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REVIEW ARTICLE

Hydrogel-based methods for engineering cellular microenvironment with spatiotemporal gradients

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Abstract

Natural cellular microenvironment consists of spatiotemporal gradients of multiple physical (e.g. extracellular matrix stiffness, porosity and stress/strain) and chemical cues (e.g. morphogens), which play important roles in regulating cell behaviors including spreading, proliferation, migration, differentiation and apoptosis, especially for pathological processes such as tumor formation and progression. Therefore, it is essential to engineer cellular gradient microenvironment incorporating various gradients for the fabrication of normal and pathological tissue models *in vitro*. In this article, we firstly review the development of engineering cellular physical and chemical gradients with cytocompatible hydrogels in both two-dimension and three-dimension formats. We then present current advances in the application of engineered gradient microenvironments for the fabrication of disease models *in vitro*. Finally, concluding remarks and future perspectives for engineering cellular gradients are given.

Keywords

Biomolecules, cellular microenvironment, disease models, spatiotemporal gradients, stiffness

History

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Introduction

Cellular behavior is known to be influenced by surrounding three-dimensional (3D) cellular microenvironment, which contains multiples of neighboring cells, the extracellular matrix (ECM) and soluble factors. The spatial and temporal control of cellular microenvironments including various physical and chemical cues has been demonstrated to maintain the proper cellular functions and mediate the pathological changes. For instance, changes in the mechanical properties of ECM can induce structural rearrangements of the cytoskeleton and immobilized proteins, which in turn can generate a cellular response called mechanotransduction (DuFort et al., 2011; Hoffman et al., 2011; Ross et al., 2013). In addition, the altered extracellular proteinases (e.g. matrix metalloproteinases) mediate a multitude of changes in the cellular microenvironment and induce tumor formation (Kessenbrock et al., 2013; Plaks et al., 2013). Therefore, recreation of the physical and chemical properties of *in vivo* tissues and reconstruction of the complex interactions between cells and their microenvironment is necessary for engineering functional tissues, which hold great potentials for applications such as tissue reconstructions and disease model fabrications.

Over the past few decades, researchers have found that the ECM provides the spatiotemporally regulated physical and biochemical cues to guide cellular behaviors such as the proliferation, differentiation, migration and apoptosis (Han et al., 2014; Hazeltine et al., 2013; Metallo et al., 2007). These cues are mostly in format of spatiotemporal gradients, which play an important role in tissue development, homeostasis and disease progression (Chung et al., 2005; Mak et al., 2011). For example, gradients of matrix stiffness (ranging from 50 Pa to 2000 Pa) are of importance in guiding cell differentiation and migration (Grevesse et al., 2013; Polacheck et al., 2014). In addition, gradients in ECM porosity and pore size as well as the surrounding cell concentrations can also connect mechanically mismatched tissues such as bone-cartilage interfaces and dentino-enamel junctions. For various molecular factors (e.g. growth factors, chemokines and cytokines) in the cellular microenvironment, spatiotemporal gradients of such molecules can also induce a variety of biological phenomena such as morphogenesis, chemotaxis and wound healing (Kothapalli et al., 2011; Warren et al., 2013; Wu et al., 2014). Thus, the recreation of such natural cellular microenvironment with spatiotemporal gradient features *in vitro* would be necessary for tissue remodeling and disease model construction.

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Various approaches have been developed to engineer cellular microenvironment with spatiotemporal gradients *in vitro* to mimic the native cellular microenvironment (Claussen et al., 2013; Hopp et al., 2013; Ramalingam et al., 2013). Although there exist several reviews on the approaches for engineering chemical gradients and surface-bound gradients, most of which mainly focus on the applications in cell-biomaterial interactions and the cellular behaviors such as migration and angiogenesis in 2D microenvironment (Genzer & Bhat, 2008; Keenan & Folch, 2008; Sant et al., 2010). In this review, we essentially focus on various physical and chemical gradients generated with cytocompatible hydrogels in both 2D and 3D formats for regulating cellular behaviors. Hydrogels are ideal for engineering cellular gradient microenvironment due to their tunable properties (e.g. mechanical, chemical, and biocompatible) and responses to environmental stimulus (e.g. heat, light, magnetic field, electrical potential and biological agents; Khetan et al., 2013; Li et al., 2013). In the ''Cellular gradient microenvironment *in vivo*'' section, the natural gradient cues in cellular microenvironment are presented. In the ''Engineering cellular microenvironment with various gradients *in vitro*'' section, materials incorporating multiple physical and chemical gradients for investigating cell behaviors and cell–matrix interactions are discussed. In the ''Engineering cellular microenvironment with gradients for biofabrication of *in vitro* disease models'' section, current applications of engineered gradient microenvironment in biofabrication of disease models *in vitro* are presented. Finally, concluding remarks as well as future perspectives for the development of engineering cellular gradient microenvironment *in vitro* are given.

Cellular gradient microenvironment *in vivo*

A cellular microenvironment composed of various physical and chemical gradients *in vivo* has been demonstrated to play an important role in regulating cell fate and tissue/organ development. For instance, for the development of human pituitary gland, differentiation of primordial pituitary cells into endocrine cell types and the cellular juxtaposition related to one another are regulated by overlapping gradients of soluble molecules including fibroblast growth factors, bone morphogenic proteins and molecules from the Wingless Int and sonic hedgehog families (Scully & Rosenfeld, 2002). Another classic example is the formation of bone canalicules enabling the efficient mechanical functions of organs (Peyrin, 2009). Here, we presented the natural physical and chemical gradient cues in the cellular microenvironment.

Physical gradients

Physical gradients exist widely in the native cellular microenvironment such as the physical properties of ECM including stiffness, stress/strain distribution, porosity and topographical cues. For instance, it is becoming increasingly obvious that each tissue has a specific mechanical property (e.g. stiffness) due to the existence of stiffness gradients in cellular microenvironment, from soft tissues including neurons, lung, breast and heart (ranging from 100 Pa to 1000 Pa) to hard tissues such as bone (2 GPa; Figure 1A). Another example is the physical gradients inherent in hard tissues such as teeth and bones. Teeth consist of gradients in the composition and mineral density, which cause microscale gradients in mechanical properties (Barani et al., 2012). For bone structure, the gradients in porosity range from compact (5–40% for healthy bone) to spongy (porosity 40–80% for cancellous bone), which induce the formation of cortical bone with a high compressive resistance and cancellous bone with an elastic properties, respectively. The former accounts for 80% of bone mass, whereas the latter accounts for the remaining 20%. However, cancellous bones have nearly 10 times the surface area of those of cortex bones (Baker et al., 2013; Caffarelli et al., 2012; Mallinson et al., 2013). In addition, stress/strain gradients also exist in the native cellular microenvironment, which have shown to influence the morphology and migration of fibroblasts, tumor cells, endothelial cells and mesenchymal stem cells. For example, shear stress gradients generated by changes in vascular wall sizes can induce the cardiovascular pathologies such as intracranial aneurysms and atherosclerosis, due to its strong relevance to atherogenesis (Dolan et al., 2011, 2013).

