stem cell differentiation

Adult mesenchymal stem cells (MSCs) can be isolated from bone marrow or fat tissue, and further expanded *in vitro* with their multipotency maintained. When properly induced and controlled, the MSCs could differentiate down to various lineages for the applications of tissue engineering of bone, cartilage, muscle, fat and other tissues. Great promise has shown using MSCs to promote tissue growth, disease tissue repair, and new tissue formation. Some of the engineered *in vitro* microenvironments have particularly drawn researchers’ attention due to their ease of use, diversity, and complexity, to control the lineage commitment of the stem cells.

Growth factors and other chemical cues are often directly added to cell culture medium to induce differentiation. Recently, studies have shown success in incorporating these molecules locally during the scaffold fabrication process without the growth factors to diffuse into other regions, and such incorporation could be patterned to mimic the *in vivo* highly localized molecule distribution. For example, locally bond growth factor FGF-2 to PEGDA scaffolds were created by
covalently conjugating acrylated-PEG to heparin sulfate [69]. Besides supplementing chemical cues to biomaterials, modification of synthetic scaffold internal structure is also a way to engineer the materials to control the fate of the stem cells. A study published by Mastrogiacomo and colleagues [70] demonstrated methods to create hydroxyapatite bioceramics with different porosity, pore size distribution and pore interconnection pathway. They confirmed osteogenic differentiation and found that scaffold internal architecture could influence the overall bone deposition and the pattern of blood vessels invasion.

It is clear that biomaterials, especially 3D synthetic scaffolds are great candidates for studying controlled stem cell differentiation for the application of tissue engineering and regenerative medicine, due to their excellent ability to be customized to have various biochemical and mechanical properties. However, as previously mentioned in section 1.3.4, current widely used \textit{in vitro} biomaterial systems are often static, which cannot mimic the dynamic microenvironment \textit{in vivo} when stem cells choose their fate. In this thesis, the programmable foam and fibrous 3D SMP scaffolds as dynamic \textit{in vitro} microenvironments were employed to explore the stem cell differentiation capacity when the scaffolds dynamically change their internal architecture.

\textbf{1.7 Low oxygen tension and its application in cartilage tissue engineering}

Previously mentioned synthetic 3D scaffolds, as 3D \textit{in vitro} cell culture platforms to be employed to study cell-microenvironment interactions for the application of tissue engineering, regenerative medicine and cancer research, need to be carefully optimized to achieve desired cell growth and phenotype. Low oxygen tension, as a microenvironmental factor critical to many \textit{in vitro} culture approaches, are often associated with small pore size and low interconnectivity [71]
which are considered as one of the limitations of the scaffold design and fabrication technique. Low oxygen microenvironment in 3D scaffold may not be optimal for the regeneration of tissue such as bone [72], but could be beneficial for cartilage tissue engineering.

Articular cartilage tissue, different than bone, has very limited self-healing ability due to lack of blood vessels for blood supply and limited mobility of chondrocytes. But the tissue can be easily damaged by bad fall or accident. Small damage does not heal itself and it often gets worse over time. Many patients with severe articular cartilage damage have to go thought total joint replacement surgery eventually. Such surgeries are painful, involves inflammatory reactions, potentially need subsequent surgical treatments, and associate with high cost. Therefore, cartilage tissue engineering has become a hot topic with the goal to use native cells, biomaterials, and appropriate culture conditions to create engineered cartilage tissue to repair the damage.

*In vitro* expanded chondrocytes are a promising and frequently studied source of cells for cartilage tissue engineering, but present several challenges. One of the most significant and well recognized challenges is dedifferentiation during monolayer expansion. Dedifferentiation is often associated with chondrocytes losing their characteristic gene and protein expression profiles [73]. This can lead to poor biochemical and biomechanical properties of cartilage engineered from these cells, when compared to native cartilage. So far, many approaches [74–79] have been investigated to minimize the dedifferentiation and maintain the chondrogenic potential. Hypoxic *in vitro* culture of articular chondrocytes has received significant attention due to the fact that low oxygen tension is present *in vivo* during chondrogenesis and in adult articular cartilage. Therefore hypoxic culture condition could potentially provide a biomimetic approach to better maintain chondrogenesis *in vitro*. 

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To explore the effect of hypoxic *in vitro* culture condition on chondrogenesis, the mature human primary chondrocytes dedifferentiation behavior in a scaffold-free 3D culture system was analyzed. Although low oxygen might be beneficial for cartilage tissue engineering, low oxygen and related limited nutrient diffusion are still pressing issues often times associated with pore size, and interconnectivity during scaffold design and fabrication which need to be further optimized.

### 1.8 Scope of dissertation

This dissertation focuses on exploring cell polarization, cell motility, stem cell differentiation, and mature cell de-differentiation behavior when influenced by *in vitro* culture microenvironments. The aim of this work is to provide better understanding of how complex *in vitro* microenvironment would influence cell behavior. These new findings could potentially provide insights for improving current *in vitro* cell culture models for the application of cancer cell study, tissue engineering, and regenerative medicine. The outline of the chapters is described below.

The current chapter (*Chapter 1*) presents an overview of current challenges and questions in the area of interactions between cells and the surrounding microenvironment and how current techniques limit the further exploration of such interactions *in vitro*. *Chapter 2* presents a new 3D fibrous dynamic system and corresponding methods to study stem cell migration behavior in such dynamic system. *Chapter 3* further explores cancer cell motility and polarization using the same 3D fibrous dynamic scaffolds discussed in *Chapter 2*. In *Chapter 4*, the same fibrous dynamic scaffold and another 3D dynamic foam scaffold were used to investigate the stem cell osteogenic differentiation capacity during and following the programmed scaffold architectural change.
After examining the effect of dynamic scaffold change on cell polarization, cell motility, and stem cell differentiation in Chapter 2 – 4, the focus of switches to a scaffold-free system in which the mature chondrocytes de-differentiation was studied under reduced oxygen tension in Chapter 5. Low oxygen tension has been often associated with scaffolds’ poor interconnectivity and small pore size, but could be beneficial for cartilage tissue engineering. Finally, all of the research findings, discussion and proposed future work are summarized in Chapter 6.

1.9 References


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Figure 1-1. One-way shape memory cycle of an SMP. The asterisk indicates the beginning of the cycle with zero force applied to the SMP and temperature increased to above its transition temperature. (I) when the temperature is kept above the SMP’s transition temperature, the SMP is deformed; (II) temperature decreases to fix the temporary strain; (III) unload the SMP and the strain is observed to examine the fixing; (IV) temperature increases to be above the SMP’s transition temperature, SMP recovery occurs with the strain decreases to almost zero. Reprinted with permission from Reference [48].
Chapter 2: Exploiting Shape Memory to Study the Effect of Change in Fiber Alignment on Cell Motility†

2.1 Synopsis

Cell motility has been widely studied to improve understanding of tissue development and disease progression. However, most of the in vitro models employed in cell motility studies are physically static and cannot mimic the often dynamic nature of the microenvironment in vivo. In this chapter, we adapted and modified a previously developed cytocompatible shape memory polymer (SMP) 3D scaffold, which can change shape and internal architecture upon activation, to study the effect of scaffold architectural change on cell motility. Our goal was to test the hypothesis that a change in internal scaffold architecture could control cell directional motility. More specifically, that a decrease in fiber alignment would result in a change from directional motility along the fiber orientation to non-directional motility; while an increase in fiber alignment would result in a change from non-directional to directional motility along the fiber orientation. Furthermore, an increase in fiber alignment would cause increased cell velocity. To test the hypothesis, we cultured murine mesenchymal stem cells on the SMP 3D scaffolds, performed live imaging, and analyzed cell motility before and after scaffolds architectural change.

†Adapted (in part) from J. Wang, A. Quach, C.E. Turner, and J.H. Henderson, 2016, (in preparation)
2.2 Introduction

Extracellular matrix (ECM) architecture plays a critical role in guiding cell motility during tissue development and disease progression. During tissue development, fibrillar fibronectin is necessary to maintain cell polarity and guide morphogenic movements [1–3]. For example, local ECM architecture can guide cells to migrate towards elongate collagen fibers where tissue branching occurs [3]. During disease progression, such as in pregnancy associated breast cancer, radially aligned collagen fibers provide “tracks” for cancer cells to invade into the surrounding stroma [4–6]. Collagen alignment is considered to facilitate tumor cell invasion, and is being studied as a marker for patient diagnosis.

Diverse in vitro biomaterial models have been developed to study the architectural effects of the surrounding microenvironment on cell behaviors, such as cell motility. These in vitro biomaterial models include naturally occurring polymeric 3D matrices and synthetic polymeric 2D substrate or 3D scaffolds. For example, collagen gels have been used extensively as a natural polymeric 3D matrix to study cell motility. Friedl and colleagues [7] showed that highly invasive melanoma cells in 3D collagen matrices follow the protrusion, attachment, and contraction three-step model of cell motility. Such invasive motility results in reorganization of the ECM. Dubey and colleagues [8] found that magnetically aligned collagen fibrils can guide Schwann cell invasion into aligned collagen gel matrix. Such findings may provide improved methods of directing and enhancing axonal growth for the application of nerve repair. In terms of synthetic polymeric systems, electrospun scaffolds have been widely used as in vitro models due to their fibrous architecture, which can mimic the fibrillar structure of many native ECMs. For example, Johnson and colleagues [9] used aligned and randomly oriented electrospun scaffolds to
quantitatively study glioma cell motility on different fiber architectures. They found that cells would move along the highly aligned fiber in the aligned fiber architecture, while cells showed non-directional motility on randomly oriented fibers. Shao and colleagues [10] employed a polycaprolactone (PCL) electrospun mesh with a specific peptide sequence (E7) conjugated as a “MSC-homing device” to recruit mesenchymal stem cells (MSCs) for the application of tissue regeneration.

The strength of existing ECM models is that they provide relevant fiber microarchitecture and biochemical composition, but these models have significant limitations related to their fundamentally static nature. These existing models have proven successful in studying the response of cells to static matrices in which fiber alignment does not change. However, the study of the response of cells to changes in fiber alignment currently requires that cells cultured in the matrix actively remodel the matrix [5,11]. Such cell-derived matrix remodeling can also result in changes in matrix biochemical composition. Many physical properties, including stiffness, are strongly coupled to the biochemical composition of the matrix. As a result, cellular remolding of model matrices leads to changes in multiple physical properties, which are hard to predict, control, and characterize. Thus, the coupling of fiber alignment to biochemistry in current in vitro models confounds analysis of the role of fiber alignment in tissue development and disease progression.

To address the limitations of current in vitro ECM models, the objective of the present study was to develop a synthetic biomaterial scaffold that can undergo programmed increases or decreases in fiber alignment on command, and to test the hypothesis that a decrease in fiber alignment would result in a change from directional motility along the fiber orientation to non-directional motility; while an increase in fiber alignment would result in a change from non-
directional to directional motility along the fiber orientation. Furthermore, an increase in fiber alignment would cause increased cell velocity. The approach was to adapt and modify a 3D shape memory polymer (SMP) nano-fibrous scaffold. Such 3D fibrous SMP scaffold was chosen for its ability to memorize a permanent shape and internal architecture – highly aligned or unaligned, be manipulated and then fixed to a temporary shape and internal architecture – unaligned or highly aligned respectively, and then later recover to the permanent shape and internal architecture – highly aligned or unaligned respective, by a triggering event, such as heating [12–14]. Following recent breakthroughs in the area of cytocompatible SMPs [15–18], the SMP used in the present work was one recently demonstrated to be suitable for shape change under cytocompatible conditions, by increasing the incubation temperature from 30 °C to 37 °C when hydrated [18]. Murine mesenchymal stem cell line C3H10T1/2 was chosen as they demonstrate a classic fibroblastic motility [19]. Analysis of cell motility was enabled by a recently developed cell tracking algorithm, ACT/E [20].

2.3 Methods and Materials

2.3.1 Study design

Scaffolds representing four different architectures, two static and two dynamic, were used in this study. The two static scaffolds were prepared by electrospinning unidirectional aligned fibers (as-spun static aligned scaffolds, “A”) or randomly oriented fibers (as-spun static unaligned scaffolds, “U”), and these static scaffolds will not change fiber alignment when incubation temperature increases. The two dynamic scaffolds were prepared by programming the as-spun aligned scaffolds into a temporary architecture in which unidirectional alignment is disrupted,
mimicking the as-spun randomly oriented architecture, and by programming the as-spun unaligned scaffolds into a temporary architecture in which unidirectional alignment is increased, mimicking the as-spun unidirectional aligned architecture (detailed description in section 2.3.3). The two dynamic scaffold architectures dynamically increase unidirectional alignment (“U-to-A”) and dynamically decrease unidirectional alignment (“A-to-U”), respectively, when warmed from 30 °C to 37 °C under cell culture conditions, which is the trigger for shape memory thermal triggering. These four types of scaffolds with different architectures (A, U, U-to-A, and A-to-U) were used as a complete set of samples for scaffolds characterization, cell culture and time-lapse experiments described in following section 2.3.3, 2.3.4 and 2.3.5. Two sets of samples – “before recovery” set and “after recovery” set were prepared (Figure 2-1), with each set containing three technical replicates per scaffold architecture. Cells were seeded on both sets of samples at the same time and then cultured at 30 °C for overnight to allow cell attachment. The first set of samples, the “before recovery” set, was then analyzed by time-lapse imaging at 30 °C to quantify cell motility in the scaffolds’ temporary architectures before the scaffolds were triggered to change fiber alignment. The second set of samples, the “after recovery” set, was allowed to recover at 37 °C, then analyzed by time-lapse at 37 °C to quantify cell motility in the scaffolds’ permanent architectures after scaffolds were fully recovered. Resultant time-lapse videos were processed and analyzed as described in section 2.3.6.

2.3.2 TPU synthesis

To produce a shape memory 3D electrospun scaffold capable of dynamically increase or decrease fiber alignment on command, a thermoplastic polyurethane featuring shape memory was
synthesized as previously described [18]. Briefly, poly-DL-lactic acid (polyol) was synthesized by ring-opening polymerization of 3,6-dimethyl-1,4-dioxane-2,5-dione (Sigma-Aldrich) with 1,4-butandiol (Sigma-Aldrich) as the initiator and a small amount of catalyst tin(II) 2-ethylhexanoate (Sigma-Aldrich). The polyol was then reacted with hexamethylene diisocyanate (HDI) and polyhedral oligomeric silsesquioxane (POSS) diol (Hybrid Plastics) in the presence of a small amount of catalyst dibutyltin dilaurate (Sigma-Aldrich). The final molar ratio of polyol and POSS diol was kept at 1:3. The resulting thermoplastic polyurethane was precipitated with cold hexanes and vacuum dried under room temperature to remove residual solvent. One TPU batch, which had a molecular weight of 130 kg/mol, was used for this entire study. The dry state glass transition temperature of this batch of TPU was 47 °C.

2.3.3 Scaffold preparation

2.3.3.1 Electrospinning

To prepare a fibrous 3D scaffold capable of dynamically change internal architecture by increasing or decreasing fiber alignment on command, an electrospun scaffold was fabricated as follows. First, an electrospinning solution of 12.5 % (w/v) was prepared using freshly dissolved TPU in dimethylformamide (DMF; Sigma-Aldrich) and chloroform (Fisher Scientific) at the ratio of DMF: chloroform = 1:2 (v/v). Electrospinning parameters were optimized to produce nanofibers with consistent diameter of approximately 400 nm. Briefly, the electrospinning solution was loaded in a 10 ml syringe with a 25 gauge blunt tip needle. The feeding rate of the electrospinning solution was set as 0.2 ml/h. The syringe needle was positively charged to 18 kV and the rotating
collecting drum was negatively charged to 0.5 kV. The distance between the tip of the needle and the collecting drum was constant at 10 cm. The duration of electrospinning was 16 h.

To prepare a static control scaffold with an “as-spun” unaligned fiber architecture, the speed of the rotating collecting drum was set as the relatively low rate of 400 rpm. To prepare a static control scaffold with an “as-spun” highly aligned fiber architecture, the speed of the rotating collecting drum was set at the higher rate of 4000 rpm. Scaffolds were subsequently dried in a vacuum oven at room temperature for at least 48 h to ensure full removal of solvent. Because amorphous polymer chains can be stretched during the electrospinning process, a thermal treatment was applied, as previously described [18], to release molecular-level strain and stabilize the scaffold. Briefly, scaffolds were immersed in 65 wt % Pluronic F 127 (Sigma-Aldrich) hydrogel solution and heated at 70 °C for 3 h. After thermal treatment, scaffolds were extensively washed in deionized water for 24 h to ensure full removal of Pluronic, and then dried in a vacuum oven at room temperature overnight.

### 2.3.3.2 Scaffold programming

To prepare programmed “U-to-A” scaffolds capable of a dynamic increase in fiber alignment upon thermal triggering, as-spun aligned scaffolds were stretched uniaxially to 100 % strain in the direction perpendicular to the fiber alignment direction at 50 °C using a DMA. The temporarily unaligned architecture was fixed by cooling the sample to 0 °C. To prepare programmed “A-to-U” scaffolds capable of a dynamic decrease in fiber alignment upon thermal triggering, as-spun unaligned scaffolds were stretched uniaxially to 100 % strain at 50 °C using a dynamic mechanical analyzer (DMA; TA Instruments). The temporarily aligned architecture was
fixed by cooling the sample to 0 °C. Programmed scaffolds could be triggered to recovery back to their permanent architectures at 37 °C when hydrated.

2.3.3.3 Scaffold characterization

Scaffolds were characterized to assess their shape memory functionality, fiber architecture, and fiber diameter before and after triggered shape recovery. Scaffold shape memory functionality at dry state was assessed by a one-way shape-memory cycle using a DMA. Briefly, scaffolds were heated to 50 °C, which is above the materials glass transition temperature, and held isothermal for 5 min. Scaffolds were then uniaxially stretched to 100 % strain. Such temporary strain was fixed by cooling the scaffolds to 0 °C. Scaffolds were then heated back to 50 °C to allow recovery. Temperature, strain, and stress were recorded during stretching, fixing, and recovery to examine the fixing ratio and recovery ratio of the scaffolds [18].

Scaffold shape memory functionality was also tested when scaffolds were hydrated under simulated cell culture conditions. Briefly, scaffolds were programed to 100 % strain using a DMA. Programmed scaffolds were hydrated and immersed in DI water, and incubated at 30 °C or 37 °C for 24 h. Sample length was measured every 45 min in the first 6 h, then at 10 h and 24 h. Strain versus time was analyzed to examine the scaffold’s stability at 30 °C and the duration of recovery at 37 °C under hydrated and simulated cell culture condition.

To analyze fiber architecture and fiber diameter before and after shape memory recovery under hydrated condition, two sets of scaffolds – “before recovery” set and “after recovery” set, with each set including A, U, U-to-A and A-to-U scaffolds, were prepared as described in section 2.3.2. The “before recovery” set of scaffolds was used to assess fiber architecture and fiber
diameter before recovery; while the “after recovery” set was used to assess fiber architecture and fiber diameter after recovery. Specifically, the “before recovery” set of scaffolds was hydrated, incubated in 30 °C water for 24 h, and then dried in vacuum oven at room temperature. In contrast, the “after recovery” set of scaffolds was hydrated, incubated in 30 °C water for 24 h and then also incubated for an additional 24 h in 37 °C before being dried at room temperature. Both sets of scaffolds, with their temporary architectures and recovered permanent architectures, respectively, were then gold sputter coated for analysis by scanning electron microscopy (SEM). SEM images acquired at 1000X were used to analyze fiber alignment and fiber diameter, as follows (n = 9 images from one scaffold architecture group). 2D fast Fourier transform (2D FFT) analysis was performed to quantify fiber alignment in each scaffold architecture before and after recovery, as previously described [18,21]. Fiber diameter was measured on 20 randomly selected fibers from each scaffold architecture before and after recovery (n = 9 images from one scaffold architecture group).

2.3.4 Cell culture

To study cell motility behavior on the shape memory electrospun scaffolds, murine C3H10T1/2 cells (ATCC) were maintained in Basal Medium Eagle (BME) cell culture medium supplemented with 10 % (v/v) Fetal Bovine Serum (FBS), 1 % (v/v) GlutaMAX, and 1 % (v/v) Penicillin-Streptomycin. All cell culture reagents were purchased from Invitrogen. “Before recovery” and “after recovery” sets of scaffolds with each scaffold architecture containing two technical replicates were prepared.
To culture cells on scaffolds for live imaging purpose, scaffolds were first UV sterilized by the UV bulb in a biological safety cabinet for 1 h on each side. After sterilization, scaffolds were hydrated with sterile DI H₂O and loosely adhered on Sylgard coated glass slides with two strips of Sylgard gel steps on both ends of the samples (Figure 2-2) to immobilize samples from moving during imaging and also to elevate scaffolds to prevent the cells being sheared against petri dish during imaging. Briefly, monomer and crosslinker provided in the Sylgard 184 Silicone Elastomer Kit (Dow Corning) were thoroughly mixed at different ratios, 90:1 (v/v) and 30:1 (v/v) respectively, in separate glass vials. After mixing, Sylgard solution was briefly sonicated for 3 min to remove air bubbles introduced during mixing. Microscope glass slides (Fisher Scientific) were cut into approximately 25 mm × 15 mm rectangles and evenly coated with a thin layer of 90:1 Sylgard solution on top by spreading the solution with a razor blade. To keep the thickness of the Sylgard coating relatively consistent, each to-be-coated glass slide was placed in between two other glass slides in parallel with three layers of double-sided tape on top when spreading Sylgard solution with razor blade. Therefore the thickness of the Sylgard coating would be equivalent to the thickness of three layers of double-sided tape, which is approximately 36 µm total. Coated glass slides were place in 60 °C isothermal oven for overnight to cure the Sylgard. Sylgard 30:1 solution was poured into a petri dish to achieve a 3 mm height of the solution and cured in 80 °C isothermal oven for 1 h. Cured 30:1 Sylgard gel was cut into 3 mm × 13 mm strips and immersed together with Sylgard coated glass slides in 70 % ethanol for 30 min to sterilize. After ethanol sterilization, Sylgard gel strips and coated glass slides were placed in new petri dishes to air dry in a biosafety cabinet. Once Sylgard gel strips and glass slides were dried, gel strips were attached on each end of the glass slides to act as steps (Figure 2-2) to elevate the samples during imaging
on the inverted microscope, on which glass slides with strips attached are flipped upside down. Such coating shows no interference with full recovery of dynamic programmed scaffolds (Figure 2-3). Once the hydrated scaffolds were attached on Sylgard coated glass slides, the scaffold/glass slides constructs were placed in 35 mm petri dishes. A 20 µl droplet of complete cell culture medium was placed on each scaffold for 20 min to allow protein deposition on scaffolds to facilitate cell attachment before cell seeding. Cell culture medium was removed by pipetting immediately before cell seeding.

To seed cells on scaffolds, cell suspension was pipetted on scaffolds in a 20 µl droplet at a density of 4000 cells/cm$^2$. Cell seeded scaffolds were cultured under 30 °C for 2 h to allow cell attachment. After cells were attached, 3 ml of complete cell culture medium was added to petri dishes to fully cover the scaffolds. Cell seeded scaffolds were then further cultured at 30 °C overnight before time-lapse imaging.