Chemical gradients

Gradients of various morphogens such as chemokines, transcription factors and cytokines have long been known as crucial regulatory components during tissue development, homeostasis, inflammation and regeneration (Bokel & Brand, 2013; Rogers & Schier, 2011; Figure 1B). The spatial distribution of proteins provides the biochemical cues to induce the organized formation of tissues (Maruthamuthu et al., 2011). One classic example is the formation of neural tissues. Initially, young neurons send out membrane protrusions, which are known as axons, to innervate target cells. Subsequently, the formed axon bodies reside at significant distances from where the neurons are born. Finally, the growth and extension of neuron axons will be guided by secreted biomolecule gradients. For instance, callosal commissural neurons are specialized spinal neurons that play a vital role in relaying sensory information to the brain (Keenan & Folch, 2008; Matsumoto & Nagashima, 2010; Rabe et al., 2009). The ventral extension of commissural axons through the spinal cord is guided by gradients of molecules including netrin-1, semaphorins and ephrins, which are secreted by cells at the ventral midline forming a high-ventral to low-dorsal gradient. Natural gradients of diffusible signaling proteins can also regulate cell migration and differentiation during physiological process such as mitosis, embryogenesis and capillary sprouting (Ridley et al., 2003; Sant et al., 2010). Chemical gradients are also involved during distorted tissue formation such as metastatic tumors (Huh et al., 2011). In this process, cancer cells must escape from the original tumor and recruit endothelial cells to generate their own capillary vessels to feed the newborn tumor sites. Gradients of biomolecules such as angiopoietin-like 3 and CXCL-8 secreted by metastatic cancer cells help to induce angiogenesis and cancer cell proliferation (Ogura et al., 2013; Wu et al., 2012).

Temporal changes in cellular microenvironment also modulate cell processes such as gene expression (Sant et al., 2010). The activation of different morphogens target genes occurs

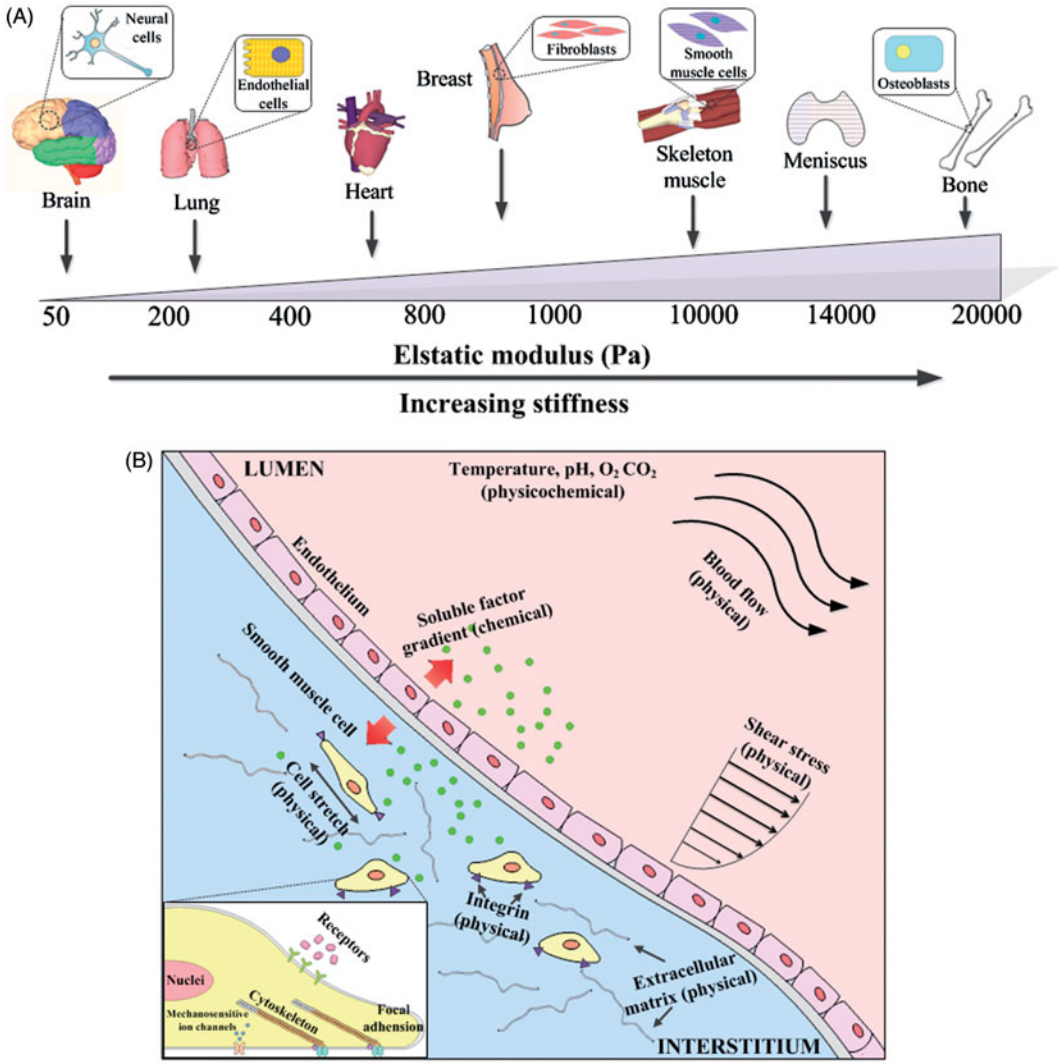

Figure 1. Schematics of cellular gradient microenvironment in native tissues. (A) Mechanical properties of various tissues and cells *in vivo*. (B) Cellular microenvironment consists of physical, biochemical and physicochemical gradient factors. For instance, the endothelium that lines blood vessels is exposed to hemodynamic shear stress gradients (external physical force) that stimulate biochemical response, releasing nitric oxide (NO) with gradients. NO diffuses to neighboring smooth muscle cells (SMCs), where it regulates cell contraction and relaxation.

at different times. Studies are underway to understand how chemical gradient signals are converted into dynamic spatial gene expression patterns, for instance, in neural tube patterning (Kutejova et al., 2009). Incorporating temporal gradients into synthetic ECM could enhance gene expression, cellular migration and cellular recruitment and therefore functional tissue generation *in vitro*.

Engineering cellular microenvironment with various gradients *in vitro*

Various approaches have been developed to engineer cellular microenvironment incorporating various spatiotemporal gradients on 2D surfaces or in 3D geometry. These can be achieved using biomaterials with physical and chemical gradients. In this section, we will highlight various physical and chemical gradients generated with cytocompatible hydrogels for studying and regulating cellular behaviors. We classify the investigations to 2D and 3D depending on whether cells are seeded on hydrogel surface or encapsulated in hydrogels.

Engineering 2D cellular gradient microenvironment

Stiffness gradients

The most common mechanical cues that cells experience *in vivo* is the stiffness gradients of ECM, ranging from ~0.1 kPa in soft tissues like brain tissues to ~20 kPa in hard tissues like bones (Huang et al., 2012). The stiffness here is characterized by the elastic modulus of tissues, reflected as the slope of linear portion of stress–strain curves of the loading tissues. Various *in vitro* studies have shown that cellular behaviors including proliferation, migration, cytoskeletal organization and differentiation can be regulated by changing the stiffness gradients of the substrates (Mason et al., 2013; Pathak & Kumar, 2012). For instance, fibroblasts seeded on hydrogels will move from soft regions of the hydrogel to the hard ones which are formed by a gradient acrylamide and bis-acrylamide with elastic modulus ranging from 140 to 300 kdyn/cm^2 (Lo et al., 2000). Furthermore, differentiation of stem cells can also be regulated on the hydrogel surface composed of spatiotemporal stiffness gradients (Dingal & Discher, 2014; Discher et al., 2005).

Mesenchymal stem cells (MSCs) cultured up to 21 days on a hydrogel containing a physiological gradient of 1.0 ± 0.1 kPa/mm undergo directed migration, or durotaxis, up stiffness gradients rather than remain stationary (Tse & Engler, 2011). Temporal assessment of morphology and differentiation markers also indicated that MSCs migrate to stiffer matrix and then differentiate into a more contractile myogenic phenotype. These observations emphasize the importance of ECM stiffness properties as fundamental regulators of stem cell fate and demonstrate that variation in these properties can have a profound influence on stem cell behavior. Therefore, it is necessary to engineer cellular microenvironment with stiffness gradients for investigating cellular physiological process *in vitro*.

A variety of approaches have been utilized to generate hydrogels with stiffness gradients including microfluidics, photolithography and micropattern (Cheung et al., 2009; Discher et al., 2005; Isenberg et al., 2009). For example, a microfluidic device composed of microchannels (20 mm in length and 500 μm in width) has been developed to generate stiffness-gradient hydrogels. In this method, two kinds of hydrogels (gelatin and hyaluronic acid) were perfused in the inlet of microchannels in the opposite direction and then crosslinked at room temperature (Figure 2A). Since the advection and diffusion of solutions is a linear process, hydrogel with a stiffness cross-gradient were generated in the microchannel (from 2 kPa to 10 kPa). Smooth muscle cells were then seeded on the hydrogel surface and it was found that cells prefer low stiffness to high stiffness and a cellular attachment gradient can be formed after 2 days of culture (Du et al., 2010). Another chitosan-gelatin hydrogel containing stiffness cross-gradient properties has also been fabricated using microfluidic methods for inducing cellular spreading and migration along the stiffness gradients toward the stiffer region of hydrogel surface (known as durotaxis; He et al., 2011). Furthermore, the rate of adult skeletal-muscle stem-cell proliferation increased with increasing substrate stiffness (Boonen et al., 2009). These results demonstrate that microfluidic technique brings a broad range of methods for exposing cells to engineered stiffness gradients and studying their regulation on cell response to cell–biomaterial interface.

Photolithography approaches can be also used to fabricate hydrogels with elastic modulus gradients by exposing polymer precursors to variable amounts of UV utilizing gradient grayscale masks. For instance, the grayscale mask method generated elastic modulus gradients in poly (ethylene glycol) diacrylate hydrogels ranging from 2.5 kPa to 10 kPa over 16 mm (Wong et al., 2003). Recently, new photodegradable hydrogels make it possible to manipulate in real-time the cellular microenvironment which affected cytoskeletal organization, differentiation, cell signaling and process extension, leading to dynamic studies on cell connectivity, migration and cell–matrix interaction (Geckil et al., 2010; Kloxin et al., 2009; Xu et al., 2011a). In one study, valvular interstitial cells (VICs) were cultured on such photodegradable poly(ethylene glycol) (PEG) hydrogels exhibiting elasticity gradients in the range from 7 to 32 kPa (Kloxin et al., 2010). By the third day of culture, pronounced smooth muscle actin (αSMA) stress fibers were observed indicating significant myofibroblast differentiation of the seeded VICs in the direction of higher elasticity modulus. The hydrogel sheet was then irradiated *in situ* to reduce its elastic modulus to 7 kPa. By the fifth day of culture, VICs no longer exhibited detectable αSMA in their cytoskeleton, indicating cytoskeletal reorganization and VIC deactivation from the myofibroblast phenotype. However, a major challenge in such studies is separating the effects of matrix stiffness from those of ligand density. Thus, development of hydrogel systems in which matrix stiffness and ligand density can be independently controlled is still needed.

Another approach to fabricate stiffness gradient gels is micropattern technique, which allows the generation of large stiffness gradients across the material surface. Recently, a novel material has been developed for high precision and decoupled control of the ECM pattern and local apparent stiffness gradients (Tseng & Di Carlo, 2014). Specifically, a structural backbone region composed of a high aspect-ratio and photopatterned KMPR (exoy) rein was covalently grafted on a soft (65:1) polydimethylsiloxane (PDMS) layer by oxygen plasma and subsequent silanization with allyl groups (Figure 2B). The stiffness of PDMS surface ranging from 1 kPa to 16 kPa and the local stiffness control at sub-cellular locations across a single patterned cell was used for studying how a cell integrated disparate mechanical signals to arrive at an overall response. When the cells were patterned on X, square and I ECM patterns along with PDMS substrate stiffness gradients, the cellular actin cytoskeleton has been found to polarize in order to match the underlying substrate. Hydrogels incorporating stiffness gradients, which are fabricated by photolithography, recently are also used for studying human mesenchymal stem cell (hMSC) memory functions (Yang et al., 2014). Researchers have cultured hMSCs on soft poly (ethylene glycol) hydrogels with altered stiffness in different culture periods. the activation of the Yes-associated protein and transcriptional coactivator with PDZ binding domain as well as the pre-osteogenic transcription factor RUNX2 in human mesenchymal stem cells cultured on soft PEG hydrogels (2 kPa) depended on previous culture time on stiff tissue culture polystyrene (3 GPa). These results suggest a temporal role in cellular mechanotransduction that involves the history of a cellular microenvironment, and hydrogel stiffness significantly affects a host of cell behaviors including adhesion, migration, proliferation, and differentiation. Such fabrication approaches can be utilized for engineering various biomimetic hydrogels incorporating controlled spatial and temporal stiffness gradients and enable the subsequent study of their effects on cell behavior.

Stress/strain gradients

Stress/strain gradients in tissues result from various physiological processes *in vivo*, such as inflammation, drainage toward lymphatics, muscle contraction, which drive fluid flow stress through ECM and locally elevated stresses caused by leaky microvessels and tumor growth (Douville et al., 2011; Jang et al., 2011). Several approaches have been developed recently to generate stress/strain gradients on cell seeding substrates (Dolan et al., 2013; Nemir et al., 2010; Simmons et al., 2011). For example, a microfluidic cell culture system has been developed to apply a hydrostatic flow