2.3.5 Cell staining and time-lapse imaging

To image cells for tracking analysis, cell nuclei were stained with Hoechst 33342 nuclear dye (Invitrogen) at the final concentration of 0.01 µg/ml in cell culture medium and imaged for 24 h. Such significantly low nuclear dye concentration, when compared to the manufacture’s recommendation (0.2 – 2 µg/ml), was previously tested [20] to ensure cell viability for long timescale imaging. Briefly, to acquire time-lapse videos on the “before recovery” set of samples, samples were stained at 30 °C for 20 min, and then the scaffolds/glass slide construct was taken out, flipped, and attached upside down to a new 35 mm petri dish. This new dish was then filled
with 3 ml of medium with 0.01 μg/ml nuclear dye to ensure full immersion of cell seeded scaffolds. This set of “before recovery” scaffolds were imaged for 24 h at 30 °C.

Nineteen hours after the “before recovery” set of imaging was initiated, the “after recovery” set of scaffolds were moved from 30 °C incubator to 37 °C incubator to trigger the recovery of the programmed strain-aligned and strain-unaligned scaffolds. When the “after recovery” set of scaffolds were fully recovered after 5 h in 37 °C incubator, they were immediately stained, as described above, and imaged at 37 °C for 24 hours to acquire time-lapse videos of cells on scaffolds after shape memory recovery.

Images were acquired using a Leica DMI 6000B inverted microscope with 10X objective, on which samples were incubated in a temperature and CO₂ controlled (5 % CO₂) live cell incubator (INC-2000, 20-20 Technology Inc.). Imaging exposure time was set as 60 ms, with 5 min interval for 24 h. Z-stacks of images were taken at each position from a relative -40 μm to 40 μm with 20 μm increment. Eight different positions per scaffold architecture group were imaged for each biological replicate. Cell seeding and subsequent live imaging were independently repeated three times (n = 3).

2.3.6 Cell tracking and motility analysis

To analyze cell motility before and after scaffold architectural change, resultant images within each z-stack were first processed using the Extended Depth of Field ImageJ macro [22] to project cells on different focal planes onto one common plane. Time lapse videos consisting of 288 frames per video representing 24 h of real time were then analyzed by ACTIVE. ACTIVE, which is a freely available automated cell tracking algorithm developed by our lab, can accurately
and efficiently track the displacement of a large population of individual cells for a long period of time.

Full tracks, diffusion plots, mean square displacement (MSD), decomposed MSD, and average velocity results output from ACT\textsuperscript{IV}E (described in details below) were used to qualitatively and quantitatively assess cell motility, as previous described [20]. Briefly, full tracks produced by ACT\textsuperscript{IV}E are used to qualitatively show cell motility directionality over the period of imaging. Each line with of a different color represents a cell’s path during the entire imaging period of time. Diffusion plots are used to qualitatively assess cell motility directionality. In these plots, cell final positions are plotted with each cell’s initial position normalized to the same origin. For aligned architectures, the coordinate plane was rotated to have the principle fiber alignment direction parallel with the x direction. MSD was calculated as

\[
MSD(\Delta t) = \sum_{i=1}^{N} \frac{(r(t+\Delta t)-r(t))^2}{N}
\]

(Eq 2-1)

Where \(\Delta t\) time-interval, \(r\) is the distance at a specific time point, and \(N\) is the total number of cells [20]. MSD was plotted as \(\log_{10}\) distance\(^2\) (\(\mu m^2\)) versus \(\log_{10}\) t (min) by ACT\textsuperscript{IV}E. Decomposed MSD was analyzed in the principle fiber alignment direction (x) and the direction perpendicular to the principle fiber alignment (y). MSD short timescale and long timescale intercepts and slopes, as well as decomposed short and long timescale intercepts and slopes in both x and y directions were further assessed to quantify cell motility. Generally, a slope close to 1 suggests diffusive motility, while a slope close to 2 suggests ballistic motility. The intercept of the long timescale fitted MSD line was additionally used as an indicator of how fast the cells move, where higher intercepts suggest faster movement. Average velocity was calculated to quantify cell motility and
allow for comparisons between scaffold architectures. Briefly, cell velocity was calculated using finite differences theorem [23]. An accuracy value of 8 was used, where x and y frame velocities were obtained via weighted analysis of cell displacements over 9 frames (4 frames prior and 4 frames after the selected frame). Cells with missing displacement information (e.g. cells moved out of the field of view) were not included in the analysis.

2.3.7 Cell body and cell nuclear alignment and quantification

To quantify cellular alignment and nuclear alignment when the C3H/10T1/2 cells were cultured on the scaffolds of four different architectures, actin cytoskeleton images from phalloidin staining and nuclear images from DAPI staining were acquired with a 20X objective. Actin and nuclear images were analyzed using ImageJ built-in function “Analyze Particles” and cellular alignment and nuclear alignment, respectively, were quantified as previously described [17], with the long axis of the cells and of the nuclei, which are oval in shape, used in determining alignment. Briefly, after all angle values were collected as Analyze Particles output, a truncated standard deviation of angles was then calculated and the final angle that yielded the minimum truncated standard deviation was computed as previously described [17]. For this analysis, a truncated standard deviation of 52° would represent randomly distributed angles, while a standard deviation of 0° would represent perfectly aligned angles. Therefore, a decrease in angular standard deviation indicates an increase in alignment. The truncated angular standard deviation was thus used to quantify the cellular angular distribution or nuclear angular distribution and to compare the cellular alignment or nuclear alignment when cells were exposed to different scaffold architectures.
2.3.8 Statistics

Data used for statistical analysis were first tested for normal distribution using Shapiro-Wilks testing. All data sets passed the assumption of normal distribution, and appropriate parametric tests were used as follows. Fiber diameter among different groups before and after recovery was analyzed by two-factor ANOVA. To compare the time effect on cell motility, MSD short timescale slope and long timescale slope were compared using a paired student t-test within each group before and after recovery. To compare the dynamic scaffold architectural change effect on cell motility, MSD short timescale slope and long timescale slope and intercept were compared before recovery and after recovery using a paired student t-test. To analyze cell motility directionality on each scaffold architecture before and after recovery, decomposed MSD x- and y-intercepts were compared within each architecture using a paired student t-test. To compare the cell average velocity change before and after recovery for each architecture, paired student t-test was performed to test for significant difference between the mean of each group. Statistical significant difference was determined at $p < 0.05$ for all the above tests.

2.4 Results

2.4.1 Scaffold properties

The shape memory espun scaffold demonstrated excellent shape memory functionality in terms of temporary shape fixing and triggered shape recovery. In a dry state, as shown in a one-way shape-memory cycle (Figure 2-4), the 3D SMP espun scaffold showed a high fixing ratio of 99%, indicating that 99% of the strain was maintained by cooling down the sample below glass
transition temperature when the applied force was removed. Upon trigger, the scaffold showed a high recovery ratio of 99 %, indicating that the scaffold recovered 99 % of the initial strain after shape memory recovery. Fixing ratio and recovery ratio were calculated as described previously [18].

When hydrated under simulated cell culture environment, the scaffolds remained highly stable at 30 °C, showing only approximately 5 % pre-recovery of the programmed strain within 24 h. When hydrated scaffolds were triggered to recover at 37 °C, full recovery completed in 5 h with only about 1 % programmed strain left unrecovered (Figure 2-3).

Internal architectural change of the scaffold before and after recovery was visualized by SEM (Figure 2-5), and alignment was further quantified by 2D FFT image analysis (Figure 2-6). Fiber alignment along a principle direction was indicated by two distinct peaks at 90° and 270° in the 2D FFT plots, while unaligned fiber architecture was indicated by no apparent peak in 2D FFT plots. To briefly summarize the results from SEM imaging and quantitative 2D FFT image analysis, static groups showed no change of fiber alignment before and after temperature change. Dynamic strain-aligned scaffold changed from highly aligned to unaligned architecture when triggered by increase of temperature, while strain-unaligned scaffold changed from unaligned to aligned architecture upon trigger. Fiber diameter measurement (Table 2-1) in all four groups before and after recovery showed no significant change of fiber diameter due to shape memory recovery.
2.4.2 Qualitative and quantitative analysis of cell motility

2.4.2.1 Cell tracks

Qualitative analysis of cell tracks over 24 h (Figure 2-7) indicate that unidirectional fiber alignment promotes polarized cell motility, that random fiber alignment results in non-polarized motility, and that dynamic increase and decrease in unidirectional fiber alignment results in on-command increase and decrease of polarized cell motility, respectively. Briefly, on static aligned scaffolds (A) cells moved preferentially along the direction of fiber alignment both before and after thermal triggering, while on static unaligned scaffolds (U) cells moved randomly without a preferential direction both before and after thermal triggering. On dynamic scaffolds that increase in unidirectional alignment (U-to-A), polarized cell motility increased after thermal triggering, while on dynamic scaffolds that decrease in unidirectional alignment (A-to-U), polarized cell motility decreased after thermal triggering.

2.4.2.2 Diffusion plots

Diffusion plots (Figure 2-8), another way to qualitatively show cell motility directionality, showed preferential cell motility direction when cells are on aligned architectures and non-preferential motility direction on randomly oriented fiber architectures. Cell final positions are plotted with the initial position normalized to the same origin and the x direction represents the principle fiber alignment direction for aligned architectures. Diffusion plots also qualitatively showed that dynamic change of fiber alignment could change the cell motility directionality.
Diffusion plots showed an elongated distribution on as-spun static aligned scaffolds (A), indicative of polarized motility along the direction of fiber alignment, a circular distribution on as-spun static unaligned scaffolds (U), indicative of non-polarized motility without preferential directionality, and a change from more circular distribution to more elongated distribution and from more elongated distribution to more circular distribution on dynamic scaffolds that increase unidirectional alignment (U-to-A) and decrease unidirectional alignment (A-to-U), respectively, indicative of on-command increase and decrease of polarized cell motility, respectively.

### 2.4.2.3 Mean Squared Displacement (MSD)

MSD, which analyzes cell displacement distance in time interval $\Delta t$, showed no statistically significant difference of short timescale slope or long timescale slope between before recovery and after recovery within each scaffold architecture (Table 2-2). This indicates that the temperature change used to trigger scaffolds shape recovery did not significantly affect cell motility. Long timescale slopes are significantly lower than corresponding short timescale slopes among all scaffolds, indicating that overtime the cells moved more diffusively (Table 2-2).

### 2.4.2.4 Decomposed Mean Squared Displacement (MSD)

Decomposed MSD (Figure 2-9) in the principle fiber alignment direction (x) and the direction perpendicular to the principle fiber alignment direction (y) enables quantitative analysis of cell motility directionality. Decomposed MSD analysis provided qualitative and quantitative confirmation of the motility behaviors qualitatively observed in the cell tracks and diffusion plots. Qualitatively, cells on static aligned control scaffolds (A) showed non-overlapping decomposed MSD curves, which indicates that cells moved with polarized motility along the direction of fiber
alignment both before and after thermal triggering. Cells on static unaligned control scaffolds (U) showed overlapping decomposed MSD curves, which indicates cells moved randomly with non-polarized motility both before and after thermal triggering. Cells on the scaffold architecture that dynamically increases unidirectional alignment (U-to-A) showed a change from overlapping to non-overlapping decomposed MSD curves, which indicates polarized cell motility increased after thermal triggering. Cells on the scaffold architecture that dynamically decreases unidirectional alignment (A-to-U) showed a change from non-overlapping to overlapping decomposed MSD curves, which indicates polarized cell motility decreased after thermal triggering.

Quantitatively, to assess cell directional motility, x-long timescale intercept and y-long timescale intercept within each scaffold architecture were compared within each scaffold architecture (Table 2-3). Briefly, static aligned scaffold and strain aligned scaffold before triggering shape recovery and static aligned scaffold and strain unaligned scaffold after recovery, which all had highly aligned fiber architecture, had significantly higher x-long timescale intercept than y-long timescale intercept. This indicates that cells on aligned fiber architecture preferentially moved along the principle fiber alignment direction (x). When comparing the motility directionality change before and after recovery in dynamic programmed scaffolds (Table 2-4), strain aligned scaffold showed significantly decreased difference in x and y long timescale intercept (x-long timescale intercept – y-long timescale intercept); while strain unaligned scaffold showed significantly increased difference in x and y long timescale intercept. This shows that, by dynamically decreasing fiber alignment, cells lost their motility directionality; while by dynamically increasing fiber alignment, cells acquired preferential motility directionality along the principle fiber alignment direction.
2.4.2.5 Average velocity

Analysis of average velocity allows quantitative comparison of cell motility velocity when cells were exposed to different fiber architectures (Figure 2-10). Before recovery, average velocity among different groups showed no significant difference. After recovery, the U-to-A group showed significantly higher velocity compared to A and U groups. When comparing before and after recovery within the same scaffold architecture, A and U-to-A groups showed significantly increased average velocity when temperature changed from 30 °C to 37 °C. U-to-A and A-to-U scaffolds showed a slight increase that was not statistically significant. This indicates that when fiber alignment dynamically increases in the strain unaligned group, cells moved faster on highly aligned fibers. Temperature increase caused a significant velocity increase in A and U groups but did not cause a significant change in A and U groups. This suggests that temperature increase might induce increased cell metabolism that may cause increased cell velocity, but not at a level detectable by the current analyses (p values are 0.149, 0.086 and 0.974 from paired t-tests with n = 3 when comparing the average velocity before and after temperature change in A, U, and A-to-U scaffolds, respectively). When comparing the fold change of velocity after recovery compared to before recovery within a scaffold architecture group (velocity fold change = after recovery velocity / before recovery velocity), the A group had a 1.320-fold increase, the U group had 1.254-fold increase, the A-to-U group had the lowest increase of 1.070-fold, and the U-to-A group had a 1.525-fold increase. The lowest fold increase in strain aligned group suggest that cells moved slower when fiber alignment decreased. As this cell velocity change induced by decrease in fiber alignment is not significant, it may also suggest that cells that were pre-exposed to aligned fibers would keep on moving at higher velocity comparing to the naïve cells.
2.4.3 Cell body and cell nuclear alignment

Cell staining images (Figure 2-11) and corresponding cell body and cell nuclear alignment analysis (Figure 2-12) showed that not only can the fiber architecture direct cell body and cell nuclear alignment but also that the dynamic change of fiber architecture can change the cell body and nuclear alignment. Specifically, cells aligned along the aligned fibers when exposed to aligned fiber architecture, and cells randomly oriented when exposed to unaligned fiber architecture. When fiber architecture dynamically changed from aligned to unaligned, cell bodies lost their alignment and become randomly oriented after the scaffold architecture change; in contrast, when fiber architecture dynamically changed from unaligned to aligned, cells changed from randomly oriented to highly aligned along the fiber alignment orientation.

2.5 Discussion

In the present study, we investigated cell motility in an in vitro dynamic microenvironment, by using a 3D shape memory electrospun fibrous scaffold system that can dynamically change fiber architecture upon triggering at body temperature. Building upon prior work that employs SMP espun scaffolds to direct cell body alignment, this is the first study that qualitatively and quantitatively investigated cell motility behavior in a dynamic SMP in vitro system. We found scaffold fiber architecture can direct cell directional motility, as has previously been shown, but we also found that the change of fiber architecture can direct a change of cell directional motility and velocity.

Such dynamic system, which not only supports the study of cell motility but also can change cell motility direction on command, can be further applied and investigated for various
application. One promising application of the present SMP scaffold system is the study and potential treatment of cancer metastasis. \textit{In vivo}, increased ECM density and fiber alignment are associated with increased tumor formation and metastasis [24,25]. Highly aligned collagen fibers are thought to provide “tracks” for cancer cells to invade into the surrounding tissue in breast cancer [5]. With the help of this dynamic SMP 3D fibrous scaffold, further investigations could be made to study cancer cell motility in such dynamic systems in which the cells motility directionality and velocity could be directed by the change of the scaffold architecture. Cancer cell invasive phenotype and cell body and internal organelle polarization could be studied to determine the extent to which matrix architecture affects cancer cell invasiveness. Such finding would be significant for better understanding of cancer cell motility, metastasis, and tumor formation. More complex scaffold architecture could also be incorporated, such as locally varied fiber alignment, to study preferential cell directional motility to remote locations with different matrix architectures. Such complex scaffold architecture may be a promising candidate that mimics the invasion of cancer cells into stroma and subsequent metastasis events \textit{in vivo}.

Stem cell motility, for example, has been extensively studied, with the goal to deliver stem cells to a specific site to promote local tissue repair by inducing stem cells to differentiate down specific lineages. Another promising application of the present SMP system would be the study of stem cell homing, a technique that attracts stem cells to sites of injury, and that has particularly drawn recent attention. Guided stem cell migration could not only direct stem cells to traffic towards the desired site, but at the same time potentially also deliver molecules as chemical cues to induce differentiation or deliver drugs for specific treatments [26–28]. Although the \textit{in vivo} cell homing mechanism and cascade events are not fully understood, several \textit{in vitro} studies have
investigated biomaterial scaffolds for cell homing. Schantz and colleagues employed a 3D polycaprolactone scaffold that incorporated cytokines to guide site-specific cell homing [29]. They found mesenchymal stem cells preferentially migrated towards scaffold in response to cytokine SDF-1, and such growth factor deliverable system further showed promise of tissue formation due to guided cell homing. Kim and colleagues [30] used 3D printed poly-ɛ-caprolactone and hydroxyapatite scaffolds with interconnected micro-channels. With the incorporation of Stromal-derived factor-1 (SDF1) and bone morphogenetic protein-7 (BMP7), significantly more endogenous cells were recruited within the scaffolds comparing to control scaffolds without the incorporation of growth factors. However, without incorporation of growth factors, cell motility directed by scaffold architecture, or particularly the dynamic change of scaffold architecture, remains largely unknown. Our dynamic SMP 3D scaffolds that can change cell motility directionality on command can potentially be employed for directed stem cells migration studies for the application of tissue regeneration. Specifically, with highly aligned scaffold architecture, stem cells may preferentially migrate along the fiber alignment direction, then be further recruited to the injured sites that could be strategically position at the ends of highly aligned fibers, even without using chemokines.

The electrospinning technique employed in the present study shows great advantage of creating fibrous 3D scaffolds with tunable fiber diameter. However, due to the limitation of the duration of the electrospinning process, the resultant scaffolds are thin and may not be of clinically relevant size. As cells were seeded on scaffolds and cultured overnight before time lapse imaging in the present work, cells did not have sufficient time to fully penetrate through the entire depth of the scaffolds. Due to such experimental limitations, in the present study we did not analyze cell
motility in the z direction – the direction parallel with the scaffold depth. Though multiple cells layers were captured during imaging, the cells located at different focal planes were projected to the same focal plane during post-processing. However, with thicker scaffolds and longer pre-culture time, cells could infiltrate more into the scaffolds and our imaging system and ACT\textsuperscript{IV}E analysis algorithm could, with modification, be capable of analyzing cell motility three dimensionally.

As previously mentioned, for before recovery and after recovery groups, scaffolds were imaged at 30 °C and 37 °C respectively. Such temperature difference might potentially have effect on cell metabolism and general cell velocity. However the quantification analysis of MSD and average velocity did not show significant effect of such temperature difference on cell motility. In the future, after the “after recovery” scaffolds were fully recovered, imaging temperature could be changed back to 30 °C to eliminate imaging temperature difference.

2.6 Conclusion

We developed an SMP 3D fibrous scaffold with nano-sized fibers that can dynamically change fiber alignment upon triggering at body temperature. Cell motility behavior was investigated and it was found that the cell motility can be directed by dynamic change of the scaffold internal architecture. We found that cells would move preferentially along fiber alignment direction when exposed to aligned fiber architecture, while cells would move randomly without specific direction when exposed to unaligned fiber architecture. Moreover, when fiber alignment dynamically increases, cell motility would change from random to highly aligned pattern, with significantly increased velocity. When fiber alignment dynamically decreases, cell motility would
change from highly oriented along fiber alignment direction to random motility. Such scaffolds could be further used for in depth study of directed stem cell migration for the application of tissue engineering, cell homing, regenerative medicine, and the study of cancer cell migration and metastasis for the application of cancer therapy.

2.7 Acknowledgements

The authors gratefully thank Ling-Fang Tseng and Andy Quach for their help with TPU synthesis, and Megan Brasch for technical questions about ACT/VE.

2.8 References


[5] Provenzano PP, Inman DR, Eliceiri KW, Trier SM, Keely PJ. Contact guidance mediated three-dimensional cell migration is regulated by Rho/ROCK-dependent matrix


Table 2-1. Scaffolds fiber diameter (nm) measurements.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>U</th>
<th>U-to-A</th>
<th>A-to-U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>396.2 ± 163.47</td>
<td>433.76 ± 163.12</td>
<td>515.76 ± 149.96</td>
<td>451.48 ± 156.09</td>
</tr>
<tr>
<td>After</td>
<td>422.56 ± 130.13</td>
<td>408.58 ± 109.89</td>
<td>365.3 ± 165.90</td>
<td>426.68 ± 224.91</td>
</tr>
</tbody>
</table>

Average fiber diameter (nm) was calculated from 20 measurements from each image of total 5 images per scaffold architecture. Three technical replicates were used per scaffold architecture. No significant difference was found by two factor ANOVA test with $p = 0.63$. 
Table 2-2. Cell motility MSD parameters analysis.

<table>
<thead>
<tr>
<th></th>
<th>Short Timescale</th>
<th>Long Timescale</th>
<th>Long Timescale</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>Slope</td>
<td>Intercept</td>
</tr>
<tr>
<td>Before</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.1871 ± 0.0128</td>
<td>1.0266 ± 0.2008</td>
<td>-0.2918 ± 0.5984</td>
</tr>
<tr>
<td>U</td>
<td>1.1134 ± 0.1044</td>
<td>0.9055 ± 0.2067</td>
<td>-0.0786 ± 0.2911</td>
</tr>
<tr>
<td>U-to-A</td>
<td>1.2662 ± 0.0782</td>
<td>0.8813 ± 0.1331</td>
<td>0.3280 ± 0.4309</td>
</tr>
<tr>
<td>A-to-U</td>
<td>1.1997 ± 0.1749</td>
<td>1.2005 ± 0.3082</td>
<td>-0.3861 ± 0.2729</td>
</tr>
<tr>
<td>After</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.2539 ± 0.1691</td>
<td>0.9767 ± 0.2419</td>
<td>0.2824 ± 0.5682</td>
</tr>
<tr>
<td>U</td>
<td>1.2892 ± 0.0093</td>
<td>0.8776 ± 0.2599</td>
<td>0.5003 ± 0.6621</td>
</tr>
<tr>
<td>U-to-A</td>
<td>1.1719 ± 0.3299</td>
<td>0.8951 ± 0.1266</td>
<td>0.7234 ± 0.4754</td>
</tr>
<tr>
<td>A-to-U</td>
<td>1.2124 ± 0.0995</td>
<td>1.1467 ± 0.1722</td>
<td>-0.2003 ± 0.0471</td>
</tr>
</tbody>
</table>

Short timescale slopes, long timescale slopes, and long timescale intercepts listed in Table 2-2 were calculated by ACTIVE by line fitting of \( \log_{10} \) MSD – \( \log_{10} t \) curve. Data are reported as average ± standard deviation. No significant difference was found between before and after recovery within each scaffold architecture (A, U, U-to-A, and A-to-U) by paired student’s t-tests. Long timescale slopes are significantly lower than corresponding short timescale slopes among all scaffolds both before and after recovery, tested by paired student’s t-test with \( p = 0.0033 \).
Table 2-3. Cell motility decomposed MSD parameters analysis.

<table>
<thead>
<tr>
<th></th>
<th>X Short Timescale Slope</th>
<th>X Long Timescale Slope</th>
<th>X Long Timescale Intercept</th>
<th>Y Short Timescale Slope</th>
<th>Y Long Timescale Slope</th>
<th>Y Long Timescale Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>A</td>
<td>1.2336 ± 0.9516 ± 0.4056 ±</td>
<td>1.0038 ± 1.0424 ± -1.3416 ±</td>
<td>0.0732 0.646 0.5726 a</td>
<td>0.2025 0.3570 0.3887 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>1.1090 ± 0.8491 ± 0.4303 ±</td>
<td>1.1159 ± 0.9268 ± -0.6476 ±</td>
<td>0.1148 0.2415 0.3433</td>
<td>0.0942 0.2038 0.3930</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U-to-A</td>
<td>1.2474 ± 0.8827 ± -0.2436 ±</td>
<td>1.2728 ± 0.8640 ± -0.0889 ±</td>
<td>0.1154 0.1186 0.3912 0.0774 0.1542 0.5685</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A-to-U</td>
<td>1.2359 ± 1.2107 ± -0.8858 ± 1.0177 ± 1.1136 ± -1.5628 ±</td>
<td>0.2039 0.2839 0.3365 b 0.2676 0.4532 0.4437 b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After</td>
<td>A</td>
<td>1.3174 ± 0.9439 ± 0.1042 ±</td>
<td>1.0683 ± 1.0546 ± -0.9615 ±</td>
<td>0.1662 0.2392 0.6128 c 0.1912 0.2077 0.3180 c</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>1.2857 ± 0.8318 ± 0.1090 ±</td>
<td>1.2885 ± 0.9216 ± -0.1083 ±</td>
<td>0.0287 0.2651 0.7909 0.0258 0.2381 0.5996</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>U-to-A</td>
<td>1.2231 ± 0.7941 ± 0.6595 ±</td>
<td>1.0531 ± 1.0057 ± -0.3644 ±</td>
<td>0.3460 0.2778 0.6266 d 0.3099 0.0870 0.2652 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A-to-U</td>
<td>1.2225 ± 1.1326 ± 0.6941 ±</td>
<td>1.2003 ± 1.1441 ± -0.7799 ±</td>
<td>0.0880 0.2194 0.2198 0.1128 0.1294 0.1006</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Highlighted groups that share the same superscript label are significantly different according to student’s paired t-tests ($p < 0.05$).
Table 2-4. x- and y- long timescale intercept differences in decomposed MSD in dynamic programmed scaffolds.

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-to-A</td>
<td>-0.1547 ± 0.2400</td>
<td>1.0239 ± 0.3789</td>
</tr>
<tr>
<td>A-to-U</td>
<td>0.6770 ± 0.2679</td>
<td>-0.0142 ± 0.1420</td>
</tr>
</tbody>
</table>

x- and y- long timescale intercept difference was calculated as x-long timescale intercept – y-long timescale intercept. The x- and y- intercept differences were significantly different ($p < 0.005$) in U-to-A scaffold ($p = 0.0195$), and A-to-U scaffold ($p = 0.0382$) according to Student’s paired t-tests.
**Figure 2-1.** Study Design. Static aligned (A) and static unaligned (U) scaffolds were fabricated by electrospinning. A and U scaffolds were further programmed to U-to-A and A-to-U scaffolds, which have temporarily unaligned and temporarily aligned architectures, respectively. Two sets of samples, “before recovery” set and “after recovery” set, were prepared with each set including four architectures, A, U, U-to-A and A-to-U. Cells were seeded on both sets at the same time. The “before recovery” set was imaged at 30 °C for 24 h. The “after recovery” set was triggered to fully recover at 37 °C then imaging at 37 °C for 24 h.
Figure 2-2. Schematic of Sylgard coated glass slides with Sylgard gel strips as steps attached on both ends. Hydrated scaffolds were loosely adhered on the coated slides to prevent the sample from moving during imaging. Sylgard steps were used to elevate samples when the entire construct was flipped upside down during imaging.
Figure 2-3. Recovery traces of hydrated scaffolds at 30 °C or at 37 °C either free floating in water or loosely attached on Sylgard coated glass slides suggest that Sylgard does not interfere with shape memory recovery. Stretched scaffolds (about 100 % strain) were hydrated and immersed in 30 °C or at 37 °C water either free floating or loosely attached on Sylgard coated glass slides. Strain was measured every 45 min for the first 6 h, then at 10 h and 24 h, and plotted against time. At 30 °C, free floating scaffolds (▲) and scaffolds loosely attached on Sylgard coated glass slides (▲) recovered only about 5 % of the programmed strain in the first 3 h and remained stable for the reminder of the 21 h. At 37 °C, free floating scaffolds (●) and scaffolds loosely attached on Sylgard coated glass slides (●) recovered rapidly in the first 1 h, and continued to recover the rest of the programmed strain till the end of 5h then remained stable for the next 19 h. At both 30 °C and 37 °C, scaffolds free floating and attached on Sylgard showed no difference in recovery traces. All recovery traces were fit well by a double 4 parameter exponential decay in SigmaPlot. Error bars were plotted as the standard deviation from 3 replicates per group (n = 3).
**Figure 2-4.** One-way shape memory cycle (left) and strain versus time plot (right) of the 3D electrospun SMP scaffold. Left: the shape memory cycle (red solid line) starts at 50 °C, as indicated by the asterisk (*). The SMP scaffold is plasticized at 50 °C, which is above its glass transition temperature (47 °C), and is uniaxially stretched to 100 % strain. The stress is held constant to maintain the strain while temperature is decreased to 0 °C (below glass transition temperature). The stress is then removed and the temporary shape of the scaffold is fixed, which is indicated by the unchanged strain. The temperature is then increased back to 50 °C at the rate of 10 °C/min and held isothermally to trigger the shape memory recovery. During recovery, the strain decreases back to below 0.1 %, indicating the scaffold has recovered back to its permanent shape. The black dashed lines are projected one-way shape memory cycle on the temperature-strain plane and the temperature-stress plane, respectively. Right: strain (%) versus time (min) is plotted using the same one-way shape memory cycle data set. When the shape memory cycle starts at 50 °C, as indicated by the asterisk (*) which corresponds to the same time point indicated by the asterisk (*) in one-way shape memory cycle plot on the left, the strain starts to increase from 0 % to 100 % as the scaffold is being stretched. The strain was maintained at 100 % and then decreased to almost 0 % when full recovery was achieved. The strain vs. time data is presented here to allow visualization of the strain recovery that occurs during the isothermal hold at 50 °C, which cannot be observed in the shape memory cycle at left.
Figure 2-5. Scanning Electron Microscopy (SEM) reveals scaffolds architecture. Static aligned (A) scaffolds showed highly aligned fibers and static unaligned (U) scaffolds showed randomly oriented fibers without specific alignment. Both static controls (A and U) showed no change of fiber orientation before and after temperature trigger. Programmed U-to-A scaffolds had unaligned fiber architecture before recovery and recovered back to highly aligned fiber architecture upon trigger. Conversely, the A-to-U scaffolds showed highly aligned fibers before recovery. Once it was triggered to recover, the fiber lost their alignment and became unaligned. Double headed arrow indicates fiber alignment direction. Scale bar is 40 µm.
Figure 2-6. 2D fast Fourier transform (2D FFT) analysis of fiber alignment. Two distinct peaks at 90° and 270° suggests highly aligned fiber architecture along a principle direction; while no apparent peak suggests random fiber orientation without certain alignment. Static aligned (A) scaffolds before and after recovery both showed highly aligned fiber architecture. Static unaligned (U) scaffolds showed no apparent peak both before and after recovery conditions, indicating no alignment. In U-to-A scaffold, before recovery no apparent fiber alignment was seen. Then upon trigger, U-to-A scaffold recovered back to highly aligned architecture. Conversely, in A-to-U scaffolds, fibers were highly aligned before recovery and recovered back to unaligned upon trigger.
**Figure 2-7.** Qualitative analysis of cell tracks. Cell tracks were plotted by ACT/IV/E with each line represents a cell’s moving path over 24 h. Cells on static aligned (A) control scaffolds moved along the fiber alignment direction both before and after recovery. Cells on static unaligned (U) control scaffolds moved randomly without a specific direction both before and after recovery. The cells on U-to-A scaffolds moved randomly without preferential directionality before recovery when fibers were unaligned, then switched to directional motility along fiber alignment orientation after the switch from unaligned to aligned architecture. Conversely, cells on A-to-U scaffolds moved along the fiber alignment before recovery when fibers were highly aligned, then changed to random motility after fibers recovered from highly aligned to unaligned. The cartoon immediately adjacent to each track plot indicates the associated fiber architecture, either aligned or unaligned. Double-headed arrow indicates the principle fiber alignment direction in the scaffold, if one existed.
Figure 2-8. Diffusion plots of cell final positions with initial position normalized to the same origin. Principle fiber alignment direction was rotated to be parallel with x direction. Diffusion plots qualitatively show cell motility directionality when cells moved on different fiber architecture. Cells moved along the aligned fiber direction in static aligned (A) scaffolds and moved randomly without apparent directionality in static unaligned (U) scaffolds. In dynamic U-to-A scaffolds, cells moved without directionality before recovery, then moved along aligned fiber direction after scaffolds recovered to aligned architecture. While in dynamic A-to-U scaffolds, cells moved along the aligned fibers before recovery, then changed to random movement after scaffolds recovered to unaligned architecture. The cartoon immediately adjacent to each track plot indicates the associated fiber architecture, either aligned or unaligned.
Figure 2-9. Decomposed mean square displacement (MSD) analysis of cell motility. Representative decomposed MSD plots showed when cells in scaffolds with aligned fiber architecture (A-before, A- after, U-to-A- after, A-to-U- before), cells showed directional motility indicated by separated decomposed MSD curves, as well as apparently higher x-intercept than y-intercept. When cells were in scaffolds with unaligned fiber architecture (U- before, U- after, U-to-A- before, A-to-U- after), cells did not have preferred direction of motility indicated by overlapped decomposed MSD curves and no difference in x- and y-intercept. By increasing fiber alignment (in U-to-A), cells acquired preferred motility direction; while by dynamically decreasing fiber alignment (in A-to-U), cells lost their motility directionality. The cartoon immediately adjacent to each track plot indicates the associated fiber architecture, either aligned or unaligned.
Figure 2-10. Average cell velocity. Average cell velocity (µm/min) was compared within each scaffold architecture before and after recovery, and also among different scaffold architectures either before or after recovery. Static aligned (A) scaffold and U-to-A scaffold showed significantly increased cell velocity after temperature trigger. Before recovery, no difference was shown among different scaffold architectures. After recovery, cells in static aligned (A) scaffold had significantly higher velocity comparing to static unaligned (U). And U-to-A had significantly higher cell velocity comparing to A, and U. Asterisks indicate significant difference ($p < 0.05$; $n = 3$).
**Figure 2-11.** Representative cell body and cell nuclear staining images before and after scaffolds shape changing recovery. Cell body aligned along the highly aligned fibers in aligned (A) scaffold before and after recovery, A-to-U scaffold before recovery, and U-to-A scaffold after recovery. Cell body was randomly oriented without specific alignment in unaligned (U) scaffold before and after recovery, A-to-U scaffold after recovery, and U-to-A scaffold before recovery. Change of fiber alignment also changed cell body alignment in dynamic SA and SU scaffolds. White double-headed arrows indicate fiber principle alignment direction, if one existed.
Figure 2-12. Angular histograms qualitatively show cell body and cell nuclear alignment on different scaffold architectures. C3H10T1/2 cell body (solid line box) and cell nuclei (dashed line box) showed the same trend of alignment. Static aligned scaffolds (A) showed narrow cell body and cell nuclear angular distribution, which indicates that both cell body and cell nuclei aligned along the unidirectional fiber alignment direction before and after thermal triggering. Static unaligned scaffolds (U) showed much broader cell body and cell nuclear angular distribution, which indicates that both cell body and cell nuclei had no apparent preferential alignment before and after thermal triggering. U-to-A scaffolds showed a change from broad to narrow cell body and cell nuclear angular distribution, which indicates that both cell body and cell nuclei increased preferential alignment along the unidirectional fiber alignment direction. A-to-U scaffolds showed a change from narrow to broad cell body and cell nuclear angular distribution, which indicates that both cell body and cell nuclei decreased alignment. The red line represents the mean resultant vector length (R). The cartoon on top right corner of each angular histogram plot indicates the associated fiber architecture, either aligned or unaligned.
Chapter 3: The Effect of Change in Fiber Alignment on Cancer Cell Polarization and Directional Motility†

3.1 Synopsis

In Chapter 2, we developed a robust 3D SMP fibrous scaffold that can change internal scaffold architecture – fiber alignment – on command under cytocompatible condition. We studied murine mesenchymal stem cells C3H10T1/2 motility on such dynamic scaffolds when dynamic shape and internal architecture change of the scaffolds occurred. We demonstrated that such SMP system could be used for in vitro models for cell motility study. In this chapter, we further examined a cancer cell line – human fibrosarcoma cells HT-1080 – on such SMP 3D fibrous scaffolds. Cell directional motility and polarized morphology were investigated to test the hypothesis that highly aligned fiber architecture would induce polarized morphology of cancer cells and further guide their directional motility. Furthermore, the dynamic change of fiber alignment would also change the cancer cell polarized morphology and directional motility. To achieve this goal, we cultured human fibrosarcoma HT-1080 cells on SMP 3D fibrous scaffolds, performed live imaging and immunohistochemistry staining, and analyzed cell alignment, polarized morphology, and directional motility before and after scaffolds architectural change.

3.2 Introduction

As mentioned in Chapter 2, directional cell motility is a critical step in cancer invasion.

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and metastasis. The extracellular matrix (ECM), as a major component of cellular microenvironment with unique physical and biochemical properties, plays an important role in regulating cancer cell directional motility. The dynamics of the ECM architecture, particularly, has shown to severely affect cancer cell polarized motility and subsequent invasion and metastasis. For example, linearized collagen fibers are common in cancer, and are often found in areas where active tumor invasion is observed [1,2]. In pregnancy associated breast cancer, radially aligned collagen fibers are thought to provide “tracks” for cancer cells to invade into the surrounding stroma [3–5]. Therefore, significant attention has been focused on exploring how cancer cell directional motility is regulated by controlling the ECM architecture.

Different types of cells migrate in various ways under different mechanisms. Actin cytoskeleton reorganization via cell-ECM interactions remains the primary mechanism of cell polarized motility and is essential for most types of cancer cell migration. When cancer cells are cultured on 2D substrates *in vitro*, a defined cell polarization and repositioning of the Golgi apparatus are necessary for directional motility, which requires the activation of Rac and Cdc42 at the leading edge and Rho at the trailing edge (Rac, Cdc42, and Rho are the most well studied actin assembly regulators that belong to the Rho family small GTPases) [6,7]. The Golgi apparatus is often repositioned to stay ahead of the nucleus in the direction of migration [8,9], but can also be positioned to the sides or behind the Golgi when cells are constrained by substrate geometry [10]. While in 3D matrices, cancer cells exhibit two typical modes of migration with distinct morphologies. One is mesenchymal migration, in which cancer cells show elongated and polarized morphology and have integrin-dependent adhesion to the ECM. In this migration mode, the cancer cells utilize proteolysis to degrade the surrounding ECM and generate paths to move. The other
migration mode is amoeboid migration. In this migration mode, cells display a non-polarized rounded morphology and move in an integrin-independent manner. They move through the ECM by squeezing themselves into gaps in the ECM and do not require the degradation of the ECM [11–13]. These two modes of migration can interconvert spontaneously, which makes the current invasion-targeted therapeutics insufficient to inhibit invasion and metastasis [14–16]. However, in 3D matrices which are more biomimetic of the in vivo microenvironment (compared to 2D substrates), how the dynamic change of the ECM architecture affect the cancer cell polarity, directional motility, and the repositioning of the Golgi apparatus is poorly understood, as most currently used 3D models are static [17–21]. Therefore, a 3D in vitro cell culture platform which can mimic the dynamic change of the ECM architecture is in great need to explore such unknown questions and to further understand the effect of the dynamics of the ECM architecture on cancer cell polarization and directional motility. Such information could help achieve better control of cancer cell motility via the cytoskeleton reorganization and provide the possibility of regulating cancer cell invasion and metastasis.

A custom synthesized and fabricated thermoplastic polyurethane 3D SMP electrospun scaffold, as previously investigated in Chapter 2, appears to be a promising candidate for studying cancer cell polarization, and directional motility. Such scaffold can dynamically change the internal scaffold architecture, which can mimic the in vivo microenvironment dynamic architectural change when cancer invasion and metastasis happens. Specifically, this fibrous scaffold mimics the architecture of the collagen fibers in ECM, and can dynamically change fiber alignment on commend under cytocompatible condition, either from highly aligned to unaligned, or from unaligned to highly aligned. Comparing to the most widely used 3D in vitro models such
as collagen gels, such scaffold is de-coupled from the cellular remodeling, which could change matrix biochemical composition and physical properties [4,22] and makes it extremely difficult to analyze the role of fiber alignment only in cancer progression.

In this present study, we employed the 3D SMP fibrous scaffold, which has been proved to be capable of studying cell motility in dynamic in vitro microenvironment (Chapter 2), to investigate how human fibrosarcoma HT-1080 cells would change their morphology and directional motility when exposed to dynamic increased or decreased matrix fiber alignment. Analysis of cell body and nuclei alignment, polarized morphology, and directional cell motility were performed to test the hypothesis that highly aligned scaffold architecture would induce polarized cell body and nuclei, and directional motility aligned along the fiber orientation, while unaligned scaffold architecture would produce rounded cell and nuclei morphology, and non-directional motility. More importantly, we also hypothesized that the dynamic increase of fiber alignment would induce cells and their nuclei to change from non-polarized to polarized morphology and subsequent motility pattern from non-directional to directional. In contrast, the dynamic decrease of fiber alignment would induce cells and their nuclei to change from polarized to non-polarized morphology, accompanied by the change from directional motility to non-directional motility.
3.3 Methods and Materials

3.3.1 Study design

Scaffolds representing four different architectures, two static – static aligned (A) and static unaligned (U), and two dynamic – U-to-A and A-to-U, were used in this study as described in details in section 2.3.1. The dynamic A-to-U scaffold can change fiber architecture from temporarily aligned to permanently unaligned by increasing incubation temperature from 30 °C to 37 °C when hydrated; while the dynamic U-to-A scaffold can change fiber architecture from temporarily unaligned to permanently aligned. The two static scaffolds will not change fiber alignment upon trigger. The static aligned scaffold will remain the aligned architecture, while the static unaligned scaffold will remain the unaligned architecture. The “before recovery” set and “after recovery” set of scaffolds with each set containing all four different scaffold architecture groups (see details in section 2.3.1), were prepared for cell culture (section 3.3.3), immunohistochemistry staining (section 3.3.4) for cell alignment (section 3.3.5) and polarization analysis (section 3.3.6), and time-lapse imaging (section 3.3.7) for cell directional motility analysis (section 3.3.8) (Figure 3-1).

3.3.2 Scaffolds preparation

To prepare 3D SMP fibrous scaffolds to study cell polarization and directional motility, a thermoplastic polyurethane (TPU) featuring shape memory functionality was first synthesized as previously described in section 2.3.1. One batch of TPU with molecular weight of about 130 K was used and dissolved for electrospinning to fabricate static aligned and static unaligned
scaffolds. Two static control scaffolds – static aligned and static unaligned scaffolds, and two
dynamic experimental scaffolds – strain aligned and strain unaligned scaffolds were prepared as
previously described in section 2.3.3.

3.3.3 Cell culture

To study cancer cell polarization and motility behavior on the shape memory electrospun
scaffolds, human fibrosarcoma HT-1080 cells (kindly provided by Dr. Christopher E. Turner from
SUNY Upstate Medical School) were maintained in Minimum Essential Medium (MEM)
supplemented with 10 % (v/v) Fetal Bovine Serum (FBS), 1 % (v/v) GlutaMAX, and 1 % (v/v)
Penicillin-Streptomycin. All cell culture reagents were purchased from Invitrogen. “Before
recovery” and “after recovery” sets of scaffolds with each scaffold architecture containing two
technical replicates were prepared.

To culture cells on scaffolds for cell polarization and motility analysis, scaffolds were UV
sterilized, hydrated and loosely adhered on Sylgard coated glass slides (see details in section 2.3.4)
before cell seeding. To seed cells on scaffolds, cell suspension was pipetted on scaffolds in a 20
µl droplet at the density of 4000 cells/cm² for time-lapse imaging experiments, and the density of
2500 cells/cm² for immunohistochemistry staining experiments. Cell seeded scaffolds were
cultured under 30 °C for 5 h to allow cell attachment. After cells were attached, 3 ml of complete
cell culture medium was added to petri dishes to fully cover the scaffolds. Cell seeded scaffolds
were then further cultured at 30 °C overnight before followed time-lapse imaging and
immunohistochemistry staining.
3.3.4 Immunohistochemistry staining and imaging

To examine the effect of fiber alignment on cell body and nuclear alignment, cell body and nuclear polarized morphology, immunohistochemistry staining was performed to fluorescently label Golgi body by GM 130 antibody (provided by kindly provided by Dr. Christopher E. Turner from SUNY Upstate Medical School), actin cytoskeleton by Alexa Fluor 568 Phalloidin (Invitrogen). Cell nuclei were stained with and DAPI (Invitrogen).

Briefly, samples were taken out of the incubator and cell culture medium was aspirated. Samples were fixed and permeabilized at the same time with freshly made 4 % paraformaldehyde and 1 % Triton X-100 in PBS at room temperature for 15 min. After fixing and permeabilization, samples were rinsed with PBS for three times, then quenched with 0.1 M Glycine in PBS for 20 min at room temperature. After quenching, samples were rinsed with PBS three times, and blocked with 3 % bovine serum albumin (BSA) at 4 °C overnight. After blocking, primary GM 130 mouse Golgi antibody at 1:50 dilution ratio made in 3 % BSA was added to the samples and incubated at 30 °C for “before recovery” samples and 37 °C for “after recovery” samples for 2 h. After primary antibody incubation, samples were rinsed with PBS with 0.05 % Tween (PBST) three times. Then the secondary antibody Anti-Mouse 488 at 1:100 dilution ratio and phalloidin 568 at the 1:50 dilution ratio made in PBST were added to the samples and incubated at room temperature for 1 h in dark to avoid photobleaching. After secondary antibody incubation, samples were rinsed with PBST three times, then with DI water twice. Samples were briefly stained with DAPI (1:1000 fold dilution in DI water) for 1 min at room temperature. After a brief rinse with DI water once, samples were mounted with Gelvatol and stored at 4 °C overnight allowing Gelvatol become fully dried.
before imaging. All immunohistochemistry solutions were kindly provided by Dr. Christopher E. Turner’s laboratory at SUNY Upstate Medical University.

Mounted samples were warmed to room temperature at least 30 min before imaging to avoid focal shifting. Images were acquired using Leica DMI 6000B inverted microscope with 20X and 40X objectives. Z-stacks of images were taken at each position from relative -20 µm to 20 µm with 8 µm increment. Images at twenty different positions per sample were taken. A sample size of six (n = 6) was used for statistical analysis.