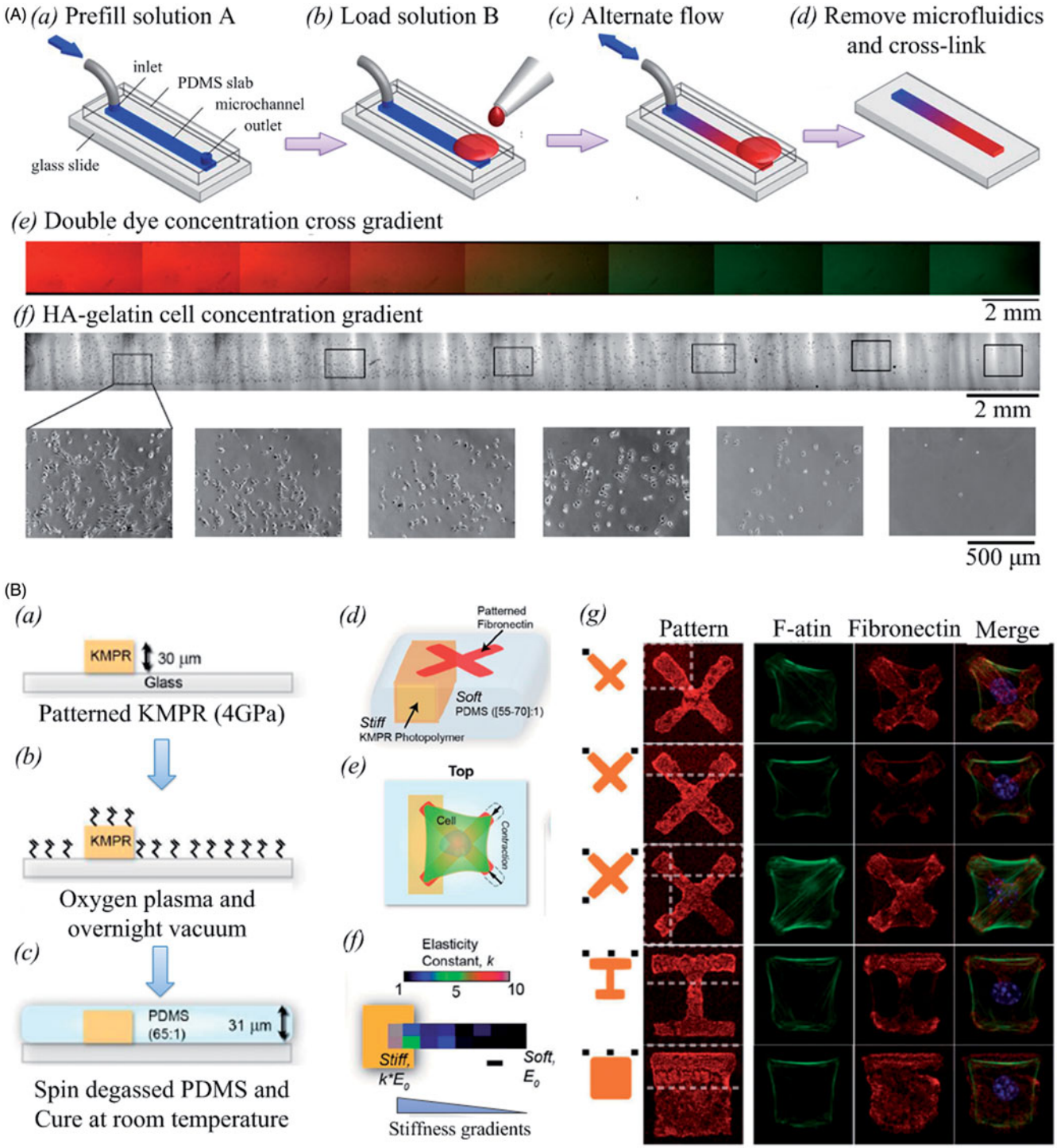

Figure 2. Approaches for engineering 2D cellular microenvironment with programmable gradients. (A) Protocol for generating gradient hydrogels with convection. (*a*) The channel is prefilled with solution A. From Du et al. (2010). (*b*) Solution B is loaded into the opposing port. (*c*) The flow is pumped back and forth in the channel until the gradient is the desired length. (*d*) The microfluidic system is removed and the gradient in pre-polymer is cross-linked. (*e*) Fluorescence images of dye gradients generated in hydrogels. (*f*) Phase images (top: lower magnification; bottom: higher magnification) of smooth muscle cells cultured on a HA–gelatin cross-gradient hydrogel. (B) Generation of stiffness gradients on PDMS. From Tseng & Di Carlo (2014). (*a–c*) Fabrication protocols for PDMS substrate with gradient resin patterning. (*d–f*) Complex substrates encoding large stiffness gradients with aligned extracellular matrix patterning. (*g*) Single-cell actin cytoskeleton polarizes to match the stiffness gradients of the underlying matrix. Shown are typical and average cell actin substructure for X, I and square fibronectin shapes with varying stiffness stimuli from the substrate.

stress gradient across collagen gel region, in which breast cancer cells were seeded on the gels (Polacheck et al., 2011). It was demonstrated that the flow stress gradient significantly affects the directional bias of tumor cell migration and the migratory response depends on both the flow rate and the cellular density. Another example is the study of C2C12 cellular orientation by using an array consisting of integrated strain gradients ranging from 2% to 6% (Simmons et al., 2011). The results showed that the actin stress fibers of C2C12 cells realigned with a circumferential preference at

higher strain levels demonstrating the usefulness of the integrated strain arrays for mechanotransduction studies. Future biological studies could utilize this platform to examine the role of cell–matrix junction proteins in transmitting strain from the substrate to the cells and cell–cell junction proteins in transmitting strain to the neighboring cells.

Porosity and pore size gradients

Porosity and pore size of ECM have an important effect on cell viability and affinity by regulating cell binding, intercellular signaling, movement and the effective nutrients and metabolites transfer (Thomas et al., 2013). Various scaffolds fabricated with programmable porosity and pore size gradients have been utilized for tissue remodeling and regenerative medicine (Rockwood et al., 2011; Wu et al., 2011; Zhao et al., 2011). For instance, biocompatible and biodegradable bone scaffolds with pore size ranging from 350 to 410 μm can significantly enhance the growth of osteoblasts, while differentiation of osteoblasts and bone formation occurs most rapidly in pores with sizes of 260–320 μm (Oh et al., 2007). In another study, poly(D,L-lactide) scaffolds were fabricated using stereolithography with a gradient in porosity (35–85%) and pore size (250–500 μm; Melchels et al., 2011). Human articular chondrocytes were seeded into the scaffolds and the alive cells mainly distributed in the region with pore sizes ranging from 400 μm to 450 μm after 7 days of culture, suggesting the proper pore architectures for cell growth and transport of nutrients and metabolites. In addition, spatiotemporal gradients of microstructure in hydrogel scaffold have also been developed for drug delivery and tissue regeneration applications (Li et al., 2009). For instance, chitosan, instead of inorganic species, was utilized as building blocks to construct a chitosan hydrogel with gradient concentric multilayers, which was inspired by the Liesegang ring phenomenon (Li et al., 2011b). In this study, gradient concentric multilayered structure in hydrogel was formed through a protonated chitosan/NaOH reaction system by alternate soaking in water and NaOH solution. The total layer number and the average width of concentric multilayer in chitosan hydrogel are 45 and 105.9 ± 84.5 μm, respectively. The interspaces between concentric multilayers and pores of the wall layer can be utilized to upload different drugs, such as hydrophobic drugs in the interspace and water-soluble drugs in the wall layer. Another example is the fabrication of chitosan hydrogel within gradient distribution of hydroxyapatites for bone tissue engineering (Li et al., 2011a). Based on the chelation properties of the amino group, a chitosan hydrogel obtained via physical crosslinking was used as template for ion assembly. Gradient structural bone-like apatite induced by chitosan hydrogel was achieved via ion assembly within a few hours under ambient conditions, in which amino groups of chitosan acted as anchors between chitosan and apatite nucleation. These results suggest that hydrogel scaffolds incorporated with microstructural gradients (e.g. porosity), which are inherent *in vivo* such as the composition in bone varies from compact (porosity 10–30%) to spongy (porosity 30–80%; Sant et al., 2010), could be explored to fabricate bone-like tissue constructs *in vitro* especially for tissue remodeling and regenerative medicine investigations.