3.3.5 Quantification of cell body and nuclear alignment

To quantify cell body and nuclear alignment when cells were cultured on scaffolds with different fiber alignment, actin cytoskeleton images from phalloidin staining and nuclear images from DAPI staining acquired with 20X objective as previously described [23] were analyzed using ImageJ built-in function Analyze Particles. Briefly, images were threshold adjusted in ImageJ to achieve a binary image to isolate cell body or cell nuclei, respectively, and to remove background. Once the binary image was created, the Analyze Particle built-in function was used to fit an ellipse to each cell body or nucleus and determine the corresponding angle, ranging from 0° to 180°. Unfocused or overlapped cell body or nuclei were excluded for analysis.

Cell body and nuclear alignment quantification was performed as previously described [23]. Briefly, after all nuclear angle values were collected, 0° was used as an arbitrary reference angle. A value of 90° was subtracted from each nuclear angle to adjust the range of angles to -90° to +90°, centered around 0°. The truncated standard deviation of nuclear angles was then calculated as discussed previously described [23,24]. A truncated standard deviation of 52° represents
randomly distributed nuclear angles; while a standard deviation of 0° represents perfectly aligned nuclear angles. Smaller angular standard deviation indicates more aligned angular angles. The truncated angular standard deviation was used for statistical analysis to quantify the cell body or nuclear angular distribution and to compare the cell body or nuclear alignment when cells were exposed to different scaffold architectures. The final angle that yielded the minimum standard deviation was used to compare if the cell body and nuclei were aligned in the same angle, which corresponds to the principle scaffold fiber alignment angle.

Cell body and nuclear alignment was also quantified by the mean resultant vector length (R), calculated by averaging the vector summation of cell angles treated as unit vectors over the total number of cells [25]. Perfectly aligned angles would produce an R of 1.0, while randomly distributed angels would have an R of ~0.63 [26]. The R values were compared before and after recovery within each scaffold architecture to assess the cell body and nuclear alignment.

3.3.6 Quantification of cell body and nuclear morphology

To quantify cell body and nuclear morphology when cells were cultured on scaffolds with different fiber alignment, actin cytoskeleton images from phalloidin staining and nuclear images from DAPI staining acquired with 20X objective as previously described were analyzed using ImageJ built-in function Analyze Particles. Again, the images were threshold adjusted, ellipse fitted, and the total area, circularity, and aspect ratio values of cell body or nuclei, respectively, were calculated by Analyze Particles function. Total area represents the total area of the cell body, or nuclei, respectively. The circularity with a value of 1.0 indicates a perfect circle, while a value approaching 0.0 indicates an increasingly elongated morphology. The aspect ratio is calculated as
the ratio of the major and minor axis of a cell body or nucleus, respectively. Collectively, a larger total area, a smaller circularity, and a higher aspect ratio together indicate a more polarized morphology. Unfocused or overlapped cell body or nuclei were excluded for analysis.

3.3.7 Time-lapse imaging

To image cells for tracking analysis, cell nuclei were stained with Hoechst 33342 nuclear dye (Invitrogen) at the final concentration of 0.01 µg/ml in cell culture medium. The “before recovery” and “after recovery” sets of scaffolds were image at 30 °C for 24 h or 37 °C for 24 h, respectively, as previously described in section 2.3.5. Cell seeding and subsequent live imaging were independently repeated three times (n = 3).

3.3.8 Cell tracking and motility analysis

To analyze cell motility before and after scaffold architectural change, resultant images from live imaging were process and the resultant time-lapse videos were analyzed by ACTIV. Full tracks, diffusion plots, mean square displacement (MSD), decomposed MSD, and average velocity results output from ACTIV were used to qualitatively and quantitatively assess cell motility, as previous described in section 2.3.6.

3.3.9 Statistics

To quantify the cell body and nuclear alignment affected by scaffold fiber alignment, statistical analysis was performed on cell body and nuclear angular standard deviation respectively. All architectural groups were compared before or after recovery using one way ANOVA tests.
Within the same architectural group, angular standard deviation was compared between before and after recovery using paired t-tests.

To quantify the cell body and nuclear morphology and polarization, total area, circularity, and the aspect ratio values of cell body and the nuclei were compared among architectural groups before or after recovery using one-way ANOVA tests or Kruskal-Wallis tests. Within the same architectural group, the values were compared between before and recovery using paired t-tests or Wilcoxon signed-rank tests.

To compare the cell motility affected by time, and scaffold architectural change, cell tracking MSD parameters, decomposed MSD parameters, and average velocity were analyzed by statistical analysis mentioned in section 2.3.8. Statistical significant difference was determined at $p < 0.05$ for all the above tests.

3.4 Results

3.4.1 Cell body and nuclear alignment

Representative merged fluorescent images of cell actin cytoskeleton, nuclei, and Golgi apparatus acquired by 40X objective (Figure 3-2) qualitatively show that human fibrosarcoma HT-1080 cells aligned along the fibers and displayed elongated morphology when exposed to highly aligned scaffold architecture and became randomly distributed and showed rounded morphology when exposed to unaligned scaffold architecture.
Angular histograms of both cell body and nuclei from angular distribution analysis (Figure 3-3) show that both cell body and nuclei have narrow angular distribution, suggesting that they both aligned along the fibers when the scaffolds possess highly aligned fiber architecture. In contrast, cell body and nuclei showed much broader angular distribution, which suggests that neither cell body nor nuclei had specific alignment in certain direction when the scaffolds have unaligned fiber architecture. More importantly, when programmed A-to-U scaffolds dynamically changed fiber architecture from temporarily aligned to permanently unaligned, cell body and nuclei angles changed from a narrow distribution to a broad distribution, indicating that cell body and nuclei lost their alignment along the fibers. Conversely, when programmed U-to-A scaffolds changed fiber architecture from unaligned to aligned, cell body and nuclei angles changed from a broad distribution to a narrow distribution, suggesting that cell body and nuclei reoriented to align along the fibers.

Such alignment was further confirmed by quantitative analysis of the angular standard deviation of both cell body and nuclei (Figure 3-4). Briefly, highly aligned cell body and nuclei showed low angular standard deviation, while randomly oriented cell body and nuclei showed high angular standard deviation. Upon triggering the shape memory recovery of the programmed scaffolds, both cell body and nuclei had significantly higher angular standard deviation in A-to-U scaffolds and significantly lower angular standard deviation in U-to-A scaffolds. To assess the correlation between the cell body angle and the nuclear angle on aligned scaffold architectures, final angles of both cell body and nuclei that resulted the maximum alignment were compared to the scaffold fiber principle alignment angle (rotated to be at 90°) (Table 3-1). Each pair of the final angles from cell body and nuclei shows a difference within 10°, and a difference within 10° to the
principle fiber alignment angle, indicating that cell body alignment and nuclear alignment are in agreement with the scaffold fiber alignment angle.

3.4.2 Cell body and nuclear polarized morphology

The polarized morphology of the cell body and the nuclei from different scaffold architectures before and after recovery were assessed by quantifying total area, circularity, and aspect ratio of the cell body (Table 3-2) and nuclei (Table 3-3), respectively, and compared among different architectures before or after recovery and within the same scaffold group before and after recovery. Significantly decreased total cell body area and total nuclear area, increased cell body and nuclear circularity, and decreased cell body and nuclear aspect ratio in programmed A-to-U scaffold suggest that decreased fiber alignment induced rounded and non-polarized cells. In contrast, significantly increased total cell body area and total nuclear area, decreased cell body and nuclear circularity, and increased cell body and nuclear aspect ratio in programmed U-to-A scaffold indicate that increased fiber alignment induced more elongated and polarized cells.

3.4.3 Qualitative and quantitative analysis of cell motility

3.4.3.1 Cell tracks

Qualitative analysis of cell tracks over 24 h shown in Figure 3-5 indicate that cell directional motility was directed by fiber architecture. Briefly, the dynamic change of fiber alignment in programmed scaffolds can change the cell motility directionality as indicated in the change of cell track orientation before and after recovery in strain aligned scaffold and strain unaligned scaffold. Specifically, after A-to-U scaffold changed from aligned to unaligned
architecture, cell tracks changed from highly oriented along fiber alignment direction to randomly oriented without a preferential direction; while after U-to-A scaffold changed from unaligned to aligned architecture, cell tracks changed from randomly oriented without a preferential direction to oriented along the principle fiber alignment direction. And cells moved preferentially along the fiber alignment direction when they were exposed to highly aligned fiber architecture, and cells moved randomly without a preferential direction when there were exposed to fibers that are not preferentially aligned.

3.4.3.2 Diffusion plots

Diffusion plots (Figure 3-6), another way to qualitatively show cell motility directionality, showed preferential cell motility direction when cells are on aligned architectures and non-preferential motility direction on randomly oriented fiber architectures. More importantly, the diffusion plots also showed the change of fiber alignment could change the directional motility. Briefly, when dynamic A-to-U scaffold changed from aligned architecture to unaligned architecture, the diffusion plot showed a change from an elongated distribution of the cells along the x direction to radially distributed cell positions. Conversely, when dynamic U-to-A scaffold changed from unaligned architecture to aligned architecture, the diffusion plot showed a change from radially distributed cell positions to an elongated distribution of the cells along the x direction.

3.4.3.3 MSD

MSD showed no statistically significant difference of short timescale slope or long timescale slope between before recovery and after recovery within each scaffold architecture (Table 3-4). This indicates that the temperature change used to trigger scaffolds shape recovery
did not affect cell motility behavior. Long timescale slopes are significantly lower than corresponding short timescale slopes among all scaffolds, indicating overtime the cells moved more diffusively (Table 3-4).

3.4.3.4 Decomposed MSD

Decomposed MSD (Figure 3-7) in the principle fiber alignment direction (x) and the direction perpendicular to the principle fiber alignment direction (y) enables analysis for cell motility directionality. Preferred motility direction is indicated by the fitted line with higher intercept. Qualitatively, when cells were exposed to programmed scaffolds in which the architecture dynamically changed over time, cell motility changed from directional to non-directional when fiber alignment decreased in strain aligned scaffolds; while cells changed from non-directional to directional motility when fiber alignment increased in strain unaligned scaffolds.

Quantitatively, to assess cell directional motility, x-long timescale intercept and y-long timescale intercept within each scaffold architecture were compared within each scaffold architecture (Table 3-4). Briefly, static aligned (A) scaffold and A-to-U scaffold before triggering shape recovery and static aligned (A) scaffold and U-to-A scaffold after recovery, with all had highly aligned fiber architecture, had significantly higher x-long timescale intercept than y-long timescale intercept. This indicates that cells showed directional motility along principle fiber alignment direction (x). When comparing the motility directionality change before and after recovery in dynamic programmed scaffolds (Table 3-4), A-to-U scaffold showed significantly decreased difference in x and y long timescale intercept (x-long timescale intercept – y-long
timescale intercept); while U-to-A scaffold showed significantly increased difference in x and y long timescale intercept. This suggests that by dynamically decreasing fiber alignment, cells lost their motility directionality; while by dynamically increasing fiber alignment, cells acquired preferential motility directionality along principle fiber alignment direction.

3.4.3.5 Average velocity

Average velocity allows quantitative comparison of cell velocity when cells were exposed to different fiber architectures (Figure 3-8). Before recovery, average velocity among different groups showed no significant difference. After recovery, the U-to-A group showed significantly higher cell velocity compared to A-to-U group. When comparing before and after recovery within the same scaffold architecture, U-to-A scaffold showed significantly increased average velocity after scaffold increased fiber alignment. This indicates that when fiber alignment dynamically increases in the U-to-A group, cells moved faster on highly aligned fibers. Temperature increase induced slightly increased cell velocity in aligned and unaligned scaffolds but these increase are not statistically significantly different. The lowest velocity fold change in A-to-U scaffold (1.002-fold increase after recovery) comparing to the fold change (velocity fold change = after recovery velocity / before recovery velocity) in other groups (1.053-fold change in A; 1.135-fold change in U; 1.389-fold change in U-to-A) may suggest that cells moved slower when fiber alignment decreased. As this cell velocity change induced by decrease in fiber alignment is not significant, it may also suggest that cells that were pre-exposed to aligned fibers would keep on moving faster than the naïve cells.
3.5 Discussion

In the present study, we investigated cancer cell polarization and directional motility in an *in vitro* dynamic 3D SMP fibrous scaffold, which has been previously proved (Chapter 2) to be capable of being used for cell motility study with cells in scaffold undergoing dynamic scaffold architectural change. Building upon our prior work, the work of the present chapter further expands the application of this dynamic 3D scaffold into the study of cancer cell morphology and directional motility. We found scaffold fiber architecture can direct cancer cell polarization and directional motility. And we also found that the change of the fiber architecture can direct the change of cell polarization, directional motility, and velocity. To the best of our knowledge, this is the first study that qualitatively and quantitatively investigates cancer cell polarization and directional motility behavior in 3D dynamic SMP *in vitro* system.

The dynamic system employed, which has shown to be able to change cell polarized morphology and directional motility, may provide a model for mimicking the collagen fiber alignment change observed *in vivo* when tumor invasion and metastasis happen. Besides cancer cell morphology, which is highly associated with the mesenchymal and amoeboid mode of motility in 3D matrices, cancer cell phenotype and signaling pathways need to be further examined to assess the effect of matrix fiber alignment on cancer cell phenotype and associated molecules, which are also key player in determining the motility modes. For example, gene markers of invasive phenotype, such as Cox-2, Cdc42, and MRCK [22,27] could be looked into to investigate if highly aligned scaffold fiber architecture would induce invasive phenotype of the cancer cells. Previous study has shown that Paxillin, a signal transduction adaptor protein that targets to focal adhesions,
regulates Golgi integrity, mesenchymal or amoeboid morphology, and polarized motility of cancer cells via inhibitory interaction with the α-tubulin deacetylase HDAC6 [21]. Such signaling pathway could also be studied in the future to achieve in-depth understanding of the effect of matrix fiber alignment on cancer cell cytoskeleton reorganization, Golgi structure and the polarized motility on a molecular level. Such continued studies mentioned above would provide more information about how change of matrix fiber alignment would control the cancer cell phenotype, and corresponding mode of motility. This information would significantly benefit the current understanding of the cell-matrix interaction during tumor invasion and metastasis, and could potentially provide therapeutic methods to control the mode of cancer cell motility to achieve more effective cancer treatments.

Instead of having uniform fiber architecture within one scaffold, more complex scaffold architecture could be incorporated, such as locally varied fiber alignment, to study preferential cell motility to remote locations with different matrix architectures. More complex scaffold architecture could include: (1) aligned fibers in one half of the scaffold and unaligned fibers in the other half with a single boundary between aligned and unaligned fibers; (2) a single isolated “island” of aligned fibers surrounded by unaligned fibers; (3) multiple isolated or interacting “islands” of aligned fibers surrounded by unaligned fibers. Such complex scaffold architecture may be a promising candidate that mimics the invasion of cancer cells into stroma and subsequent metastasis events in vivo.

In this study, individual cancer cell motility was investigated. However, collective motility of the cancer cells could also be studied using such 3D dynamic in vitro system in the future as collective invasion is common in many cancer types [28], and many cancers show predominantly
collective invasion when explanted *in vitro* [29]. Previous study has shown that in *in vitro* oral squamous cancer model, local ECM remodeling associated with collective invasion might contribute to invasive tumor growth [30]. Even though the polarity and guidance mechanisms of cancer cell collective motility are poorly understood, but the collective motility associated with ECM remodeling strongly suggest that dynamic change of the ECM architecture could play an important role in collective tumor invasion and metastasis.

Due to current experimental limitations, in the present study we did not analyze cell motility in the z direction – the direction parallel with the scaffold depth. Though multiple cells layers were captured during imaging, the cells located at different focal planes were projected to the same focal plane during post-processing. However, with thicker scaffolds and longer pre-culture time, cells could infiltrate more into the scaffolds and our imaging system and ACTIVE analysis algorithm could, with modification, be capable of analyzing cell motility three dimensionally.

3.6 Conclusion

Human fibrosarcoma HT-1080 cell polarized morphology and directional motility was investigated and it was found that the cell polarized morphology and directional motility can be directed by dynamic change of the scaffold fiber architecture. We found that when cells were exposed to highly aligned scaffold architecture, cells would display elongated and polarized morphology, align along the principle fiber alignment direction, and directional motility along the fiber alignment direction. When cells were exposed to unaligned scaffold architecture, cells would display rounded and non-polarized morphology, randomly distributed in the scaffold without
preferential alignment, and a random motility pattern without preferential direction within the scaffold. Moreover, when fiber alignment dynamically increases, cells would change their morphology from rounded non-polarized to elongated and polarized, become aligned along the fiber orientation, and display directional motility along the fiber orientation with significantly increased velocity. In contrast, when fiber alignment dynamically decreases, cell morphology would change from polarized to non-polarized, losing preferential alignment, and cells would move randomly without preferred direction. This scaffold appears to be a promising candidate as dynamic in vitro 3D cell culture platform for in depth study of cancer cell motility and metastasis for the application of cancer therapy.

3.7 Acknowledgements

The authors gratefully thank Ling-Fang Tseng and Andy Quach for their help with TPU synthesis, Megan Brasch for technical questions about ACTIV/E, Turner lab from SUNY Upstate for providing the HT-1080 cells, and Dr. Christopher Turner and Dr. Nicholas Deakin for generally providing insights on the topic.

3.8 References


[20] Deakin NO, Turner CE. Distinct roles for paxillin and Hic-5 in regulating breast cancer cell


Table 3-1. Final angles of both cell body and nuclei that resulted the maximum alignment in aligned fiber architecture groups.

<table>
<thead>
<tr>
<th>A before</th>
<th>A after</th>
<th>A-to-U before</th>
<th>U-to-A after</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell body Nuclei</td>
<td>Cell body Nuclei</td>
<td>Cell body Nuclei</td>
<td>Cell body Nuclei</td>
</tr>
<tr>
<td>98° 97°</td>
<td>99° 91°</td>
<td>92° 94°</td>
<td>89° 89°</td>
</tr>
<tr>
<td>95° 95°</td>
<td>94° 95°</td>
<td>81° 89°</td>
<td>89° 91°</td>
</tr>
<tr>
<td>96° 96°</td>
<td>97° 102°</td>
<td>89° 81°</td>
<td>94° 94°</td>
</tr>
<tr>
<td>82° 87°</td>
<td>101° 94°</td>
<td>91° 92°</td>
<td>89° 90°</td>
</tr>
<tr>
<td>91° 95°</td>
<td>93° 95°</td>
<td>99° 91°</td>
<td>90° 82°</td>
</tr>
<tr>
<td>84° 79°</td>
<td>83° 84°</td>
<td>90° 82°</td>
<td>95° 91°</td>
</tr>
</tbody>
</table>

The final angles of the cell body and nuclei that yielded the minimum standard deviation were in agreement with a difference within 10°, indicating that the cell body and the nuclei were aligned in the same direction in aligned fiber architecture groups. These final angles were also in agreement with the principle fiber alignment angle – 90° with a difference within 12°. Six replicates were used per scaffold architecture (n = 6).
Table 3-2. Cell body morphology analysis.

<table>
<thead>
<tr>
<th></th>
<th>Before Recovery</th>
<th>After Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>U</td>
</tr>
<tr>
<td><strong>Area (pixels^2)</strong></td>
<td>864.303 ± 358.897</td>
<td>493.066 ± 143.109</td>
</tr>
<tr>
<td><strong>Circ.</strong></td>
<td>0.286 ± 0.027</td>
<td>0.440 ± 0.093</td>
</tr>
<tr>
<td><strong>Aspect Ratio</strong></td>
<td>3.682 ± 0.547</td>
<td>1.869 ± 0.141</td>
</tr>
</tbody>
</table>

Total area, circularity and aspect ratio values of cell body were compared within the same architectural group (A, U, U-to-A, A-to-U) before and after recovery. Data are reported as average ± standard deviation. Highlighted groups that share the same color are significantly different with p value less than 0.05. Statistically significant differences exist among the architectural groups before or after recovery, but are not shown to highlight the key difference of interest.
Table 3-3. Cell nuclei morphology analysis.

<table>
<thead>
<tr>
<th></th>
<th>Before Recovery</th>
<th></th>
<th></th>
<th></th>
<th>After Recovery</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>U</td>
<td>U-to-A</td>
<td>A-to-U</td>
<td>A</td>
<td>U</td>
<td>U-to-A</td>
<td>A-to-U</td>
</tr>
<tr>
<td>Area (pixels²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>226.827</td>
<td>175.936</td>
<td>187.388</td>
<td>169.529</td>
<td>255.520</td>
<td>234.831</td>
<td>263.90 ±</td>
<td>119.210 ±</td>
</tr>
<tr>
<td>Circ.</td>
<td>0.618 ± 0.157</td>
<td>0.631 ± 0.157</td>
<td>0.715 ± 0.060</td>
<td>0.396 ± 0.047</td>
<td>0.682 ± 0.040</td>
<td>0.683 ± 0.092</td>
<td>0.564 ± 0.104</td>
<td>0.565 ± 0.050</td>
</tr>
<tr>
<td>Aspect Ratio</td>
<td>1.516 ± 0.094</td>
<td>1.3580 ± 0.069</td>
<td>1.439 ± 0.071</td>
<td>1.543 ± 0.191</td>
<td>1.593 ± 0.191</td>
<td>1.423 ± 0.087</td>
<td>1.730 ± 0.081</td>
<td>1.383 ± 0.043</td>
</tr>
</tbody>
</table>

Total area, circularity and aspect ratio values of cell nuclei were compared within the same architectural group before and after recovery. Data are reported as average ± standard deviation. Highlighted groups that share the same color are significantly different with p value less than 0.05. Statistically significant differences exist among the architectural groups before or after recovery, but are not shown to highlight the key difference of interest.
Table 3-4. Cell motility MSD parameters analysis.

<table>
<thead>
<tr>
<th></th>
<th>Short Timescale</th>
<th>Long Timescale</th>
<th>Long Timescale</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>Slope</td>
<td>Intercept</td>
</tr>
<tr>
<td><strong>Before</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.113 ± 0.098</td>
<td>1.039 ± 0.300</td>
<td>-0.181 ± 0.852</td>
</tr>
<tr>
<td>U</td>
<td>1.131 ± 0.115</td>
<td>1.007 ± 0.309</td>
<td>-0.228 ± 0.861</td>
</tr>
<tr>
<td>U-to-A</td>
<td>1.198 ± 0.121</td>
<td>1.092 ± 0.085</td>
<td>-0.347 ± 0.086</td>
</tr>
<tr>
<td>A-to-U</td>
<td>1.156 ± 0.107</td>
<td>1.257 ± 0.038</td>
<td>-0.564 ± 0.142</td>
</tr>
<tr>
<td><strong>After</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.346 ± 0.218</td>
<td>0.901 ± 0.196</td>
<td>0.344 ± 0.631</td>
</tr>
<tr>
<td>U</td>
<td>1.379 ± 0.194</td>
<td>1.087 ± 0.115</td>
<td>-0.018 ± 0.536</td>
</tr>
<tr>
<td>U-to-A</td>
<td>1.413 ± 0.144</td>
<td>1.131 ± 0.186</td>
<td>-0.047 ± 0.454</td>
</tr>
<tr>
<td>A-to-U</td>
<td>1.347 ± 0.206</td>
<td>1.012 ± 0.120</td>
<td>0.186 ± 0.391</td>
</tr>
</tbody>
</table>

Short timescale slopes, long timescale slopes, and long timescale intercepts listed in above were calculated by ACT/VE by line fitting of $\log_{10}\text{MSD} - \log_{10} t$ curve. Data are reported as average ± standard deviation. No significant difference was found between before and after recovery within each scaffold architecture. Long timescale slopes are significantly lower than corresponding short timescale slopes among all scaffolds both before and after recovery, tested by paired student’s t-test with $p = 0.0049$. 
Table 3-5. Cell motility decomposed MSD parameters analysis.

<table>
<thead>
<tr>
<th></th>
<th>X Short Timescale Slope</th>
<th>X Long Timescale Slope</th>
<th>X Long Timescale Intercept</th>
<th>Y Short Timescale Slope</th>
<th>Y Long Timescale Slope</th>
<th>Y Long Timescale Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before A</td>
<td>1.126 ± 0.156</td>
<td>0.974 ± 0.428</td>
<td>-0.143 ± 0.549</td>
<td>1.045 ± 0.145</td>
<td>1.003 ± 0.184</td>
<td>-0.969 ± 0.878</td>
</tr>
<tr>
<td></td>
<td>0.156</td>
<td>0.428</td>
<td>0.549a</td>
<td>0.145</td>
<td>0.184</td>
<td>0.878a</td>
</tr>
<tr>
<td>U</td>
<td>1.107 ± 0.097</td>
<td>0.921 ± 0.285</td>
<td>-0.629 ± 0.796</td>
<td>1.150 ± 0.133</td>
<td>1.024 ± 0.344</td>
<td>-0.721 ± 1.041</td>
</tr>
<tr>
<td></td>
<td>0.097</td>
<td>0.285</td>
<td>0.796</td>
<td>0.133</td>
<td>0.344</td>
<td>1.041</td>
</tr>
<tr>
<td>U-to-A</td>
<td>1.199 ± 0.151</td>
<td>1.104 ± 0.114</td>
<td>-1.231 ± 0.354</td>
<td>1.181 ± 0.085</td>
<td>1.106 ± 0.194</td>
<td>-1.305 ± 0.462</td>
</tr>
<tr>
<td></td>
<td>0.151</td>
<td>0.114</td>
<td>0.354</td>
<td>0.085</td>
<td>0.194</td>
<td>0.462</td>
</tr>
<tr>
<td>A-to-U</td>
<td>1.182 ± 0.008</td>
<td>1.271 ± 0.028</td>
<td>-0.715 ± 0.283b</td>
<td>1.073 ± 0.220</td>
<td>1.147 ± 0.173</td>
<td>-1.301 ± 0.164b</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0.028</td>
<td>0.283b</td>
<td>0.220</td>
<td>0.173</td>
<td>0.164b</td>
</tr>
<tr>
<td></td>
<td>1.314 ± 0.173</td>
<td>0.927 ± 0.273</td>
<td>0.234 ± 0.533c</td>
<td>1.294 ± 0.316</td>
<td>0.942 ± 0.173</td>
<td>-0.776 ± 0.709c</td>
</tr>
<tr>
<td></td>
<td>0.173</td>
<td>0.273</td>
<td>0.533c</td>
<td>0.316</td>
<td>0.173</td>
<td>0.709c</td>
</tr>
<tr>
<td>After A</td>
<td>1.389 ± 0.193</td>
<td>1.041 ± 0.205</td>
<td>-0.397 ± 0.735</td>
<td>1.363 ± 0.190</td>
<td>0.949 ± 0.303</td>
<td>-0.290 ± 1.059</td>
</tr>
<tr>
<td></td>
<td>0.193</td>
<td>0.205</td>
<td>0.735</td>
<td>0.190</td>
<td>0.303</td>
<td>1.059</td>
</tr>
<tr>
<td>U</td>
<td>1.432 ± 0.142</td>
<td>1.041 ± 0.316</td>
<td>-0.251 ± 0.669d</td>
<td>1.366 ± 0.168</td>
<td>1.175 ± 0.128</td>
<td>-0.813 ± 0.464d</td>
</tr>
<tr>
<td></td>
<td>0.142</td>
<td>0.316</td>
<td>0.669d</td>
<td>0.168</td>
<td>0.128</td>
<td>0.464d</td>
</tr>
<tr>
<td>U-to-A</td>
<td>1.338 ± 0.190</td>
<td>1.023 ± 0.179</td>
<td>-0.380 ± 0.580</td>
<td>1.344 ± 0.216</td>
<td>0.996 ± 0.070</td>
<td>-0.305 ± 0.306</td>
</tr>
<tr>
<td></td>
<td>0.190</td>
<td>0.179</td>
<td>0.580</td>
<td>0.216</td>
<td>0.070</td>
<td>0.306</td>
</tr>
</tbody>
</table>

Highlighted groups that share the same superscript label are significantly different ($p < 0.05$).
**Figure 3-1.** Study Design. Static aligned (A) and static unaligned (U) scaffolds were fabricated by electrospinning. A and U scaffolds were further programmed to strain-aligned (SA) and strain-unaligned (SU) scaffolds which have temporarily aligned, and temporarily unaligned architecture, respectively. Two sets of samples, “before recovery” set and “after recovery” set, were prepared with each set including A, U, SA, and SU scaffolds. Cells were seeded on both sets at the same time. To acquire time-lapse videos, the “before recovery” set was imaged at 30 °C for 24 h, and the “after recovery” set was triggered to fully recover at 37 °C then followed by imaging at 37 °C for 24 h. Samples for immunohistochemistry staining went through the same culture condition as time-lapse samples, then fixed for staining and subsequent imaging.
Figure 3-2. Representative merged fluorescent images of cell actin cytoskeleton (red), nuclei (blue), and Golgi apparatus (green) acquired by 40X objective. The images qualitatively indicate that human fibrosarcoma HT-1080 cells aligned along the fibers and displayed elongated morphology when exposed to highly aligned scaffold architecture (before recovery: A, A-to-U; after recovery: A, U-to-A), and randomly distributed and showed rounded morphology when exposed to unaligned scaffold architecture (before recovery: U, U-to-A; after recovery: U, A-to-U). Change of fiber alignment also changed cell body alignment in dynamic U-to-A and A-to-U scaffolds. The cartoon indicates the current fiber architecture, either aligned or unaligned. White double-headed arrows indicate fiber principle alignment direction. Scale bar = 100 µm.
Figure 3-3. Angular histograms qualitatively show nuclei and cell body alignment on different scaffold architectures. Both cell body (top) and cell nuclei (bottom) angular histograms showed narrow distribution in highly aligned scaffold fiber architectures (before recovery: A, A-to-U; after recovery: A, U-to-A) indicating the nuclei and cell body were aligned along the fiber direction (rotated to be at 90° as the reference angle), and nuclei and cell body angular histograms showed broad distribution in unaligned fiber architectures (before recovery: U, U-to-A; after recovery: U, A-to-U) indicating the nuclei and cell body were randomly distributed without certain alignment. The dynamic decrease of fiber alignment in A-to-U scaffold induced broader distribution of the angles of both nuclei and cell body; while the dynamic increase of fiber alignment in U-to-A scaffold induced narrower distribution of the nuclei and cell body angles. The red line represents the mean resultant vector length (R). The cartoon indicates the current fiber architecture, either aligned or unaligned.
Figure 3-4. HT-1080 cell body and cell nuclei angular standard deviation comparison within the same scaffold architecture before and after thermal triggering. HT-1080 cells showed that the cell body (left column) and cell nuclei (right column) angular standard deviation decreased in dynamic U-to-A scaffolds after thermal triggering, which indicates that body cell body and cell nuclei reoriented to be preferentially aligned along the fiber unidirectional alignment direction when fiber unidirectional alignment increased. Cell body and cell nuclei angular standard deviation increased in dynamic A-to-U scaffolds after thermal triggering, which indicates that body cell body and cell nuclei reoriented to be preferentially aligned along the fiber unidirectional alignment direction when fiber unidirectional alignment increased. Boxes display interquartile range with black median center line, red lines indicate mean, and capped whiskers indicate 95% confidence interval of the mean. Red shading represents the range of unidirectionally aligned architecture; while blue shading represents the range of unaligned architecture. Asterisks indicate significant difference (p < 0.05; n = 6). Statistically significant differences exist among the architectural groups before or after recovery, but now shown to highlight the key difference of interest.
Figure 3-5. Qualitative analysis of cell tracks of HT-1080 cells. Cell tracks were plotted by ACTIVE with each line representing a cell’s moving path over 24 h. For HT-1080 cells, on static aligned scaffolds (A), cells moved preferentially along the unidirectional fiber alignment direction both before (top row) and after (bottom row) thermal triggering. On static unaligned scaffolds (U), cells moved randomly without a preferential direction both before and after thermal triggering. On dynamic scaffolds that increase in unidirectional alignment (U-to-A), cells showed increased directional cell motility after thermal triggering. On dynamic scaffolds that decrease in unidirectional alignment (A-to-U), cells showed decreased directional cell motility after thermal triggering. The cartoon on top right corner of each track plot indicates the associated fiber architecture, either aligned or unaligned. Double-headed arrow indicates the principle fiber alignment direction in the scaffold, if one existed.
**Figure 3-6.** Diffusion plots of cell final positions with initial position normalized to the same origin. Principle fiber alignment direction was rotated to be parallel with x direction. Diffusion plots qualitatively show cell migration directionality when cells moved on different fiber architecture. In dynamic A-to-U scaffolds, cells moved along the aligned fibers before recovery, then changed to random movement after scaffolds recovered to unaligned architecture. While in dynamic U-to-A scaffolds, cells moved without directionality before recovery, then moved along aligned fiber direction after scaffolds recovered to aligned architecture. Cells moved along the fiber alignment direction in scaffolds with highly aligned fiber architecture (before recovery: A, A-to-U; after recovery: A, U-to-A); and moved randomly without a specific direction in scaffolds with unaligned fiber architecture (before recovery: U, U-to-A; after recovery: U, A-to-U). The cartoon indicates the current fiber architecture, either aligned or unaligned.
Figure 3-7. Decomposed mean square displacement (MSD) analysis of HT-1080 cell motility. For HT-1080 cells, static aligned scaffolds (A) had non-overlapped decomposed MSD curves, which indicates that cells showed directional motility preferentially along the unidirectional fiber alignment direction both before (top row) and after (bottom row) thermal triggering. Static unaligned scaffolds (U) had overlapped decomposed MSD curves, which indicates that cells showed non-directional motility both before and after thermal triggering. Dynamic scaffolds that increase in unidirectional alignment (U-to-A) showed a change from overlapped to non-overlapped decomposed MSD curves, which indicates that cells had increased directional motility. Dynamic scaffolds that decrease in unidirectional alignment (A-to-U) showed a change from non-overlapped to overlapped decomposed MSD curves, which indicates that cells had decreased directional motility. The cartoon on top right corner of each MSD plot indicates the associated fiber architecture, either aligned or unaligned.
Figure 3.8. Average cell velocity. Average cell velocity (µm/min) of HT-1080 cells was compared within each scaffold architecture before (open circles) and after (closed circles) thermal triggering, and also among different scaffold architectures either before or after thermal triggering. For HT-1080 cells, cells on dynamic scaffolds that increase in unidirectional alignment (U-to-A) scaffolds showed significantly higher average velocity after thermal triggering comparing to before thermal triggering. After thermal triggering, cells on U-to-A scaffolds showed significantly higher average velocity than the cells on dynamic scaffolds that decrease in unidirectional alignment (A-to-U).
Chapter 4: Preservation of Osteogenic Differentiation Capacity of Stem Cells in Deployable Shape Memory Polymer Scaffolds†

4.1 Synopsis

In the previous two chapters, we established a robust 3D fibrous SMP electrospun fibrous scaffold that can change internal architecture upon triggering at body temperature, and we studied cell motility when fiber alignment dynamically changed. In this chapter, the objective was to determine the effect of such scaffold dynamic internal architectural change on stem cells osteogenic differentiation capacity. SMP constructs have recently been studied for their potential as fillers to treat bone defects. SMP constructs can be delivered non-invasively into the defect site and, upon triggering, expand in situ to tightly fit the defect. Approaches for incorporating stem cells into the SMP scaffolds have been investigated in the literature to promote new bone tissue growth at defect site. However, it is not known whether SMP scaffold shape change would interfere with stem cell differentiation potential, as stem cells are sensitive to the change of their microenvironment. In this chapter, the osteogenic differentiation capacity of human adipose-derived mesenchymal stem cells was assessed before and after changes in scaffolds shape and internal architecture. Both an electrospun fibrous SMP scaffold and a foam SMP scaffold were employed to test the hypothesis that the dynamic change of deployable SMP scaffolds would not hinder stem cell osteogenic differentiation.

4.2 Introduction

Costs associated with grafting of bone defects exceed $100 billion annually worldwide [1]. For treatment of many defects, autografts, allografts remain the gold standard, but the source is limited. Synthetic biomaterials for use as bone grafts have been widely studied in an effort to overcome the limitations of autografts and allografts, and to further enhance the bone tissue regeneration at the defect site. For instance, approaches were investigated to incorporate components such as hydroxyapatite [2,3], β-tricalcium phosphate [4,5], and growth factors, such as bone morphogenetic proteins [6,7] to improve osteoinductivity to enhance stem cells to differentiate into osteoblasts. Incorporation of stem cells directly into synthetic bone grafts has been shown to improve new bone growth in the graft [8,9]. Prior studies have demonstrated significant bone regeneration using stem cell-based bone tissue engineering constructs when compared to cell-free constructs [10,11].

Shape memory polymers (SMPs) particularly, as a class of smart synthetic biomaterials, have recently been studied for their potential for minimally invasive delivery, conformal fitting to defect margins, and defect stabilization in the treatment of bone defects. SMPs can undergo programmed changes in shape from a temporarily fixed shape to a permanently memorized shape when exposed to an external stimulus, such as heat [12–18]. For example, two previous in vitro studies have demonstrated the ability of the SMP scaffolds to support osteoblast adhesion, proliferation, and osteogenic gene expression [14,18]. Other studies have also employed SMP scaffolds to fill or to stabilize bone defect site [16,19], and to support stem cell proliferation in SMP for the application of tissue engineering [20]. Collectively, these studies have demonstrated
the potential for employing programmable SMP scaffolds in treatment of bone defects. However, none of these studies investigated the effect of programmed changes in shape on the behavior of cells seeded in the SMP scaffolds.

Despite interest in SMPs for treatment of bone defects, the extent to which stem cell osteogenic differentiation capacity is preserved following programmed shape change has not yet been determined. The extent to which stem cell differentiation would be affected by the dynamic architectural change of the surrounding microenvironment remains unknown. Here, we hypothesized that stem cell osteogenic differentiation could be preserved during and following programmed architectural changes in SMP scaffolds. To test this hypothesis, stem cell osteogenic differentiation capacity was assessed before and after SMP triggered scaffold shape change of expanding SMP foam scaffolds and contracting SMP electrospun fibrous scaffold. To characterize differentiation capacity, mineral deposition, protein production, and gene expression were compared between active shape-changing and static control scaffolds.

4.3 Methods and Materials

4.3.1 Scaffolds Preparation†

A modified porogen-leaching technique was used to fabricate porous foam scaffolds. For this study, 6.0 g of salt with 300-500 µm diameter was added to a 20 mL glass vial. The glass vial was placed in a Styrofoam box (9.25 × 6.25 × 8.25 in), and a beaker with 2 L of water at approximately 42 °C was placed in the Styrofoam box. The box was closed and after 72 h the

†All foam scaffold preparation and characterization was performed by Dr. Richard M Baker
vials were removed and placed into a vacuum oven to dry. Following drying, a fused puck of salt remained, to which the polymer solution was added. To fabricate foam scaffolds with a hydrated Tg of 42 °C, a comonomer wt% ratio of 98-2 (tBA-BA) was used, with a constant 5 wt% tetraethylene dimethacrylate (TEGDMA) crosslinker and 1 wt% DMPA photoinitiator. An example comonomer solution would consist of: 2 mL tBA (1.75 g), 40 μL BA (37.5 mg), 82.6 μL TEGDMA (89.4 mg), and 18.8 mg of DMPA. The composition enabled programmed scaffolds to remain stable during cell seeding conditions at 30 °C and recover once heated to the triggering temperature of 37 °C. This solution was mixed and added to a fused salt template. The fused salt template was photo-cured for 2 h using UV light, after which the glass vial was broken and the cured foam was removed. Foams contained a solid skin on each surface that was removed with a razor blade. Following skin removal, foams were placed in 60 °C water for 48 h to extract the salt. Foams were rinsed thoroughly and placed in methanol for 12 h to extract unreacted monomer and photoinitiator. Foams were then dried in a vacuum oven for 24 h.

To coat foam scaffolds with polydopamine to achieve robust cell attachment, scaffolds were rinsed in deionized water 3 times, followed by a 1 h submersion in dopamine solution. The dopamine solution consisted of dopamine hydrochloride at a concentration of 2 mg/mL in 10 mM Tris buffer with a pH of 8.5. Submersion of the scaffolds led to polymerization of the dopamine on the scaffolds. Scaffolds were then vigorously rinsed in deionized water 3 times to remove non-attached polydopamine. Following polydopamine coating, scaffolds turned from white to a dark brown/black color. Cylindrical foam plugs with a 3.5 mm diameter and 4–4.5 mm height were used. As-prepared foam scaffolds were used as the static control samples and contained a cuboidal pore morphology. Active shape-changing foam scaffolds were programmed into a temporary
compressed state by uniaxially compressing the foam plugs with 30% strain using calipers at room temperature. Upon unloading, foams elastically recovered approximately 5% strain, resulting in a fixed compressive strain of 25%. Pore morphology of compressed foams was more elongated in the direction perpendicular to the compression direction.

Aligned fibrous scaffolds were fabricated by electrospinning the same custom-synthesized thermoplastic polyurethane (TPU) as described previously in Chapter 2. Electrospinning parameters were adjusted to produce fibers in about 1 µm in diameter. Briefly, TPU was dissolved at a concentration of 30% (w/v) in dimethylformamide (Sigma-Aldrich) and chloroform (Fisher Scientific) at a 1:2 (v/v) ratio. The polymer solution was loaded in a syringe with a 22 G stainless steel blunt needle used as a spinneret. The polymer solution was pumped at a rate of 0.4 mL/h through the spinneret, which was positively charged to 15.5 kV. The rotating drum was negatively charged to 0.5 kV with a 10 cm distance from the spinneret to the rotating drum surface. To produce aligned electrospun microfibers, the rotation speed of the drum was 4000 rpm. The total duration of the electrospinning process was 12 h, which resulted in an electrospun scaffold with a thickness of approximately 100 µm.

To prepare static aligned control fibrous scaffolds, molecular-level strains created during the electrospinning process were released using a thermal treatment protocol with a modified technique ("tube-in-tube") to ensure fiber alignment was maintained. Briefly, an aligned electrospun fibrous scaffold was wrapped around a 15-mL conical tube (inner tube) while the fiber alignment direction was parallel to the circumference of the tube. The 15-mL conical tube was immersed in a 50-mL conical tube (outer tube) which contained 65% (w/v) Pluronic F-127 hydrogel; the tube-in-tube setup was thermal-treated at 70 °C for 3 h, where the Pluronic solution
is a gel and the TPU is above its Tg. In this state, the TPU can relax its stress without changing shape. After the thermal treatment, the scaffold was washed rigorously in deionized water at 4 °C, where the Pluronic solution is a liquid, for 24 h to remove the hydrogel. To program active shape-changing scaffolds that change from temporary unaligned fibers to permanent aligned fibers, aligned scaffolds were uniaxially stretched along the direction perpendicular to the fiber alignment in a dynamic mechanical analyzer (DMA; TA Instruments Q800) to 100% strain at 60 °C, and fixed in this temporary shape by cooling to 0 °C. Prior to cell studies, static and active foam and fibrous scaffolds were sterilized using mono-nitrogen oxides†.

4.3.2 Scaffolds Characterization

4.3.2.1 Shape Memory Recovery Kinetics‡

The ability of active foams and espun scaffolds to maintain a stable programmed shape at 30 °C (cell seeding temperature) and be subsequently triggered to recover at 37 °C was quantified. Foams were programmed with a 25% compressive strain, and espun scaffolds were programmed with a 100 % tension strain and hydrated with phosphate buffered saline (PBS) in a 30 °C water bath for 5 d, simulating cell culture conditions. Scaffold recovery was measured during the 5 d period using calipers to determine the stability of the programmed shape at 30 °C. After 5 d, samples were moved to a 37 °C water bath, triggering recovery, and the shape recovery was measured. Changes in length were measured using a digital caliper (n = 4).

†All mono-nitrogen oxides sterilization protocol and experiments were developed and performed by Dr. Ling-Fang Tseng.
‡Shape memory recovery kinetics of foam scaffolds were analyzed by Dr. Richard M Baker. Shape memory recovery kinetics of fibrous scaffolds were analyzed by Dr. Ling-Fang Tseng.
4.3.2.2 Scanning Electron Microscopy

Scaffold architectural change was imaged using scanning electron microscopy (SEM) at three different time points. Active and static foam and espun scaffolds were hydrated and incubated at 30 °C for 5 days and subsequently triggered to change the internal architecture by moving the scaffolds to 37 °C for additional 2 days and 16 days; the static scaffolds did not change length throughout this period of incubation. After each time point, the scaffolds were dried in a vacuum oven at room temperature and sputter-coated with gold for imaging (n = 3). Images were acquired in secondary electron mode using a JOEL JSM-5600 scanning electron microscope. SEM images from espun scaffolds were further analyzed by using 2D Fourier fast transform (2D FFT) imaging analysis technique to quantify fiber alignment before and after transition.

4.3.3 Cell Culture

4.3.3.1 Cell Expansion

Human adipose-derived stem cells (hASCs) were used to investigate the effect of scaffold shape and architecture changes on osteogenic differentiation. Prior to scaffold experiments, hASCs (Cat# R7788-115) were expanded in basal growth medium: MesenPro RS medium with 2 % MesenPro RS growth supplement, 1 % GlutaMAX, and 1 % penicillin/streptomycin in a 37 °C humidified incubator with 5 % CO₂. Cells were cultured on a T-175 flask with 30 ml of complete growth medium. The medium was changed every four days and cells were passaged at 80 % confluence using TrypLE Express solution. Cells were used at passage 6 for scaffold experiments. All cell culture products were purchased from Life Technologies (Grand Island, NY).
4.3.3.2 Cell Seeding on Scaffolds

To determine whether changes in foam architecture would affect stem cell osteogenic differentiation capacity, hASCs were seeded in static and active foams and espun fibrous scaffolds that underwent a two-stage cell culture process. During the first stage, cells were cultured in foams or espun scaffolds at a cytocompatible temperature (30 °C) that does not trigger recovery of active scaffolds; at the second stage, scaffolds were heated to 37 °C to trigger recovery of the active scaffolds. Prior to cell seeding, sterilized scaffolds were immersed in antibiotic-antimycotic solution (Sigma-Aldrich) overnight at 4 °C to ensure the sterility, followed by rinsing in PBS.