In addition to biomimetic hydrogel scaffolds, poly(ethylene glycol) dimethacrylate (PEGDMA) hydrogels consist of porosity gradients have also been utilized in microfluidic electrophoresis applications such as on chip protein sizing (Lo et al., 2008). Both linear and nonlinear porosity gradients (60–95%) were created through the diffusion, microfluidics and photolithography. Cell spreading and proliferation behaviors were enhanced when cells were encapsulated in hydrogels with porosity ranging from 70% to 85%. Although using porosity and pore size to regulate cellular behaviors have been studied for several decades, it is still an active research area due to the emergence of new synthesized biomimetic materials and fabrication approaches such as microfluidics, stereolithography and photolithography.

Chemical gradients in hydrogels

Chemical gradients in native cellular microenvironment can induce the cellular processes such as angiogenesis and chemotaxis (Fischer-Colbrie et al., 2005). Various gradients of chemical cues such as soluble growth factors (e.g. growth factors, toxins, chemokines and chemoattractants) and proteins (e.g. adhesion ligands and metalloproteinases) have been incorporated with hydrogels to investigate cellular adhesion, differentiation, proliferation and migration *in vitro* (Huh et al., 2011; Young & Beebe, 2010; Zelzer et al., 2008). For instance, a gradient of angiogenic vascular endothelial growth factors distributed in collagen scaffolds was generated by diffusing growth factors in scaffolds for 2 h (Chung et al., 2009). Sprouting structures was formed after seeding endothelial cells on collagen scaffolds for 2 days, while those in a blank scaffold without growth factor gradients were less migrated. In another study, a gradient of cell-adhesion ligands (Arg-Gly-Asp-Ser) (RGD) of poly(ethylene glycol) dimethacrylate hydrogels was generated using microfluidic methods. Specifically, a high concentration (3.8 mg/ml) of ligand solutions was pumped into a channel embedded with a ligand solution at a lower concentration (0.5 mg/ml). The fabricated gradient hydrogels were used to study the cell–material interactions (He et al., 2010). Using this method, linear, exponential and other geometrical gradients could be potentially achieved through different microfluidic designs.

In a recent study, endogenous gradients of chemokine CCL21 within mouse skin have been identified and the guidance of dendritic cells toward lymphatic vessels was achieved (Weber et al., 2013). Investigators utilized mature dendritic cells (DCs) that migrate from the dermal intertitium into afferent lymphatic vessels (LVs) as a model platform for studying chemokine gradient effect. Quantitative imaging reveals depots of CCL21 within DCs and steeply decaying gradients within the perilymphatic interstitium. These gradients match the migratory patterns of the DCs, which directionally approach vessels from a distance of up to 90 μm. Interstitial CCL21 is immobilized to heparan sulfates, and its experimental delocalization or swamping the endogenous gradients abolishes directed migration. These findings functionally establish the concept of haptotaxis, directed the migration along the immobilized gradients in tissues.

Hydrogels incorporated with spatiotemporal chemical gradients have also been used for guiding stem cell

differentiation (Huh et al., 2011; Lutolf et al., 2009). One predominant approach to fabricate chemical gradient hydrogels is based on microfluidic systems, which are able to generate precise concentration mixtures and allow real-time observation of cells (Liu et al., 2014; Xu et al., 2011b). For instance, proliferation and differentiation of human neural stem cells (hNSCs) were observed while cells were cultured in a microfluidic device applying with a continuous concentration gradient of mixture containing epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF2; Chung et al., 2005). hNSCs in the low growth factor compartment showed increased astrocyte differentiation compared to cells in the high growth factor compartment. These results indicate that such microfluidic devices could be utilized for engineering tissue constructs composed of cell type gradients (e.g. neurons and neuroepithelial cells in pallium) and tissue regeneration *in vitro*. Other examples of chemical gradients include the morphogen gradients which control stem cell differentiation during embryogenesis and the chemical gradients subsequently guide axonal growth in nervous tissue (Baumann, 2014; Kicheva et al., 2012). Chemical gradients have also been generated in biocompatible hydrogel scaffolds for inducing stem cell differentiation. For example, gradient distributions of microspheres with recombinant insulin-like growth factor (rhIGF-I) and human bone morphogenic protein 2 (rhBMP-2) in silk and alginate scaffolds have been utilized to study the osteochondrogenic differentiation of hMSCs (Wang et al., 2009). The results showed that hMSCs cultured on silk scaffolds exhibited chondrogenic (cartilage-like) and osteogenic (bone-like) differentiation along concentration gradients of rhBMP-2 and even the cross-gradients of rhBMP-2/rhIGF-I. Differentiation of hMSCs did not follow the gradients of rhBMP-2 and rhIGF-I in the alginate scaffolds, likely due to the range and slope of the gradients were too small or the rapid diffusion of growth factors degraded the gradients too quickly. This novel technique offers control over growth factor distribution and temporal release within hydrogels.

Hydrogels within chemical gradients find widespread applications as facile and rapid screening platforms for investigating multiple cellular processes and even guiding stem cell fate. Such gradient hydrogels are now being integrated with tools from developmental biology and genetics to fabricate more complex and biologically relevant tissues *in vitro* (Sant et al., 2010).

Engineering 3D cellular gradient microenvironment

Most existing engineered gradients incorporated with hydrogels or biomimetic scaffolds are grafted onto 2D surfaces of materials. Few literatures have reported on gradients generated in 3D microenvironment and even few studies exist on the behavior of cells encapsulated in such 3D gradient microenvironments. One such study used a diffusion source-sink device to generate gradients of density of cells encapsulated in hydrogels (Frisk et al., 2008). Following exposure to chemical and physical gradients, biological samples are generally analyzed to characterize their response to surrounding 3D microenvironments. The analysis is often complicated for samples encapsulated in 3D matrices which may obscure the sample. In this study, the use of reversible hydrogels could address this challenge, which allows encapsulated cells to be washed out for further analysis following the experiment. Recently, several studies have focused on the cellular behavior under both physical and chemical gradients generated in 3D cellular microenvironment. For instance, a simple method to fabricate 3D biocompatible hydrogels with a range of static strain gradients has been reported (Hsieh et al., 2014). In this study, NIH 3T3 cells were suspended in a photocrosslinkable gelatin methacrylate (GelMA) hydrogel precursor in a glass-supported microfluidic chip and a convex flexible PDMS membrane was employed on the top of the chip (Figure 3A). Following UV crosslinking through a photomask with a concentric circular pattern, the cell-laden hydrogels were formed with a height gradient from the center (maximum) to the boundary (minimum). When the convex PDMS membrane retracted back to a flat surface, it applied compressive gradient forces on the cell-laden hydrogels and the compressive strain gradients ranging from 10% to 80% generated due to the various designed cross-sectional areas. The concentric circular hydrogel patterns confined the direction of hydrogel elongation, and the compressive strain on the hydrogel therefore resulted in an elongation stretch in the radial direction to guide cell alignment. The results have shown that NIH 3T3 cells have a radial alignment encapsulated in 5% w/v GelMA hydrogels applied with >50% compressive strain, while those encapsulated in hydrogels applied with 5–15% strain exhibited a circle alignment after 5 days of culture. These results suggest that alignment behavior of cells seeded on 2D substrate with various strain features, which predominantly align perpendicular to the direction of principal strain direction (known as stretch-avoidance or strain-avoidance; Li et al., 2014), is totally different from the situation in 3D.

In another study, linear physical gradients within a photocrosslinkable alginate hydrogel was fabricated for regulating behaviors of hMSCs encapsulated in alginate hydrogels after 2 weeks of culture (Jeon et al., 2013). Increasing the degree of the alginate methacrylation increases resultant hydrogel crosslinking density, which has been previously demonstrated to increase the compressive modulus of hydrogels (Huang et al., 2012). High cell viability (>85%) was observed when cells were encapsulated in hydrogels with linear gradients ranging from 10 kPa to 150 kPa within centimeter scale. These results suggest that the stiffness and hydrogel crosslinking density of hydrogel matrix are all important in guiding cell viability and growth, which can be enhanced in a matrix microenvironment that permits the diffusion of both nutrients and wastes (Huang et al., 2011). Such processes are mainly influenced by the permeability and porosity of hydrogel matrix, which can be easily regulated by changing hydrogel crosslinking density (Asthana & Kisaalita, 2013). They subsequently fabricated alginate hydrogel with gradients of RGD peptides (2–18 mg/g). A higher number of alive cells was observed in regions of higher RGD concentration compared with regions of lower RGD concentrations as demonstrated by the live/dead staining (Figure 3B). Hydrogels incorporated with heparin-binding growth factors BMP-2 (a potent osteogenic growth factor) and TGF-β1

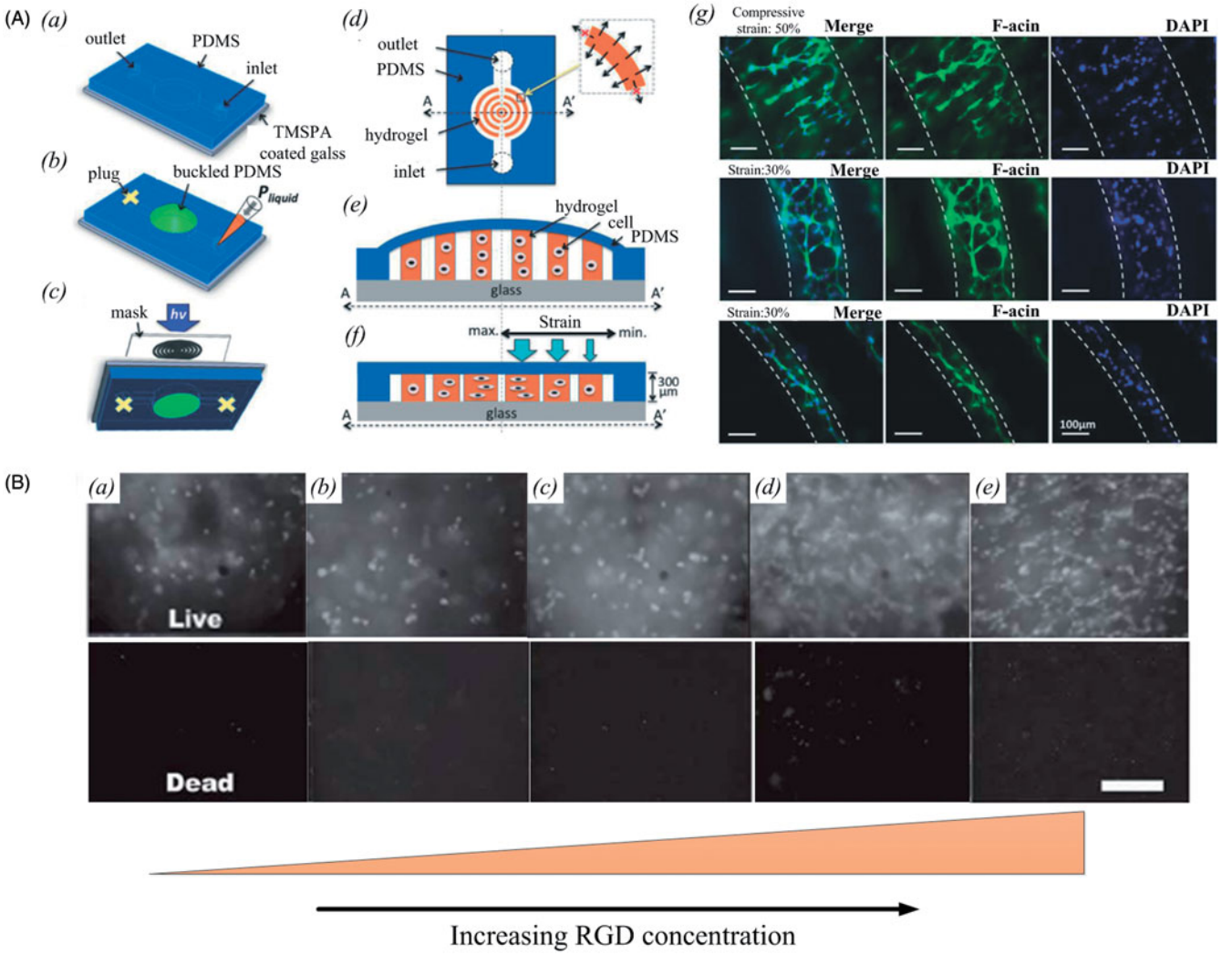

Figure 3. Approaches for engineering 3D cellular microenvironment with programmable gradients. (A) (*a–f*) Schematic of the fabrication processes for gradient strain hydrogels in a microfluidic chip. From Hsieh et al. (2014). (*a*) PDMS was bonded to the TMSPMA-coated glass. (*b*) A PDMS plug was used to block the outlet and application of liquid pressure to obtain convex PDMS deformation. (*c*) After UV patterning through the glass bottom, the uncrosslinked hydrogel with cells was washed out and (*d*) concentric circles were formed in the chip with (*e*) a height gradient along the radius. (*f*) The inlet and outlet were unplugged, and the PDMS membrane became flat and applied gradient force on the cell-encapsulated hydrogels. (*g*) Actin–DAPI staining images and the alignment frequency of the NIH/3T3 cells in the gradient chips at day 3. (B) Characterization of RGD gradient alginate hydrogels and response of human marrow stromal cells in RGD gradient hydrogels. From Jeon et al. (2013).