To facilitate cell seeding into the foam scaffolds, a vacuum-assisted seeding approach was employed. Prior to cell seeding, foams were first equilibrated in growth medium for 20 min. Samples were then placed in transfection tubes containing 3 mL of cell suspension at a cell concentration of 500,000 cells/mL. To each transfection tube, 30 foam scaffolds were added. To perform vacuum-assisted seeding, a 1-mL syringe with a 16 G needle tip was attached to house vacuum. Air from each transfection tube was evacuated by pulling house vacuum for 30 s, three times. By evacuating air, cell suspension was forced into the interconnected pores of the foams, improving cell seeding into each foam scaffold. The scaffolds were incubated at 30 °C for 5 h allowing cells to attach to the scaffolds. During this seeding period, transfection tubes were lightly agitated every 30 min to keep cells in suspension. After 5 h, extra basal growth medium was added to each sample, and samples were incubated for 24 h at 30 °C.

For espun scaffolds, the scaffolds were hydrated in basal growth medium at room temperature for 20 min and transferred to tissue culture plates. Droplets of cell suspension were
laid on each scaffold with a cell density corresponding to 80,000 cells/cm² for the total scaffold area of 0.8 cm². The scaffolds were incubated at 30 °C for 5 h allowing cells to attach to the scaffolds and extra basal growth medium was added into each well.

After 24 h incubation at 30 °C, the basal growth medium was switched to osteogenic differentiation medium: BGJb medium (Life Technologies), 10 mM HEPES (Life Technologies), 10 mM β-glycerophosphate (Sigma-Aldrich), 1 nM dexamethasone (Sigma-Aldrich), 150 μM L-ascorbic acid phosphate magnesium salt n-hydrate (Wako), 10 % fetal bovine serum (Gibco), and 1% penicillin/streptomycin (Life Technologies). The osteogenic medium was changed every 2 d. After incubation at 30 °C for 5 d, foams were incubated at 37 °C, triggering expansion, for 23 d. Osteogenic specific characteristics were characterized at three time points: before triggering (30 °C for 5 d); 2 d post-triggering (37 °C 2 d); and 23 d post-triggering (37 °C 23 d). Three independent replicates consisting of cell expansion through the foam experiments were performed (n = 3).

4.3.4 Assessing Calcium Deposition

4.3.4.1 Xylenol Orange and Nuclear Staining

Xylenol orange (XO) staining is a non-destructive staining method to detect calcium deposition [21]. XO powder (398187, Sigma-Aldrich) was dissolved in deionized water and filtered to make a sterile stock solution of 20 mM and stored at 4 °C. The scaffolds were cultured at the final concentration of 40 μM XO in the medium overnight before imaging at three different time points. The XO-containing medium was replaced with fresh Hoechst-containing medium (0.1 mg/ml; Hoechst 33342, Life Technologies) 30 min before imaging to stain for cell nuclei.
Fluorescent images of XO-stained scaffolds and Hoechst-stained nuclei were taken via N3 and A4 filter cubes, respectively (Leica DMI 4000B).

4.3.4.2 Scanning Electron Microscopy and Energy-dispersive X-ray Spectroscopy†

To qualitatively visualize mineral deposition, scanning electron microscopy (SEM) was performed on the same samples used for microcomputed tomography (μCT) analysis. Scaffolds were sputter-coated with gold, and images were acquired at secondary electron mode. Representative images were taken at different areas to visualize cells and mineral deposition at three different time points. Moreover, elemental composition analysis of the mineral deposition on the scaffolds was conducted using energy-dispersive x-ray spectroscopy (EDS); and images of the analyzed areas were acquired at backscattered electron mode.

4.3.4.3 Microcomputed Tomography (μCT)‡

Three-dimensional analysis of mineral deposition was assessed using microcomputed tomography (μ-CT 40, Scanco Medical). Scaffolds were rinsed with PBS three times and fixed with 10% neutral buffered formalin overnight. Fixed scaffolds were rinsed with deionized water before drying in an evacuated desiccator overnight. Dried scaffolds were scanned at a nominal voxel size of 6 μm, and images were acquired at 55 kV, 144 μA, and 200 ms integration time. Mineral volume was measured for each scaffold by applying a threshold of 121.67 Hounsfield Units (HU) for foams and –21.48 HU for fibrous scaffolds. A larger threshold was applied for

†EDX scanning and analysis was performed with the help of Eric Ouellette.
‡All μCT experiments and analysis were performed by Dr. Richard M Baker and Dr. Ling-Fang Tseng.
foam scaffolds as they are more radio-opaque than the fibrous scaffolds. Mineral volume for each scaffold was normalized by scaffold volume and compared between groups \((n = 3)\). Three-dimensional images of mineral deposition were rendered using the 3D viewer in ImageJ.

**4.3.5 Alkaline Phosphatase (ALP) Activity†**

At the end of each time point, the foam and espun scaffolds were washed three times with tris-buffered saline (TBS) at room temperature. Cells in both types of the scaffolds were first lysed in 500 μl sterile deionized water and stored at – 20 °C. Cells were further lysed by three “freeze-thaw” cycles using liquid nitrogen and then homogenized using 20 G needles. Cell lysates were separated from scaffolds by centrifuging through nylon filters (Spin-X centrifuge filter tube, Costar). Fifty microliters of cell lysates were added in three separate wells and reacted with 150 μl of \(p\)-nitrophenyl phosphate (N1891, Sigma-Aldrich) at 37 °C for 1 h. The reaction was stopped by adding 50 μl 3 N NaOH in the wells. The absorbance was measured at 405 nm in a plate reader (Synergy 2, BioTek). Alkaline phosphatase concentration was determined using 4-Nitrophenol solution (N7660, Sigma-Aldrich) and normalized by DNA contents (PicoGreen dsDNA assay, Life Technologies).

**4.3.6 Osteogenic Characteristic Gene Expression**

**4.3.6.1 RNA Extraction and cDNA Synthesis**

To determine osteogenic gene expression, real-time qPCR was performed. Total RNA

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†All Alkaline Phosphatase (ALP) activity experiments and analysis were performed by Dr. Richard M Baker and Dr. Ling-Fang Tseng.
extraction and cDNA analysis were conducted prior to running qPCR. Scaffolds were rinsed three times with sterile PBS then frozen at – 80 °C. Total RNA was extracted using RNeasy Micro Kit (74004, QIAGEN) per the manufacturer's instructions. Total RNA was then used for reverse-transcription reaction to synthesize cDNA using QuantiTect Rev. Transcription Kit (205311, QIAGEN). RNA and cDNA quantity was assessed by absorbance readings at 260 nm using spectrophotometry. Absorbance ratios of 260/280 and 230/280 were greater than 1.8, indicating high purity of RNA and cDNA.

4.3.6.2 Real-time qPCR

To examine osteogenic differentiation, characteristic gene expression of Runx2 and osteocalcin (OC) were examined, with normalization to reference genes B2M and RPL13A for cells cultured in foam and fibrous scaffolds, respectively (n = 3). To determine the best housekeeping genes for the foam and fibrous scaffolds, respectively, ten housekeeping genes (ACTB, B2M, GAPD, GUSB, HPRT1, PGK, PP1A, RPL13A, TBP, TFRC; purchased from realtimeprimer.com) were tested on hASCs seeded in active and static scaffolds. Raw qPCR data was processed by LinRegPCR [22]. All primer information is shown in Table 4-1. The resulting primer efficiencies and Ct values were used as inputs in Bestkeeper [23] to find the most stable housekeeping gene. Real-time qPCR was performed in quadruplicate with 100 ng of cDNA in 20 µl reaction volume containing Power SYBR Green Master Mix (Invitrogen) and primers (RealTimePrimers) using Mastercycler ep realplex (Eppendorf). Fold change of gene expression was calculated as expression level normalized to the static scaffolds at the first time point. A fold
change higher than 1 indicates gene up-regulation, while a fold change lower than 1 indicates gene down-regulation.

### 4.3.7 Histology

To visualize mineral deposition in each scaffold, histological analysis was performed using Alizarin red staining. Scaffolds were first rinsed with PBS three times and fixed with 10% neutral buffered formalin overnight. Due to the high porosity of the foam scaffolds, thin histological sections could not show the extent of mineral deposition. Instead, entire foam scaffolds were stained with 2% Alizarin red with pH adjusted to 4.2 for 30 s ($n = 3$). Stained whole samples were extensively rinsed with deionized water. For fibrous scaffolds, fixed scaffolds were dehydrated using a series of ethanol solutions, embedded in paraffin, and sectioned into 3 μm thick slices. The sections were stained with 2% Alizarin red with pH adjusted to 4.2 for 2 min ($n = 2$). Sections were then fixed with Xylene and mounted. Only samples at the last time point (37 °C for 23 d) were collected for staining.

### 4.3.8 Statistics

Paired $t$-tests and one-way ANOVA with Tukey’s HSD post hoc tests were performed for comparisons between active and static groups and comparisons within group over time, respectively, when analyzed measurements of mineral volume, ALP activity, and gene expression using a commercial statistical analysis package (StatPlus:mac, AnalystSoft Inc.). Statistical significance was set at $p < 0.05$. 
4.4 Results

4.4.1 Scaffold architecture reorganization

Expanding SMP foams and contracting SMP fibrous scaffolds underwent a programmed change in architecture upon heating to 37 °C (Figure 4-1). SMP foams recovered 13% of the programmed strain following sterilization, but the programmed compressive strain remained stable at 30 °C, as no recovery of the programmed strain was observed over the 5 d period (Figure 4-1 Foam). Upon triggering recovery at 37 °C, the foam completely recovered within 24 h. We speculate that the recovery during sterilization is due to a combination of programming the foam below its glass transition temperature and exposing the foam to a humid environment during sterilization. Programmed SMP fibrous scaffolds recovered 11% of the programmed strain at 30 °C over 5 d (Figure 4-1 Fibrous). Upon triggering recovery at 37 °C, fibrous scaffolds contracted 53% in the first 4 h and had recovered 90% over the first 5 d. SEM micrographs show that although 11% strain was recovered at 30 °C, fiber architecture remained unaligned (Figure 4-2). Triggering recovery at 37 °C resulted in fibers reorganizing to a uniaxially aligned orientation, as revealed by corresponding 2D FFT image analysis results with two distinct peaks at 90 ° and 270 ° indicating aligned fiber architecture.

4.4.2 Mineralized nodule formation

Results from SEM, XO staining, and μCT show that no qualitative or quantitative difference of mineral deposition between active and static groups exist for either foams or fibrous scaffolds. SEM analysis (Figure 4-3) showed qualitatively comparable levels of calcium
deposition between *active* and *static* samples for both foams and fibrous scaffolds, and no
difference in mineral nodule formation was qualitative observed between *active* and *static* groups.
For foams, no significant mineral deposition was found at 30 °C for 5 d and 37 °C for 2 d; however,
a significant increase in the number and size of calcium nodules was observed at 37 °C for 23 d
(Figure 4-4). For fibrous scaffolds, calcium nodules were only observed at 37 °C for 23 d (Figure
4-4), with both *active* and *static* samples containing large aggregates of calcium nodules. At all
time points, cells can be seen spanning several fibers and covering the fibrous scaffolds.

XO staining also revealed that for both foams and fibrous scaffolds, calcium deposition
was qualitatively comparable between *active* and *static* samples (Figure 4-5). No detectable
calcium deposition was observed on any samples during the initial 5 d at 30 °C; as a comparison,
nuclear staining showed substantial amount of cells in the scaffolds (Figure 4-6). After triggering
at 37 °C for 2 d, small calcium nodules were detected on both *active* and *static* fibrous scaffolds.
After 23 d at 37 °C, *active* and *static* samples for both foams and fibrous scaffolds showed a
significant amount of calcium deposition, and no qualitative difference between *active* and *static*
groups was observed.

Microcomputed tomography analysis also demonstrated qualitatively and quantitatively
comparable amount of mineral deposition between *active* and *static* groups, with increasing
mineral formation being observed over time for both foams and fibrous scaffolds (Figure 4-7).
For foams, calcium nodules were observed at all time points, with noticeable increase in number
and size as time progresses. Calcium deposition appears to be concentrated near the outer pores of
the foams, likely due to a much higher cell density near the surface when compared to the center
of the scaffold. No statistical difference was observed between *active* and *static* scaffolds. Both
groups showed a trend of increased mineral deposition over time although the differences were not statistically significant (Table 4-2). For fibrous scaffolds, no calcium nodules were detected at 30 °C for 5 d, however small artifacts from the fiber mat can be observed. At 37 °C for 2 d, calcium nodules can be seen for both active and static scaffolds and appear to begin nucleating from the edges of the fiber mat. At 37 °C for 23 d, both active and static scaffolds are completely covered with calcium deposits. No statistical difference was found between active and static groups; however, when comparing within group, both groups demonstrated a significant increase in mineral deposition over time (Table 4-3).

4.4.3 Alkaline phosphatase (ALP) activity

Quantitative analysis of ALP activity showed no statistical difference between active and static groups of foams and fibrous scaffolds (Figure 4-8). ALP protein expression being an early markers of mineral nodule formation—a functional endpoint indication of osteogenic differentiation. For foams, with the exception of one replicate, there was a slight increase in normalized ALP content over time, and a trend of higher ALP content for the active scaffolds compare to the static scaffolds, albeit not significant. Normalized ALP content of the active fibrous scaffolds showed no change over time, while static fibrous scaffolds showed a trend of increasing ALP content, but not statistically significant.

4.4.4 Real-time PCR gene expression

Real-time PCR analysis of genetic markers of osteogenesis showed that both foams and fibrous scaffolds expressed the osteogenic marker, Runx2, with no significant difference between active and static groups found (Figure 4-9). Up-regulation of Runx2 expression suggests that the
hASCs differentiated toward an osteogenic lineage over time. A second characteristic marker, osteocalcin (OC; Figure 4-9), was also expressed in both foams and fibrous scaffolds with no significant difference between active and static groups for both types of scaffolds was observed. A trend of OC down-regulation was found in all groups.

4.4.5 Histology

Alizarin red staining at the final time point (Figure 4-10) showed substantial calcium deposition in both foams and fibrous scaffolds. The results indicate that qualitatively comparable level of osteogenic differentiation was achieved in both types of scaffolds with or without architecture change. For the foams, both active and static groups stained strongly positive for calcium deposition. Foams were not sectioned before staining since the high porosity of the foams resulted in sections with little material. The material itself does not absorb the staining, as foams with no cells did not stain red (Figure 4-11). Likewise, for fibrous scaffolds, both active and static samples stained strongly positive, indicating calcium deposition on the scaffolds. Several dark red circles can be seen in active and static samples, which are attributed to large mineralized nodules.

4.5 Discussion

This work is the first to demonstrate differentiation of stem cells cultured in 3D SMP scaffolds during and following programmed architectural changes in SMP scaffolds and found no detrimental effects on stem cell osteogenic differentiation capacity. Osteogenic differentiation was confirmed by mineral deposition, protein production, and gene expression assays. Comparable
levels of mineral deposition, protein production, and gene expression between active and static scaffolds were observed.

The mineral deposition assays found qualitatively and quantitatively comparable amounts of mineral deposition for both active and static scaffolds (Figures 4-3, 4-5, and 4-7). In addition, EDS analysis (Figure 4-4) showed that the nodules on the SMP scaffolds consisted of high levels of calcium and phosphorus, two key constituents of bone mineral [24]. These results suggest that human adipose-derived stem cells differentiated down an osteoblastic lineage and produced mineralized matrix during the course of the 4-week culture.

In examining osteogenic protein production, ALP activity was less conclusively indicative of osteogenic function but remained consistent between the active and static groups. Instead of increasing over time as shown in some previous studies [25], ALP activity remained unchanged or increased modestly for both SMP scaffolds (Figure 4-8). Such relatively flat ALP activity during stem cell osteogenic differentiation has been reported in prior studies [24,26]. It is possible that in the present study the peak ALP activity occurred during the 3-week gap between the second and the third time points. A lack of a significant increase in ALP activity may also be attributed to the fetal bovine serum (FBS) that was used in the induction medium, which has been reported to result in low ALP protein production in hASCs compared with several different sera [27].

Results of real-time qPCR showed no significant difference between active and static groups for both SMP scaffolds (Figure 4-9); however, a trend of up-regulation of Runx2 expression and down-regulation of OC expression over time for both SMP scaffolds were found. With respect to the down-regulation of OC over time, such down-regulation in human adipose-
derived stem cells has also been shown in two previous studies [26,28]. As with the results presented in this study, both prior studies demonstrated successful mineral deposition regardless of the reduced $OC$ expression. These aforementioned studies suggest that although ALP activity and osteogenic gene expression are indicators of osteogenic differentiation [24,26,28], mineralized matrix formation represents successful end-point outcome of osteogenic differentiation [25,29].

In this study, we have demonstrated successful induction of stem cell osteogenic differentiation following programmed architectural changes in SMP scaffolds. The SMP scaffolds used herein have previously been studied in vivo in grafting and stabilizing complex bone defects in a mouse segmental defect model [19]. The finding of the present work support the feasibility of incorporating stem cells in such SMP scaffolds for cell-based approaches to bone defect treatment.

4.6 Conclusions

In this study, we have demonstrated osteogenic differentiation of adipose-derived stem cells in two different types of programmable SMP scaffolds—foams and fibrous scaffolds—upon shape memory triggered changes in internal architectures under cytocompatible conditions. The foams transitioned from a temporarily compressed-pore to a permanently open-pore architecture; the fibrous scaffolds transitioned from a temporarily unaligned to a permanently uniaxially aligned architecture. The results indicate that stem cells demonstrate comparable level of osteogenic characteristics when in the active and static scaffolds, as indicated by mineral deposition, ALP protein production, and osteogenic gene expression. Promisingly, this present study suggests that shape memory scaffolds as deployable cell-based strategy may potentially be applied to treat complex bone defects.
4.7 Acknowledgements

The authors gratefully thank Dr. Richard M Baker and Dr. Ling-Fang Tseng for their collaboration on this work, and Professor Megan Oest for microcomputed tomography data analysis. This work was sponsored by DARPA (DP12AP00271 to JHH), and the content of the information does not necessarily reflect the position or the policy of the Government, and no endorsement should be inferred.

4.8 References


Table 4-1. Real time PCR primers table, showing all the primers target gene, accession ID, sequences, melting temperature, and produce size.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Accession</th>
<th>Forward</th>
<th>Reverse</th>
<th>Tm</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>NM_001101.2</td>
<td>5'-GGACCTCGAGCAAGAGATGG -3'</td>
<td>5'-AGCAGCTGTGGTGCCGTACAG -3'</td>
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<tr>
<td>B2M</td>
<td>NM_004048.2</td>
<td>5'-TGCTGTCTCCATGTTTGATCT -3'</td>
<td>5'-TCTCTGCTCCCCACCTCAATG -3'</td>
<td>55°C</td>
<td>87bp</td>
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<tr>
<td>GAPDH</td>
<td>NM_002046.2</td>
<td>5'-GAGTCACCGGATTTGAGTGT -3'</td>
<td>5'-TTGATTTTGGAGGAATCTCG -3'</td>
<td>55°C</td>
<td>238bp</td>
</tr>
<tr>
<td>GUSB</td>
<td>NM_000181.1</td>
<td>5'-AAACGATGCGAGTTTAC -3'</td>
<td>5'-CTCTGTCGTTGACGTGTTCA -3'</td>
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<tr>
<td>HPRT1</td>
<td>NM_000194.1</td>
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<td>5'-GGTCCTTTTCTCAGCAAGCT -3'</td>
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<td>5'-CAAACACCCAAGAAGACCACCA -3'</td>
<td>5'-CCCAAAAGAAGTTTGGCTGA -3'</td>
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<td>5'-ATGACAAAGCGGTCTTATCCA -3'</td>
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Table 4-2. Mineral volume for active and static foam scaffolds.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Active 5 d at 30 °C</th>
<th>Active 2 d at 37 °C</th>
<th>Active 23 d at 37 °C</th>
<th>Static 5 d at 30 °C</th>
<th>Static 2 d at 37 °C</th>
<th>Static 23 d at 37 °C</th>
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<tr>
<td>1</td>
<td>0.734</td>
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<td>0.032</td>
<td>0.854</td>
<td>0.449</td>
<td>0.062</td>
</tr>
<tr>
<td>2</td>
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<td>0.398</td>
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<td>0.091</td>
<td>0.295</td>
</tr>
<tr>
<td>3</td>
<td>0.005</td>
<td>0.005</td>
<td>0.502</td>
<td>0.015</td>
<td>0.009</td>
<td>0.280</td>
</tr>
</tbody>
</table>

Mineral volume (MV) to foam volume (BV) percentages for active and static foam scaffolds show a trend of increasing mineral content over time, with the exception of one biological replicate (replicate 1). This replicate had an unexpected trend that is attributed to inconsistencies in initial seeding density.
Table 4-3. Mineral volume for active and static fibrous scaffolds.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Active</th>
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<th></th>
<th>Static</th>
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<tr>
<td></td>
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Values were expressed as BV (bone volume) per apparent scaffold volume (mm$^3$/mm$^3$). No statistical difference between groups at all time points. *Within group, mineral volume at the last time point was significantly different than that of the two early time points for both groups ($p < 0.05$).
Figure 4-1. SMP foam and fibrous scaffolds are triggered to change shape and architecture under cytocompatible conditions. Programmed foam (left) scaffolds recover 13% of the programmed deformation (25% uniaxial compressive strain) following sterilization and remain stable for 5 d at 30 °C; upon heating to 37 °C foams recover all the programmed strain within 24 h. Inset SEM micrographs reveal a compressed-pore morphology that expands to an open-pore morphology upon heating to 37 °C. Fibrous (right) scaffolds that are programmed with 100% uniaxial tension recover 11% strain over 5 d at 30 °C; upon heating to 37 °C the fibrous scaffolds recover 80% strain over the first 24 h, and gradually recover the remaining strain over the next 5 d. Inset SEM micrographs show an initial bimodal fiber orientation during the 5 d at 30 °C that changes to an aligned orientation upon recovery at 37 °C. Scale bars for SEM micrographs are foam 500 μm and fibrous 50 μm.
Figure 4-2. Electrospun scaffold fiber alignment of active and static scaffolds before and after temperature transition. Active scaffolds demonstrate unaligned orientation before triggering scaffold shape recovery, as no apparent peaks were shown in the 2D FFT plot. Upon heating to 37 °C, the active scaffolds recovered back to the initial aligned architecture and retained the alignment after incubation at 37 °C for additional 2 d and 16 d, as indicated by two distinct peaks at 90° and 270° in the 2D FFT plots. Static scaffolds retained the aligned architecture before and after temperature transition; two distinct peaks at 90° and 270° in the 2D FFT plots suggest that fibers align along a principal direction. Scale bar: 20 μm.
**Figure 4-3.** Scanning Electron Microscopy (SEM) reveals significant mineral deposition in SMP foam and fibrous scaffolds. Both foam (top) and fibrous (bottom) scaffolds showed no observable difference between *active* and *static* groups was found for either foam or fibrous scaffolds. No obvious calcium nodules was observed under SEM when cultured at 30 °C for 5 d and 37 °C for 2 d after triggering of architecture change. After 23 d at 37 °C, a significant amount of calcium nodules were observed in both foam and fibrous scaffolds. Arrows highlight representative calcium deposition on each scaffold. Scale bar is 20 μm.
Figure 4-4. Energy dispersive spectroscopy (EDS) of mineral deposition shows minerals contain calcium in both foam (top) scaffolds and fibrous (bottom) scaffolds. (Left) SEM images from backscattered electrons of a magnified view of a mineralized nodule, identified by white arrows. (Right) EDS traces of mineralized nodules show a significant amount of calcium and phosphorous in both the foam scaffolds and fibrous scaffolds. Scale bars are 10 μm.
Figure 4-5. Xylenol orange (XO) staining for calcium deposition on SMP foam and fibrous scaffolds shows increasing calcium deposition over time. Both foam (top) and fibrous (bottom) scaffolds showed no observable different between active and static groups. For either foam or fibrous scaffolds, no calcium deposition is detected during the 5 d culture at 30 °C. Following triggering of architecture change at 37 °C for 2 d, small calcium nodules (white specs) are detected in the active and static control groups for both foam and fibrous scaffolds. After 23 d at 37 °C, a significant increase in the size and number of calcium nodules is detected for all groups. Scale bar is 200 μm. For foam scaffolds, histogram stretching was performed on the raw images to allow visualization of calcium nodules.
Figure 4-6. Hoechst nuclear staining for cells seeded in foam (top) scaffolds and fibrous (bottom) scaffolds. Cell nuclear staining using Hoechst 33342 at three time points show cells attached and proliferated, with cell sheets forming on both active and static scaffolds after 4 weeks of culture. Cell densities for active and static scaffolds were comparable. Scale bar is 200 μm.
3D reconstructions of microcomputed tomography (µCT) showed mineral deposition over time in foam and fibrous scaffolds. Both foam (top) and fibrous (bottom) scaffolds showed qualitatively comparable amount of mineral deposition in active and static groups. For foam scaffolds, both active and static scaffolds showed minimal minerals before transition (30 °C 5 d) and 2 d after transition (37 °C 2 d), and mineral size was small. A significant increase in mineral volume and size was found on both groups 23 d after transition (37 °C 23 d). For fibrous scaffolds, neither static nor active groups showed mineral deposition at 30 °C for 5 d; however, artifacts from the scaffolds were observed. At 37 °C for 2 d, both groups showed minimal mineral formation, and minerals appear to begin nucleating from the edges of the scaffolds. At the last time point, scaffolds from both groups were covered by mineral nodules. Scale bar: 1 mm.
Figure 4-8. Alkaline phosphatase (ALP) activity of cells in active and static foam and fibrous scaffolds. No significant difference in ALP activity was found between active and static scaffolds for both foam (top) and fibrous (bottom) scaffolds. For foam scaffolds, a trend of increased ALP activity was seen after transition (37 °C 2 d and 37 °C 23 d) compared to before transition (30 °C 5 d). But no statistically significant difference was found. For fibrous scaffolds, static scaffolds demonstrated a trend of increasing ALP activity, yet not statistically significant. Vertical lines represent averages of three replicates in the groups.
Figure 4-9. Real-time PCR (qPCR) expression of osteogenic marker Runx2 and OC for active and static foam and fibrous scaffolds. There was no difference between active and static scaffolds in both foam (top) and fibrous (bottom) scaffolds across all time points. For both foam and fibrous scaffolds, a trend of increasing (upregulation) Runx2 and decreasing (downregulation) OC over time was observed for active and static scaffolds, although significance was not achieved. Horizontal lines indicate averages over 3 independent replicates.
Figure 4-10. Alizarin red staining of calcium deposition in foam and fibrous scaffolds 4 weeks after culture. Calcium deposition (red stain) was observed in both foam (top) and fibrous (bottom) scaffolds. No apparent difference between active and static scaffolds were observed in either foam or fibrous scaffolds. Both active and static groups in foam and fibrous scaffolds showed significant amount of calcium deposition at the final time point (37 °C 23 d). For foam scaffolds, the scale bar is 1 mm. For fibrous scaffolds, the scale bar is 100 μm.
Figure 4-11. Alizarin red staining of a control foam with no cells shows no calcium deposition (lack of red stain). The sample was subjected to the same 4-week culture conditions as cell-seeded scaffolds. A negative result of staining suggests the calcium deposition observed on cell-seeded samples is attributed to the cells, and not spontaneous deposition on the foam scaffolds. Scale bar is 1 mm.
Chapter 5. Effect of Low Oxygen Tension during Expansion on Chondrogenic Potential of Osteoarthritic Chondrocytes

5.1 Synopsis

In previous chapters, we established a robust 3D fibrous shape memory electrospun scaffold that could be employed to study cell motility and differentiation in vitro. Such synthetic scaffolds can act as active complex microenvironment, and be used as an in vitro model to study various cell behavior in complex dynamic microenvironment in vivo. In this chapter, the objective is to study the cell differentiation behavior in complex scaffold-free microenvironment, to explore a non-scaffold-related factor — low oxygen, which exists in complex in vivo microenvironment but is also highly associated with scaffold systems, on cell differentiation behavior. More specifically, osteoarthritic chondrocytes phenotype was examined when they exposed to low oxygen tension during expansion then followed by normal oxygen tension in 3D pellet culture. Low O$_2$ tension in vitro culture has been studied to examine its effect on preventing dedifferentiation of primary chondrocytes, and results have been conflicting. In particular, low O$_2$ tension effects on human osteoarthritis (OA) chondrocytes are poorly understood. Here in this chapter, the effect of low O$_2$ tension during expansion on OA chondrocytes phenotype was examined.

5.2 Introduction

In vitro expanded primary chondrocytes are a promising and frequently studied source of cells for cartilage tissue engineering strategies, but present several challenges. One of the most significant and well recognized challenges is dedifferentiation during monolayer expansion.
Dedifferentiation is often associated with chondrocytes losing their characteristic gene and protein expression profiles [1]. This can lead to poor biochemical and biomechanical properties of cartilage engineered from these cells, when compared to native cartilage. Approaches studied to minimize dedifferentiation and optimize chondrogenic potential following monolayer expansion include co-culture with mesenchymal stem cells [2,3], growth factor supplementation [4,5], hypoxic (< 21 % O_2) culture [6,7], and 3D culture using hydrogel or synthetic 3D scaffolds [8,9]. Hypoxic *in vitro* culture of articular chondrocytes has received significant attention due to the fact that low oxygen tension is present *in vivo* during chondrogenesis and in adult articular cartilage. Therefore hypoxic culture condition could potentially provide a biomimetic approach to better maintain chondrogenesis *in vitro*, by controlling the oxygen percentage in incubation system in a scaffold-free system, or by designing scaffold’s pore size and interconnectivity in a 3D scaffold system to achieve optimal low oxygen inside of scaffolds [10–12].

Previous studies have shown that low oxygen expansion (1 % to 5 %) [6,7] of chondrocytes is sufficient to enhance the biochemical quality of the extracellular matrix (ECM) produced post expansion. They have demonstrated such enhanced biochemical quality by showing increased specific chondrogenic characteristic gene expression [13], collagen type II protein expression [14], and glycosaminoglycan (GAG) secretion [15]. For example, bovine chondrocytes expanded at 1.5 % O_2 and micromass cultured at 21 % O_2 showed a two-fold increase in *Col IIA1* mRNA after expansion, though no increase in *Col IIA1* mRNA or protein was observed after micromass culture. However, a two-fold increase in glycosaminoglycan content after micromass culture was observed [6]. Similarly, rabbit chondrocytes expanded at 5 % O_2 and pellet cultured at 21 % O_2 showed increased expression of *Col IIA1* type IIA mRNA after expansion and an increase in *Col IIA1* type
IIB after pellet culture. Furthermore, an increase in GAG density and total collagen content was observed in pellets formed from chondrocytes expanded at 5% O\textsubscript{2} compared to 21 % O\textsubscript{2} [7]. In the study performed by Murphy and colleagues, human healthy articular chondrocytes were expanded under 20 % O\textsubscript{2} condition, then further encapsulated in alginate beads for followed 3D cultured under 5 % O\textsubscript{2} or 20 % O\textsubscript{2} condition. Encapsulated chondrocytes that cultured under 5 % O\textsubscript{2} showed a significant increase chondrogenic characteristic gene expression, such as \textit{SOX9}, \textit{ACAN}, and \textit{Col II}, as well as GAG content [15]. However, they didn’t study the oxygen condition effect during monolayer expansion on maintaining chondrocyte phenotype. Collectively, these studies have shown that hypoxic expansion has the potential to preserve chondrogenic phenotype and cartilage tissue production upon pellet culture in bovine, rabbit and healthy human chondrocytes, but it is not known if these benefits contribute to an improvement in biomechanical properties to enhance the functionality of the engineered cartilage tissue using human primary OA chondrocytes, which is a more clinical relevant source of cell for engineered human cartilage tissue.

The objective of this study was to examine the effect of low oxygen culture condition on maintaining chondrocyte phenotype. Different from previous chapters that investigated scaffolds architectural effect on cell behavior, the goal of this study was to explore low oxygen – a non-scaffold-related factor on cell behavior in scaffold-free 3D culture environment. Our hypothesis is that hypoxic expansion of human osteoarthritic (OA) articular chondrocytes will result an increase in the characteristic gene and protein expression of the resultant cartilage pellets derived from these chondrocytes. To test these hypotheses, we expanded primary human OA articular chondrocytes under 5 % O\textsubscript{2} and 21 % O\textsubscript{2}. We then promoted neo-cartilage formation in a pellet culture assay using passage 2 (P2) chondrocytes under 21 % O\textsubscript{2}. We analyzed gene expression
with real-time PCR, GAG content with using colorimetric assay, and chondrogenic characteristic protein expression with histology staining and immunohistochemistry staining.

5.3 Methods and Materials

5.3.1 Study Design

The study design is shown in Figure 5-1. Human OA articular cartilage tissue was first harvested, dissected, and then digested to collect primary human OA chondrocytes. Collected primary OA chondrocytes were monolayer expanded under different oxygen conditions, 5 % O₂ and 21 % O₂. At the end of monolayer expansion, desired amount of cells were collected for gene expression analysis, then the rest of the cells were proceed for 3D aggregate pellet culture, under different oxygen conditions 5 % O₂ and 21 % O₂ as well. At the end of 3 weeks of pellet culture, pellets were harvested for gene expression analysis, GAG/DNA quantification, histology and immunohistochemistry staining.

5.3.2 Chondrocytes harvest, isolation, and expansion

Human articular cartilage tissue were harvested from femoral condyles of four OA patients (age from 51 to 62) undergoing total knee replacement surgery under State University of New York and Syracuse University institutional review board protocols. Cartilage tissue slices from visibly unaffected areas of the joint were collected from surgical waste. Immediately after harvesting, slices were dissected from bone, finely chopped, then digested sequentially in high-testicular hyaluronidase (Sigma), Trypsin-EDTA (Invitrogen), and Type II Collagenase (Invitrogen), as previously described [7]. After digestion, chondrocytes were collected by filtering
through 70 µm Nitex filter and centrifuged, and then expanded in complete expansion medium which composed of DMEM-LG (Invitrogen) with 10 % lot number specific fetal bovine serum (Invitrogen) and 1 % Penn/Strep (Invitrogen) under normal oxygen tension (21 % O₂) and low oxygen tension (5 % O₂) conditions. Low oxygen tension was achieved by injecting nitrogen into CO₂/O₂-control incubator (SANYO) to dilute oxygen tension to 5 %.

5.3.3 Chondrocyte pellets preparation, culture and harvest

To generate cartilage matrix for biochemical testing, when chondrocytes cultured under different oxygen tension reached confluence (at the end of P1), cells were trypsinized and pelleted down for micromass culture [7]. Briefly, 200,000 monolayer cells from each oxygen condition of each patient were collected for real-time PCR gene expression analysis; while the rest were followed with conventional chondrogenic pellet culture under normal oxygen tension (21 % O₂) at the density of 250,000 cells per pellet as previously described [7]. Fifteen chondrocytes pellets from each group were cultured for 3 weeks in complete pellet medium. Complete pellet medium is made with DMED-HG (Invitrogen) with 10 ng/ml TGF-β1(Peprotech), 1 % ITS+Premix (Becton-Dickinson), 10⁻⁷ M Dexamethasone (Sigma), 1 % sodium pyruvate (Invitrogen), 1 % 13 mM L-Ascorbic Acid Phosphate (Wako), and 1 % Penn/Strep (Invitrogen) [16]. At the end of 3 weeks of culture, chondrocyte pellets were collected and frozen down in 30 % DMSO (Sigma) in PBS (Invitrogen) in liquid nitrogen for later biochemical and biomechanical analysis. Preliminary tests showed no difference regarding biochemical and biomechanical properties between fresh and frozen pellets (data not shown).
5.3.4 Total RNA extraction, cDNA synthesis, and real-time PCR gene expression

To allow quantitative comparison of markers of chondrocyte phenotype, total RNA was extracted from monolayer chondrocytes at the end of P1 culture and from pellets at the end of 3 weeks culture from different groups using RNeasy Micro Kit (QIAGEN) per the manufacturer’s instructions. Total RNA were then used for reverse-transcription reaction using QuantiTect Rev. Transcription Kit (QIAGEN). RNA and cDNA quality and quantity was assessed by absorbance readings at 260 nm using spectrophotometry. Absorbance reading ratios of 260/280 nm and 230/280 nm were greater than 1.8, indicating high purity of RNA and cDNA.

Real-time PCR reaction was performed in quadruplicate with 20 µl as total reaction volume using iTaq Universal SYBR Green Supermix (Bio-Rad) on Mastercycler ep realplex (Eppendorf).

The most stable housekeeping genes were first screened both on monolayer cells and pellets. Briefly, ten housekeeping gene primers (ACTB, B2M, GAPD, GUSB, HPRT1, PGK, PP1A, RPL13A, TBP, TFRC purchased from realtimeprimer.com; primer sequences are shown in Table 5-1) were tested on monolayer chondrocytes that expanded under 21 % O\textsubscript{2} and 5 % O\textsubscript{2}, and resulted pellets. Raw real-time PCR data were processed by LinRegPCR [17]. The resulted primer efficiencies and Ct values were then used as input in Bestkeeper [18] to find the most stable housekeeping gene in monolayer chondrocytes and resulted pellets respectively. These most stable housekeeping genes were used as reference genes in following real-time PCR reactions.

To examine chondrocytes characteristic gene expression at the end of monolayer expansion and pellet culture respectively, SOX9, ACAN, COL IIA1 and COL IAI (Realtimeprimers.com. Primer sequences are shown in Table 5-1) primers were used together with previously screened
most stable housekeeping gene primers. Gene expression fold change was analyzed using LinRegPCR and REST 2009 (QIAGEN) [19].

5.3.5 DNA and GAG content analysis

To quantify GAG secretion per DNA content, six chondrocyte pellets from each group were digested by Proteinase K digestion buffer at 60 °C for overnight. Proteinase K digestion buffer was prepared by freshly adding 3 mg/ml proteinase K (QIAGEN) to sterile PBS with 10 % SDS (Sigma), 50 mM EDTA (Sigma), 10 mM Tris-Cl (pH 7.4) (Sigma). Quantification of GAG content was performed following a modified 1,9-dimethylmethylene blue (DMMB) method [20]. DNA content was determined using Molecular Probes picoGreen Kit (QIAGEN) per the manufacturer’s instructions. GAG content was then normalized to DNA content and was compared between groups.

5.3.6 Histological and immunohistochemical analysis

To examine cartilage matrix deposition and protein distribution, three pellets from each group were fixed by 10 % buffered formalin phosphate (Sigma), followed by series dehydration in ethanol, mounting, and cutting into 5 µm slices. For each pellet, half of the slices (from top to center) were used for histological staining, and the other half were analyzed by immunohistochemistry staining. Briefly, sections for histological analysis were deparaffinized in xylene (Sigma) and series concentration of ethanol (Sigma), then stained with Toluidine Blue (Sigma) for proteoglycans and FastGreen/Safranin-O (Sigma) for glycosaminoglycans as previously described [21].

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For immunohistochemical analysis, sections were first rehydrated, incubated with primary antibodies – anti-Col II (Developmental Studies Hybridoma Bank) and anti-Col I (Sigma) with appropriate antibody dilution in BSA (Sigma), then secondary antibody (MP Biomedicals). Fluorescent signals were developed using VECTOR VIP Peroxidase Substrate Kit (VECTOR Laboratories, INC.), and then observed on Leica fluorescence microscope.

5.3.7 Statistics

Real-time PCR and GAG/DNA were compared between 5 % O₂ and 21 % O₂ groups using paired t-tests. Statistical significance was set at $p < 0.05$.

5.4 Results

5.4.1 Real-time PCR gene expression analysis

At the end of monolayer expansion under 5 % and 21 % oxygen tension, chondrocytes showed differential chondrogenic characteristic gene express based on real-time PCR results (Figure 5-2). Monolayer chondrocytes expanded under 5 % O₂ showed slightly but not statistical significant increased expression of $SOX9$, which is a master regulator of chondrogenesis. And significantly increased of $ACAN$ and $COL IIA1$ gene expression in 5 % O₂ monolayer expanded chondrocytes when compared to chondrocytes that were expanded under 21 % O₂. Low oxygen expanded chondrocytes also showed significantly decreased $COL IAI$ expression than chondrocytes that were under 21 % oxygen tension, which indicates lower level of dedifferentiation under low oxygen condition.
However, for the micromass pellet cultured chondrocytes with prior exposure to 5 % and 21 % O\textsubscript{2}, real-time PCR results didn’t show significant difference in the expression of the chondrogenic characteristic genes (Figure 5-3). Results also showed large deviation due to variation from different patients. Gene expression ratios are shown in Table 5-2.

5.4.2 DNA and GAG quantification analysis

From GAG and DNA quantification analysis, at the end of 3 weeks of pellet culture, 5 % O\textsubscript{2} expanded chondrocytes resulted pellets showed no significant difference in GAG content (normalized to DNA content) when comparing with 21 % O\textsubscript{2} expanded chondrocytes resulted pellets (Figure 5-4). Results showed large variation from different patients.

5.4.3 Histological and immunohistochemical staining

Pellets that produced by chondrocytes expanded under 5 % and 21 % O\textsubscript{2} tension were examined by histology staining with safranin-O/fast green and toluidine blue, and immunohistochemical staining with collagen type II and collagen type I (Figure 5-5). For histology staining, pellets resulted from chondrocytes expanded under both low and normal oxygen condition showed intense staining of glycosaminoglycans by safranin-O and proteoglycans by toluidine blue. But no apparent difference of staining intensity between 5 % O\textsubscript{2} expanded chondrocytes pellets versus 21 % O\textsubscript{2} expanded chondrocytes pellets. Immunohistochemistry staining showed much higher signal of Col II compared to Col I in all groups, but no obvious difference between 5 % O\textsubscript{2} expanded chondrocytes pellets and 21 % O\textsubscript{2} expanded chondrocytes pellets was observed.
5.5 Discussion

In this chapter, we used primary human OA chondrocytes to study the effect of low oxygen expansion condition on maintaining the chondrogenic phenotype. Human primary OA chondrocytes were expanded under 5 % O$_2$ and 21 % O$_2$, then followed by 3D pellet cultured under 21 % O$_2$. We have observed significant increase in chondrogenic characteristic gene expression at the end of monolayer expansion. However, no significant increase in characteristic gene expression, protein production, or GAG content was achieved in resultant chondrocytes pellets at the end of 3 weeks of pellet culture when the chondrocytes were expanded under low oxygen tension. Statistical significance was not achieved partially due to huge sample variation from different patients. These patients were aged from 51 to 62 with OA, however, other medical conditions were unknown. Individual patient’s health condition and the severity of their OA would directly or indirectly influence their chondrocytes’ capacity to maintain or restore their chondrogenic phenotype. In the future, patients with similar age and similar severity of OA should be selected for such study, and more patients should be included as well.

To further examine the extent to which the low oxygen expansion condition on maintaining chondrogenic phenotype on monolayer expanded human primary chondrocytes, healthy chondrocytes from the same patient could be compared with OA chondrocytes, if condition permits. Low oxygen tension might have stronger effects on maintaining chondrogenic phenotype on healthy chondrocytes than on OA chondrocytes since the OA chondrocytes have lower characteristic protein profile and functionality [22–24].
Appropriate mechanical testing on the resultant chondrocyte pellets should be performed in the future to further analyze the mechanical properties, such as elastic modulus. This would allow examination of whether low oxygen expansion condition not only can enhance the biochemical properties but can improve also mechanical properties of the resultant engineered cartilage tissue to produce engineered cartilage tissue with better functionality. We have tested use of a custom built mesoindenter system [25–27] for compressive indentation test under load control model, with a glass spherical indenter tip (radius = 326 µm) attached to a stiff aluminum cantilever. However, the measurements were not consistent due to various reasons. A better testing system and model should be utilized in the future to further examine the mechanical properties of the chondrocyte pellets.

At the end of 3 weeks of pellet culture, some pellets showed a hollow center when sectioned in half using a microtome. This could due to poor nutrition and oxygen transfer during extended pellet culture that caused cell death at the center of the pellets. In the future, bioreactor could be incorporated during pellet culture to enhance nutrition and oxygen transfer [28–30].

Additionally, instead of injecting nitrogen into CO₂/O₂-control incubator to dilute oxygen tension to achieve low oxygen culture condition, 3D synthetic scaffolds could also be potentially employed to create low oxygen culture condition for the application of cartilage tissue engineering. Small pore size and low interconnectivity are often associated with low oxygen tension inside of the engineered scaffold [31], which are considered as one of the limitations of the scaffold design and fabrication technique. Such low oxygen microenvironment in 3D scaffold may not be optimal for regeneration of tissues such as bone [32], but could be beneficial for cartilage tissue engineering.
5.6 Conclusion

In conclusion, the monolayer real-time PCR results suggest that low oxygen condition (5%) may promote chondrogenic gene expression in monolayer cells. But improved ECM production, which was seen earlier in animal models, may not be relevant to tissue engineering using human OA primary chondrocytes. Further examination of the mechanical properties of the engineered pellets is needed to confirm the effects of low oxygen expansion on human OA chondrocytes and their use in tissue engineering application.

5.7 References


[19] Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR.


[26] Gilbert JL, Cumber J, Butterfield A. Surface micromechanics of ultrahigh molecular weight


Table 5-1. Real-time PCR primers table.

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<th>Reverse</th>
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<th>Product size</th>
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<td>NM_004048.2</td>
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<td>NM_002046.2</td>
<td>5'- GAGTCAAGGGAGTTTGGTCTG -3'</td>
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<td>5'- CTCTCTGCTGGGACTTCA -3'</td>
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<td>HPRT1</td>
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<td>5'- GGTCCCTTTTACCAAGCAGT -3'</td>
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<td>94bp</td>
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<td>COL2A1</td>
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<td>5'- GGACACAGAGGTTTTCAGG -3'</td>
<td>5'- CCAATGGCCACCATTTGC -3'</td>
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Table 5-2. Real time PCR gene expression ratio from four patients at the end of monolayer expansion and pellet culture.