(a chondrogenic growth factor) were also fabricated. hMSCs encapsulated in such gradient hydrogels underwent osteogenic and chondrogenic differentiation during 2-week culture and the levels of alkaline phosphatase activity and glycosaminoglycan secretion were enhanced with increasing growth factor concentration. These results indicated that such biocompatible alginate hydrogels, incorporated with chemical (e.g. growth factor) and physical gradients (e.g. stiffness), could be a valuable tool to study stem cell fate in 3D and guide tissue regeneration. Hydrogels containing gradients of micro- and nanoparticle-immobilized drugs have also been generated for high-throughput drug screening applications (Ahadian et al., 2014). Briefly, dielectrophoresis was used to manipulate gold micro- and nano-particles of different sizes within GelMA hydrogels in a rapid and facile way to generate 3D gradients in a micro chamber. The high-throughput release properties of 6-hydroxydopamine from a gold microparticle gradient and its subsequent effect on the viability of C2C12 myoblasts were then studied. The results showed that the release of 6-hydroxydopamine induced the death of C2C12 myoblasts and indicated a novel platform for high-throughput screening research from fundamental biology to cell and tissue engineering and drug discovery applications (Breslin & O'Driscoll, 2013).

By appropriate choice of input solutions and cross-linking methods, the fabrication methods outlined above can generate not only chemical gradients of soluble factors, particles, proteins, but also cell density gradients with spatiotemporal distribution. In addition, combining the concentration gradient protocols with crosslinking gradient protocols can produce gradient hydrogels with superposed physical and chemical gradients. Applications include toxin, protein or chemoattractant gradients within hydrogels encapsulating uniform concentrations of cells or a gradient in cell concentration within a 3D hydrogel with a constant nutrient source (Anderson et al., 2011; Haycock, 2011; Tibbitt &

Anseth, 2009). A variety of gradient hydrogels have been synthesized, such as concentration gradients in the cell-adhesion ligand (RGD) within a poly(ethylene glycol) diacrylate hydrogel (Seidi et al., 2011), molecular chain length gradients (from 5 to 20 kDa) photo-cross-linked to form a poly(ethylene glycol) diacrylate hydrogel with a gradient in elastic modulus (Nemir et al., 2010), collagen fibril density gradients (Du et al., 2010), and polyacrylamide hydrogels with gradients in elastic modulus or pore size (Orsi et al., 2014). Although increasing studies have focused on controlling cellular behaviors within such hydrogels composed of 3D spatiotemporal gradients, several challenges such as short-term cell culture due to the large sample size, development of precise characterization for behaviors of encapsulated cells and simple and facile gradient fabrication, still need to be addressed.

Engineering cellular microenvironment with gradients for biofabrication of *in vitro* disease models

During the last decades, researchers have found that gradient physical and chemical cues in cellular microenvironment play an important role in tumor formation and progression (Butcher et al., 2009; Paszek et al., 2005). For instance, tissue stiffness gradients have been demonstrated for promoting malignant behavior through regulating cellular growth, morphogenesis and integrin adhesions (Saez et al., 2007). A typical study has demonstrated that epithelial tumors are formed with an altered tissue stiffness gradient which reflects differences in rheology and increased cell-generated forces in the transformed cells (Paszek et al., 2005). In this study, human mammary epithelial cells (MECs) were seeded on polyacrylamide (PA) hydrogels with calibrated elastic modules ranging from 0.15 to 5 kPa. After 20 days of culture, MECs on a compliant PA gel formed growth-arrested acini with cell–cell localized β-catenin, basally polarized β4 integrin and apical–lateral cortical actin and assembled an endogenous laminin-5. Increasing matrix rigidity (from 0.1–0.4 to 0.6–1 kPa) progressively increased EGF-dependent ERK activation and colony size, hindered lumen formation and perturbed tissue polarity (absence of β-catenin/E-cadherin colocalization and triton-extractable β-catenin, disrupted localization of β4 integrin and laminin-5 and progressive filling of the spheroid lumens (Figure 4A). Intriguingly, actin stress fibers of MECs were not observed in any of these cultures until matrix rigidity increased to 5 kPa, toward that measured in tumors ($\approx$4 kPa) and in MECs on highly rigid 2D substrata. Recently, stiffness gradients (ranging from 5 to 50 Pa) were generated by edge effects at the interface of a rigid support with a soft Matrigel on OSU-2 cell migration in 3D (Rao et al., 2012). The results have shown that tumor cells in hydrogels with high elastic modulus (45–50 kPa) displayed highly organized stress fiber formation, which enables cells to generate traction forces for migration, in contrast to the cells in hydrogels with a lower stiffness. Another physical gradient that is crucial for tumor uptake and the penetration of macromolecules is vascular pressure gradient, which is induced by an enhanced permeability and retention such as leakiness of tumor vasculature and reduced lymphatic drainage (Marcucci & Corti, 2012). A recent study has noted that flow stress gradient has a significantly effect on the directional bias of breast cancer cell migration and the migratory response depends on both flow rate and cellular density (Polacheck et al., 2011).

Another tumor cellular process, migration, can also be modulated by chemical gradients such as growth factors and chemokines. A variety of microfluidic chips have been developed to study the effects of growth factor gradients on the tumor cell migration, tumor-endothelial cell interactions and tumor–stromal cell interactions (Huang et al., 2009; Song et al., 2009). For instance, an *in vitro* 3D microfluidic model of a tumor-vascular interface has been designed to integrate the live imaging, precise control of growth factor gradients and endothelial barrier measurements (Zervantonakis et al., 2012). In this study, microfluidic chips were used to explore the relationship between fibrosarcoma cell migration and the gradients of epidermal growth factors (EGFs; Figure 4B). The results have shown that tumor cells encapsulated in collagen hydrogels have a high migration speed (30 μm/h) with an applied high concentration of EGFs (250 pg/ml), in contrast to the cells with low speed (5 μm/h) in a low EGF concentration (3 pg/ml).

Tumor cells may also utilize chemokine gradients as guidance cues to enter lymphatic vessels through intercellular openings between endothelial cell junctions or, possibly, by inducing larger discontinuities in the endothelial cell layer. For instance, breast cancer cells and primary breast tumours express patterns of chemokine receptors that ''match'' chemokines specifically expressed in organs to which these cancers commonly metastasize, namely the lymph nodes, bone marrow, lung, and the liver. Furthermore, blocking one of the chemokine receptors was found to inhibit metastasis of breast cancer cells in experimental animal models (Gout & Huot, 2008; Muller et al., 2001). Similarly, the chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases in the liver. In summary, various physical and chemical gradients play an important role in guiding tumor cellular behavior and tumor progressions and more investigations are needed to engineer tumor cellular microenvironment with programmable gradients for the fabrication of *in vitro* disease models for clinical and fundamental research (Table 1).