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<td>Sox9 0.752</td>
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<td></td>
<td>patient 2</td>
<td>1.346</td>
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<td></td>
<td>patient 3</td>
<td>1.235</td>
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<td></td>
<td>patient 4</td>
<td>2.082</td>
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<td>Pellet culture ratio = expanded under 5% O$_2$/expanded under 21% O$_2$</td>
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<td>patient 2</td>
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<td>patient 3</td>
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<tr>
<td></td>
<td>patient 4</td>
<td>2.276</td>
</tr>
</tbody>
</table>

Gene expression from each patient under monolayer expansion or pellet culture condition was first normalized to the most stable housekeeping genes. Then the ratio was calculated as normalized gene expression under 5% O$_2$ condition divided by normalized gene expression 21% O$_2$ condition. A ratio greater than 1 indicating higher expression in 5% O$_2$ condition than in 21% O$_2$ condition; while a ratio less than 1 indicating lower expression in 5% O$_2$ condition than in 21% O$_2$ condition.
Figure 5-1. Study design. Primary human OA cartilage tissue were collected, dissected, and then digested to harvest primary human OA chondrocytes. Chondrocytes were monolayer expanded under 5% and 21% oxygen tension. After two passages of monolayer expansion, desired amount of cells were collected for real time PCR analysis, and the rest of the cells were proceeded to 3 weeks of 3D aggregate pellet culture under 21% oxygen tension. At the end of pellet culture, pellets were collected for gene expression, GAG/DNA quantification, histology staining, and immunohistochemical staining.
Figure 5-2. Chondrogenic characteristic genes at the end of monolayer expansion under 5 % (empty circles) and 21 % O$_2$ (filled circles) tension normalized to GAPDH. Chondrogenic characteristic genes *ACAN* and *Col II* showed significantly higher expression in chondrocytes expanded under 5 % O$_2$ tension than chondrocytes expanded under 21 % O$_2$ tension ($p < 0.05$, n = 4). *Sox9* showed slight but not significant increase in 5 % O$_2$ expanded chondrocytes than 21 % O$_2$ expanded chondrocytes. *Col I*, as an indicator of dedifferentiation, showed no difference in 5 % and 21 % O$_2$ expanded chondrocytes. Each line with two circles connected represents one patient. Results showed big variation among different patients.
Figure 5-3. Chondrogenic characteristic genes at the end of 3 weeks of pellet culture under 21 % O₂ tension with the cells previously expanded under 5 % (empty circles) and 21 % O₂ (filled circles) tension normalized to RPL13A. Chondrogenic characteristic genes Sox9, ACAN and Col II after pellet culture showed no difference between cells previously expanded under 5 % O₂ and 21 % O₂ tension. Col I, as an indicator of dedifferentiation, showed no difference in 5 % and 21 % O₂ expanded chondrocytes either. Each line with two circles connected represents one patient. Results showed big variation among different patients.
Figure 5-4. Glycosaminoglycan (GAG) content normalized to DNA content at the end of 3 weeks of pellet culture under 21 % O₂ tension with the cells previously expanded under 5 % (empty circles) and 21 % O₂ (filled circles) tension. No significant differences ($p = 0.0574$, $n = 4$) between pellets that were expanded under 5 % and 21 % O₂ tension. Each line with two circles connected represents one patient. Results showed big variation among different patients.
Figure 5-5. Histology and immunohistochemistry staining at the end of 3 weeks of pellet culture that resulted from chondrocytes expanded under 5% and 21% O₂ tension. Safranin-O staining for glycosaminoglycans in red (counter stained by FastGreen for nucleus in green) and Toluidine Blue staining for proteoglycans in purple showed intense signal in both in pellet groups that resulted from 5% and 21% O₂ monolayer tension but no difference was observed. Immunohistochemical staining for Col II in dark purple showed intense signal in both pellet groups with no apparent difference. Col I in dark purple showed weak signal in both groups indicating low level of dedifferentiation, with no apparent difference between 5% and 21% O₂ expansion tension. Scale bar is 0.5 mm.
Chapter 6: Conclusions and Future Directions

6.1 Conclusions

The work presented in this dissertation was designed to study cell behavior, such as motility and differentiation, in complex 3D in vitro microenvironments. The first part of this dissertation (Chapter 2 – 4) developed a programmable 3D shape memory electrospun scaffold that can dynamically change fiber alignment upon triggering under cytocompatible conditions. In these chapters, the programmable dynamic 3D scaffold was employed to study stem cell and cancer cell motility and stem cell differentiation for ultimate application in stem cell homing, cancer metastasis studies, and bone tissue engineering. The work builds upon the previous development of the 3D electrospun scaffold made from SMP thermoplastic polyurethane [1] and made further modification and optimization to achieve robust cell attachment and migration. The second part of this dissertation (Chapter 5) utilized a scaffold-free 3D culture system, and investigated the effect of a non-scaffold-related factor—low oxygen tension—on mature chondrocytes dedifferentiation behavior. Overall, this dissertation has examined different aspects of complex in vitro 3D microenvironments, and their effect on cell motility and differentiation behavior.

In Chapter 2, a novel 3D nano-fibrous SMP electrospun scaffold that can dynamically alter fiber alignment on command was developed and applied as an active cell culture platform to study the motility of cells, using C3H10T1/2 murine mesenchymal stems, when the cellular microenvironment changes its architecture. This system can potentially mimic the in vivo fibrous ECM network that can remodel its architecture overtime, and a key advance provided by this model is that the programmable change in architecture is de-coupled from cellular remodeling. We found
that when fiber alignment dynamically increases, cell migration changes from random to highly aligned pattern, with significantly increased velocity. When fiber alignment dynamically decreases, cell migration changes from highly oriented along the fiber alignment direction to random migration. Cells migrate preferentially along fiber alignment direction when exposed to aligned fiber architecture, while cells migrate randomly without specific direction when exposed to unaligned fiber architecture. This is the first study to date that investigated cell motility in an *in vitro* dynamic SMP scaffold. We confirmed that such a programmable 3D SMP scaffold is capable of being employed as a dynamic platform to study cell migration behavior. The platform provides a new tool for mimicking the dynamics of ECM to study cell-microenvironment interactions *in vitro*.

In **Chapter 3**, the 3D fibrous SMP electrospun scaffold was applied to study cancer cell motility and polarization. Human HT-1080 fibrosarcoma cells showed a similar motility trend as C3H10T1/2 cells showed in **Chapter 2**. The dynamic increase of the scaffold fiber alignment induced cell alignment along fiber orientation, highly polarized cell morphology, and directional motility along the fiber orientation; while the dynamic decrease of the scaffold fiber alignment induced cells losing their preferential alignment and became randomly oriented, non-polarized morphology, and motility with a random pattern without preferential directionality. The work presented in this chapter once again demonstrated the capability of an SMP electrospun scaffold to be used as an *in vitro* system to study cell motility, but it also provided a method that could potentially be used to manipulate cancer cells polarity and subsequent invasive phenotype.

**Chapter 4** employed the same chemistry as the previous two chapters but used a 3D micro-fiber SMP electrospun scaffold and a 3D SMP foam scaffold together to investigate human
mesenchymal stem cells’ osteogenic differentiation capacity when cells went through the shape memory architectural change of the scaffolds. We achieved successful osteogenic differentiation, and confirmed that the dynamic architectural change of the deployable 3D SMP scaffolds have no measureable effect on stem cell differentiation capacity, compared to the cells cultured in static scaffolds. Such findings support the potential application of such deployable scaffolds in bone critical-sized defect treatment.

Different from Chapter 2 – 4 that examined the effect of microenvironment architecture on cell behavior, Chapter 5, instead, investigated low oxygen tension in the microenvironment in a scaffold free system, with the focus particularly on cartilage tissue engineering. Low oxygen has been frequently brought up as a limitation associated with synthetic 3D scaffolds when the scaffolds have small pore size or poor interconnectivity, or both, due to the fact that low oxygen and limited nutrient diffusion can cause cell death. However, low oxygen tension during culture could be beneficial for cartilage tissue engineering as cartilage is an avascular tissue, and low oxygen presents in vivo during chondrogenesis and in adult articular cartilage. We examined the low oxygen expansion effect on engineered scaffold-free cartilage tissue, and found that low oxygen during monolayer expansion could potentially maintain chondrogenic phenotype and might be beneficial for cartilage tissue engineering. In the future, the SMP scaffolds could also be incorporated into the low oxygen tension study by controlling the dynamic change of the scaffold’s pore size and interconnectivity. Small pore size and low interconnectivity are often associated with low oxygen tension inside of the scaffold [2,3], which are considered as one of the limitations of the scaffold design and fabrication technique. However, such 3D low oxygen microenvironment could be beneficial for cartilage engineering.
In summary, the 3D dynamic programmable SMP scaffolds discussed in this dissertation showed great promise for use in active cell culture and studies of cell-microenvironment interactions for the applications of fundamental mechanobiology, cancer metastasis, tissue engineering, and regenerative medicine. Many other modifications or optimizations could be considered for a wide range of applications which will be discussed below.

6.2 Recommendations for future work

6.2.1 Shape memory electrospun scaffold

Electrospinning, in general, provides an excellent way to create a fibrous network that could mimic the *in vivo* ECM architecture with great tunability. However, insufficient cell infiltration, which could result in an uneven distribution of cells, has always been a challenge for electrospun scaffolds. To improve cell infiltration, techniques such as sacrificial fibers [4], dynamic culture [5], collagen grafting on fibers [6], and ultrasonication [7] could be introduced in the future to enhance cell penetration into the scaffold.

As discussed in Chapter 2 and Chapter 3, SMP electrospun scaffolds provide a new platform for an *in vitro* active 3D cell culture to study cell motility in a dynamic environment in real time. In the live imaging of cell motility work presented in Chapter 2 and Chapter 3, only the cell nuclei were fluorescently stained and imaged and the electrospun fibers were not visible during imaging, which makes it difficult to directly observe cell-fiber interactions. In the future, fluorescent dyes such as Rhodamine B [8] or Cell Tracker Red [5] with optimized concentration
could be incorporated into the fibers to visualize the fibers to enable the live imaging of the cell-fiber interaction.

It has been argued that electrospun scaffolds present cells with a pseudo-3D environment, instead of true 3D environment, as electrospun scaffolds are often very thin due to the time and challenge associated with electrospinning thick scaffolds [9]. Such limitation could potentially be address in the future by utilizing grounded spherical dish as fiber collector, and an array of probes to create uncompressed fibers [10], or by concurrent gel electrospray and polymer electrospinning as demonstrated in the work published by Hong and colleagues [11].

6.2.2 Shape memory electrospun scaffold for guided stem cell motility

In Chapter 2, we demonstrated the capability of employing the 3D SMP electrospun scaffold for stem cell motility studies and we found the dynamic change of the fiber alignment could direct cell motility directionality. Such dynamic scaffolds could be potentially used for the application of guided stem cell migration, or cell homing, for tissue repair and tissue regeneration. With the current experimental setup, we were only able to change the cell motility direction and not to direct the cells to move to a certain location. To achieve this goal, for more well-controlled guided migration, conjugating binding affinity peptide [12], incorporating chemotactic factor [13], or growth factors [14] in a well-defined gradient and pattern by utilizing techniques such as magnetic beads, and micro-contact printing, could be investigated in the future.
6.2.3 Shape memory electrospun scaffold for cancer cell motility and metastasis

In Chapter 3, we studied cancer cell polarization and directional motility guided by dynamic change of fiber alignment of 3D SMP electrospun scaffold. The results show that highly aligned fiber result in directional motility with increased averaged velocity. We tested the hypothesis that highly aligned fibers would provide “highways” for cells to migrate. However, how exposure of cancer cells to highly aligned fibers would further affect the cells invasiveness is still unknown. To study if the aligned fibers would also transiently or more permanently affect the invasive phenotype of the cancer cells, the internal architecture of the scaffold could be further tailored to have localized alignment surrounded by unaligned fibers. More specifically, the spatially varying fiber alignment could be designed as (1) a scaffold divided by a single boundary into half scaffold with highly aligned fibers and half scaffold with unaligned fibers; (2) a scaffold with an “island” of aligned fibers that is surrounded by unaligned fibers; (3) a scaffold with multiple, potentially interacting “islands” of aligned fibers that are surrounded by unaligned fibers. With cancer cells seeded on aligned regions or unaligned regions in different scaffold designs mentioned above, cell motility directionality, velocity, cell morphology and polarization could be analyzed in the future. Such study would help understand how cancer cells invade from highly aligned fibers into unaligned fibers, which would be biomimetic of cancer cells invading into stroma and subsequent metastasis.

To further investigate the scaffold fiber alignment effect on cancer cell invasive phenotype, signaling pathways could be studied in the future, employing techniques such as transfecting cells with small interfering RNA (siRNA). A better understanding of the interactions between the microenvironment architecture and the cytoskeletal structure, focal adhesion formation, and Golgi
body structure of the cancer cells at molecular level are essential for determining if the highly aligned fibers in the scaffold would have an impact on the invasiveness of the cancer cells. For instance, Rho and Rac are the best characterized Rho GTPases that regulate the assembly and organization of the actin cytoskeleton. They play an essential role in cancer cell migration [15]. Previous studies have shown significant association of Rho/ROCK pathway with invasion of bladder cancer [16], activation of Rac1 and Rac3 with invasiveness of human breast cancer cells [17], and focal adhesion protein Paxillin with Golgi structure and polarized cancer cell migration [18]. Collectively, transfecting cells using siRNA to knock out proteins such Paxillin, giantin, and Rac1 and observing how these signaling pathways would respond to cancer cell moving from highly aligned fibers to unaligned fibers, or moving on temporarily aligned then recovered to unaligned fibers, is worthy looking into in the future, to achieve an in-depth understanding of how highly aligned microenvironment architecture would impact on cancer cell invasiveness and subsequent metastasis.

6.2.4 Shape memory scaffolds for stem cell osteogenic differentiation and bone tissue engineering

In Chapter 4, we demonstrated successful osteogenic differentiation of human adipose-derived mesenchymal stem cells when they were cultured in 3D SMP foam and electrospun scaffolds, which underwent bulk shape and internal architectural change of the scaffolds. The results suggest that the dynamic change of the scaffold did not hinder the ASCs osteogenic differentiation, and supported the potential of applications incorporating stem cells in SMP deployable scaffolds for the application of bone defect treatment. However, because the lack of a
functional vascular supply could eventually result in decreased cell viability and implant failure following engineered bone scaffold implantation [19], approaches that could vascularize the scaffold need to be taken into consideration in future investigations. Previous studies have shown promising vascularization in engineered bone scaffold by incorporating angiogenic growth factors such as VEGF [20,21], and co-culture of endothelial cells and osteoblasts in the scaffolds [22,23]. Similar methods such as implementing growth factors and co-culture system could be employed in our present 3D SMP foam and electrospun scaffolds in the future work.

6.2.5 Mature chondrocytes dedifferentiation and redifferentiation

In Chapter 5, we examined the effect of low oxygen culture condition on maintaining mature human chondrocytes chondrogenic phenotype in a scaffold-free 3D environment in vitro. The low oxygen condition was investigated as a non-scaffold-related factor but an important factor that exists in in vivo complex microenvironment, and low oxygen condition has important impact on cell behavior. The results suggested that low oxygen condition during monolayer expansion might be beneficial for chondrogenesis, and low oxygen during expansion could potentially decrease dedifferentiation. However, the results showed huge patient variation and with the limited sample size (4 patients) further in-depth investigation on the resulting engineered cartilage tissue is required to achieve conclusive results.

With the conclusion that low oxygen culture condition would better preserve chondrogenic phenotype of mature human chondrocytes after monolayer expansion in vitro, we also conducted preliminary experiments to examine the epigenetic regulation of chondrogenic phenotype when the chondrocytes were exposed to low oxygen versus normal oxygen during expansion. It is known
that epigenetic regulation—the post translational modification of genes without altering DNA sequences—could regulate cell phenotype, cell lineage specification [24,25]. But the extent to which epigenetics regulates chondrocytes phenotype and characteristic gene expression remains largely unknown. Therefore, we investigated histone methylation on the chondrogenic transcription factor Sox9, which is essential for chondrocyte differentiation and formation, and the methods and preliminary results are shown in Appendix 1. We found that specific forms of epigenetic regulation could potentially be used as markers of dedifferentiation status.

6.3 Final remarks

This dissertation has focused on employing various 3D programmable SMP scaffolds as in vitro dynamic 3D cell culture microenvironments to study the effect of the complex dynamics and oxygen tension of the microenvironment on cell differentiation and motility behaviors. With the promising results showing that dynamic SMP scaffolds can direct cancer cell morphology, stem cell and cancer directional motility, and stem cell differentiation, SMP scaffolds, with their potential to be tailored and engineered to fit specific needs, provide a new tool for studies of cell mechanobiology, tissue regeneration, and cancer therapy.

6.4 References


Appendix 1: Histone Methylation as a Marker of Chondrocyte Phenotype

Synopsis

Maintaining articular chondrocyte phenotype is important in terms of pathological conditions and cartilage development but technically challenging. Epigenetic modifications are one factor that can effect cell phenotype regulation. However, the extent to which epigenetic marks regulate chondrocytes phenotype and characteristic gene expression remains largely unknown. In this study, we studied specific epigenetic marks, in the form of trimethylation of histone H3 Lysine 4 and 27 (H3K4me3 and H3K27me3) level, in the Sox9 promoter in human articular chondrocytes (hACs) and in human adipose-derived mesenchymal stem cells (hASCs) as well as global H3K4me3 level in hACs and in dedifferentiated hACs. The results suggest that these epigenetic marks may regulate chondrocyte phenotype, and could be used as unique marks to distinguish the hACs phenotype from other lineages and could change during dedifferentiation.

Introduction

Cells within articular cartilage tissue are recognized to possess diverse phenotype. These phenotypic differences are important because they may lead to joint diseases such as osteoarthritis (OA) [1]. Cells-based therapies designed to address such diseases require primary or stem-cell-derived chondrocytes [2]. However, when chondrocytes are cultured in monolayer, they will dedifferentiate and lose their characteristic gene expression and phenotype and eventually lose their ability to redifferentiate and produce healthy cartilaginous tissue [3].
The extent to which epigenetics regulates chondrocyte phenotype and characteristic gene expression remains largely unknown. Histone methylation has been implicated in lineage specification and phenotype maintenance in multiple cell types [4-5]. Histone lysine methylation can regulate activation or deactivation of genes [6]. Histone H3 Lysine 4 trimethylation (H3K4me3) is often associated with transcription activation, while histone H3 Lysine 27 trimethylation (H3K27me3) is often associated with deactivation [7].

To investigate the relationship between histone methylation and human articular chondrocyte (hAC) phenotype, we have performed a preliminary analysis to determine the unique H3K4me3 and H3K27me3 status in two groups of comparison. One comparison was between hACs cultured under chondroprotective hypoxic (5% O2) condition and dedifferentiated hACs under normal O2 (21% O2) condition [9]; the other comparison was between hACs and hASCs. Our hypothesis is that histone modifications in the forms of H3K4me3 and H3K27me3 distinguish the hAC phenotype from other lineages such as hASCs, and change during dedifferentiation, redifferentiation, and in health and disease.

Methods and materials

Global H3K4me3 level assessment

OA articular chondrocytes (hACs) was harvested from a 68-year-old female OA patient undergoing total knee replacement surgery according to IRB-approved human subjects research protocols. hACs were then expanded under 21% or 5% O2 tension. hACs in 21% O2 condition will
dedifferentiate gradually; while hACs in 5% O₂ condition are seen as in chondroprotective condition [9].

At the end of passage 2 culture, whole cell lysate from same number of hACs under 21% O₂ or 5% O₂ tension were used for western blot analysis, with H3 and β-actin as reference and loading controls. hACs cultured under 5% O₂ were used as control group. Two technical replicates (n = 2) were performed using cells from the same donor.

**Targeted histone methylation analysis**

hACs were harvested from a 54-year-old male OA patient as previously described for this part of study, then expanded under 5% O₂ tension. Human adipose-derived mesenchymal stem cells (hACSs) from one donor were purchased from Invitrogen, then expanded under 21% O₂ tension. hACs of passage 4 and hASCs of passage 7 were harvested for analysis.

Total RNA were isolated, and used for cDNA synthesis (Qiagen), then real-time PCR with primers of Sox9, Col1, Col2, ACAN, MMP13. bACT was used as reference gene. hASCs were used as control group. Gene expression fold change (hAC/hASC) was determined using Pfaffl method [10]. Values were obtained from triplicate qPCR replicates (n = 3) from quadruplicate wells.

Three replicates of (n = 3) chromatin immunoprecipitation assay (ChIP) were performed (Millipore) using the same groups of cells as described above. Poll II Ser²-P is associated with gene transcription activation, being used as a positive ChIP control (Poll II Ser²-P had 2 replicates due to sample degradation). DNA samples from hACs and hASCs from each ChIP replicate were
used for real-time PCR analysis with ChIP-qPCR primers of Sox9 promoter and GAPDH promoter (Qiagen). GAPDH was used as reference gene to test ChIP efficiency. hASCs were used as control group. Values were obtained from 3 ChIP-qPCR replicates from triplicate wells. Gene expression fold enrichment (hAC/hASC) was determined following as previously described [11].

**Preliminary results**

The results of global H3K4me3 level assessment (Figure A1-1) showed increased H3K4me3 level in hACs cultured under non-chondroprotective condition (21% O₂) compared to chondroprotective hypoxic condition (5% O₂). This result suggests that during dedifferentiation under 21% O₂ condition, chondrocytes have higher H3K4me3 level, which could be a mark of dedifferentiation status of chondrocytes.

In targeted histone methylation study with comparison of hACs and hASCs, we first used real-time PCR to confirm characteristic chondrogenic gene expression in hACs (Figure A1-2). Next, we compared H3K4me3 and H3K27me3 level in Sox9 promoter in hASCs and hACs. The level of H3K27me3 in Sox9 promoter was found to be 8.7-fold lower in hACs than in hASCs (Figure A1-3), suggesting that repressive chromatin in the Sox9 promoter decreases in the chondrocyte phenotype. The level of H3K4me3 at the same site of Sox9 promoter was relatively unchanged or decreased slightly. Binding of Pol II Ser²-P to the Sox9 promoter was increased 1.5-fold, indicating increased transcriptional elongation of Sox9 (standard deviation is not shown due to sample degradation).
Discussion

Our results suggest that epigenetic marks in forms of H3K4me3 and H3K27me3 may regulate chondrocyte phenotype, and could be used as unique marks distinguish the hACs phenotype from other lineages and change during dedifferentiation. In further studies, more targeted methylation marks and regions, as well as genome-wide analysis should be included, with including more biological replicates from various donors. In conclusion, the present data support our working hypothesis that epigenetic marks regulate chondrocyte phenotype during dedifferentiation. These marks would be used to identify but also potentially control chondrocyte phenotype.

References


Figure A1-1. Western blot showed increased H3K3me4 in dedifferentiating OA chondrocytes. Human OA chondrocytes monolayer expanded under 21% O$_2$ are considered being dedifferentiation comparing to cells expanded under 21% O$_2$. Dedifferentiation OA chondrocytes showed higher level of H3K4me3, indicating that increased H3K4me3 could be a mark of dedifferentiation status of chondrocytes.
Figure A1-2. Real-time qPCR confirmed chondrogenic gene expression in the human articular chondrocytes than in human adipose-derived mesenchymal stem cells, with higher expression of Sox9, Col2, ACAN, and MMP13.
Figure A1-3. ChIP-qPCR showed lower H3K27me3 enrichment, slightly lower H3K4me3 enrichment in Sox9 promoter in hACs than in hASCs.
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