Challenges and future prospective

Engineering 2D and 3D cellular microenvironment *in vitro* with spatiotemporal physical and chemical gradients can significantly enhance the understanding of cellular responses to surrounding ECM and other cells. Various approaches such as microfluidic, photolithography and micropattern have been developed to fabricate hydrogels and scaffolds incorporating with physical (e.g. stiffness, stress/strain distribution, porosity and pore size) and chemical (e.g. growth factors, chemokines, transcription factors and cytokines) gradients for regulating cellular processes (Annabi et al., 2013; Gurkan et al., 2013; Huang et al., 2011). However, most of the existing studies on cell-material interactions mainly focus on 2D surfaces of gradient hydrogels. Studies are beginning to appear on the behaviors of cell encapsulated in 3D hydrogels, which more

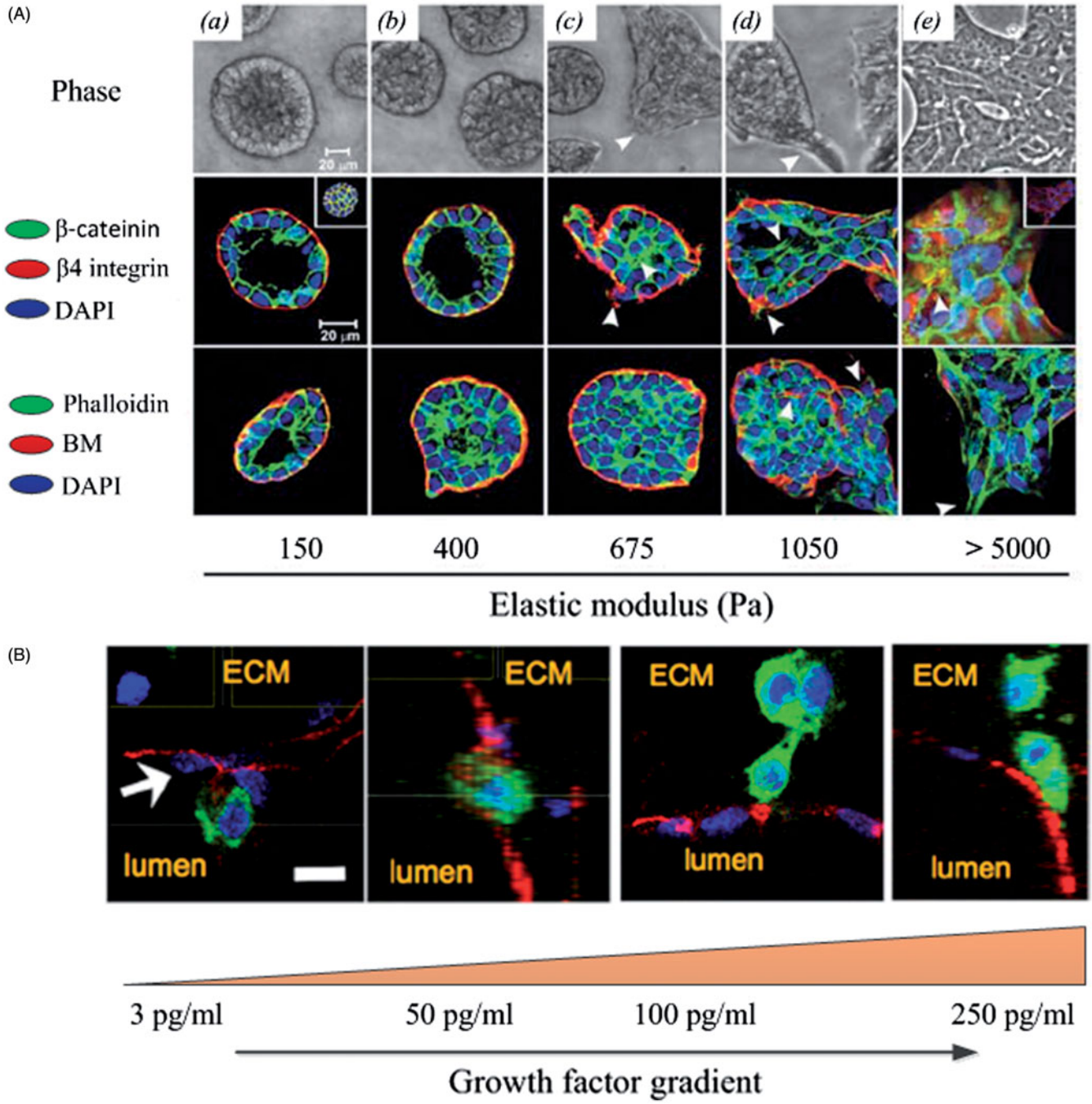

Figure 4. Engineered cellular microenvironment with gradients for *in vitro* disease model applications. (A) Phase contrast microscopy and confocal images for MEC colonies on 3D BM gels composed of stiffness gradients (150–5000 Pa), showing colony morphology after 20 days (top); β-catenin before and after triton extraction (inside colony), costained with β4 integrin (surrounding the colony) or E-cadherin (inset), costained with LN-5 and nuclei. From Paszek et al. (2005). (B) Macrophages enable tumor cell intravasation under gradients of epidermal growth factors. Scale bar, 30 μm. From Zervantonakis et al. (2012).

closely mimic the native situation. More importantly, characterizing the response of cells encapsulated in 3D matrices is also more challenging than cells seeded on 2D surfaces.

Ongoing and future study is tasked with improving gradient generation methods and integrating them with material synthesis, cell manipulation, genetics and other biological fields to regenerate the complexity of *in vivo* tissues and organs. Developed approaches of gradient distribution control and stability must be developed and integrated into tissue engineering and regenerative medicine applications. In addition, improved and standardized methods are also needed to properly correlate cell response to both 2D and 3D applied gradient and biological cues. Soon, material synthesis techniques should be sufficiently advanced to create both physiologically and pathologically relevant gradient materials to investigate complex spatiotemporal phenomena such as morphogenesis of normal and diseased tissues (Pampaloni et al., 2007; Tibbitt & Anseth, 2009). Smart biomaterials incorporating various chemical and physical gradient cues inside hydrogels could be subsequently generated for the regeneration of complex and high-order

Table 1. Different approaches for fabricating cellular gradients *in vitro*.

Gradients	Approaches	Spatiotemporal resolution	Fabricating range	Cellular application	References
Stiffness	Microfluidics, photolithography and micropattern	27–50 μm	0.05–30 kPa	Fibroblast/osteoblast/ human mesenchymal stem cell/smooth muscle cell/neurons	Cheung et al. (2009), Discher et al. (2005), Mason et al. (2013), Pathak & Kumar (2012)
Stress/strain	Microfluidics, photolithography	100–500 μm	0.05–20 kPa/2–160%	Smooth muscle cell/cardiacmyocyte/fibroblast	Dolan et al. (2013), Lee et al. (2008), Nemir et al. (2010), Simmons et al. (2011)
Porosity/Pore size	Electrospinning, phase separation, freeze-drying, stereolithography and Self-assembly	10–30 μm	5–90%/5 nm–500 μm	Fibroblast/osteoblast/ human mesenchymal stem cell/chondrocyte/ neurons	Dubruel et al. (2007), Fischer-Colbrie et al. (2005), Lo et al. (2008), Oh et al. (2007), Young & Beebe (2010)
Biomolecules	Diffusion and photolithography	200–300 μm	–	Fibroblast/endothelial cell/ mesenchymal stem cell	He et al. (2010), Huang et al. (2011), Huh et al. (2011), Weber et al. (2013)

grafting templates, which can simulate the cellular and structural characteristics of both physiological and pathological tissues.

In addition, methods to fabricate hydrogels with combined multiple physical and chemical gradients are also required to properly correlate cellular response to the applied gradient and biological cues. Meanwhile, all such methods must be made more accessible and user-friendly to a broad range of researchers. With the rapid advances in the development of manufacturing techniques and novel-material synthetic techniques, such engineered functional biomaterials incorporating various gradient cues could be then utilized for the regeneration of complex tissues and fabrication *in vitro* disease models.

Declaration of interest

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