Review Article

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Physical cues of biomaterials guide stem cell fate of differentiation: The effect of elasticity of cell culture biomaterials

Abstract: It is widely accepted that stem cells directly sense the elasticity of two-dimensional (2-D) substrates and differentiate into a distinct cell type dependent on the substrate elasticity (*direct-sensing differentiation*: soft and hard substrates promote differentiation into soft and hard tissue lineage, respectively). Biologically, native extracellular matrices (ECMs) are constantly remodeled throughout the life of individuals, which inadvertently introduce changes of mechanical properties. Therefore, *direct-sensing differentiation* might not fully take into account the responses of stem cells in the actual ECMs microenvironment. Recent investigations in three-dimensional (3-D) cell culture environment suggested the inconsistency of *direct-sensing differentiation*. Stem cells specifically differentiate not only by sensing the elasticity of materials but also by considering the cellular traction exerted to reorganize the matrices and the matrices deformation. This paper aims to expand further how the cells incorporate the elasticity cues and traction-mediated deformation in final differentiation fates of stem cells. To achieve the aim, we introduced an empirical model based on the investigations in 3-D cell culture environment. The empirical model would serve as a useful framework for future studies intended to investigate the relationship of traction-mediated deformation and commitment of stem cells for variety of tissue lineage in 2-D or 3-D cell culture environment.

Keywords: mechanosensing, stem cell differentiation, traction-mediated deformation, 2-D cell culture, 3-D cell culture

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1 Introduction

Stem cells are the potential agent to treat the degenerative disease or heavy tissue injury, owing to the capability of self-renewal [1–3] and the capacity to differentiate into functionally mature cells [4–6]. Several types of stem cell, such as embryonic stem cells (ESCs) and induced-pluripotent stem cells (iPSCs), are pluripotent, and are able to differentiate into any cell types derived from three germ layers: ectoderm (nerves and epidermal tissue), mesoderm (bone, muscle, cartilage), and endoderm (functional organ) [7–10]. Other types of stem cell, such as mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs), are more limited in the differentiation capacity with limited life span, in which MSCs are only able to differentiate into cells of mesodermal origin (fat, bone, muscle, cartilage) [11–15]. Regardless of the differentiation capacity, it is clear that stem cells are useful in medical applications, and an ideal culture system should be established to effectively expand the stem cell number and guide the differentiation of stem cells.

In the past decade, researchers have focused on improving the efficiency of stem cell differentiation by tuning the physical properties of biomaterials (elasticity [16, 17], surface topography [18, 19], architecture [20, 21]) in culturing. The biological cues (growth factors, hormones, peptides) conventionally used to differentiate the stem cells have been widely investigated [22–25], thus it is difficult to discover novel biological cues to increase efficiency of stem cell differentiation [10].

Elasticity, as one of the physical cues, was recently shown to be able to selectively induce the differentiation of stem cells into the specific lineage [8, 26, 27]. MSCs were specifically differentiated into neuron, muscle, and bone cells when MSCs were cultured on two-dimensional (2-D) substrates with elastic modulus (E) approaching the elas-

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ticity of native tissues (brain (E~0.1-1 kPa), muscle (E~ 8-17 kPa), and bones (E~40 kPa)). Such idea of elasticity-driven commitment of stem cells was replicated for other types of stem cell, such as ESCs [8, 28], or non-bone marrow-derived MSCs [29, 30]. In these studies [8, 26], the substrates made of synthetic polymers (*e.g.*, poly(acrylamide) (PA), poly(ϵ-caprolactone)) were used, which show pure elasticity.However, the cells in native tissue environment reside in extracellular matrices (ECMs) which are non-elastic and constantly remodeled (resorbed and rebuilt) [31, 32]. Therefore, the cell responses observed on 2-D elastic materials may not reflect the true mechanism on how the cells detect the physical cues of cell culture substrates in actual setting.

Unlike 2-D substrates, elasticity of three-dimensional (3-D) matrices indirectly regulates the stem cell differentiation by allowing or preventing the cellular traction-mediated deformation of matrices [31, 33, 34]. Khetan *et al.* demonstrated thatif the hydrogels were stable enough to prevent mechanical reorganization of matrices by stem cells, then the stem cells consistently adopted differentiation fate into adipocytes (fat tissue) in all range of hydrogel elasticity (~4-95 kPa) [33]. Introduction of degradable bonds in the hydrogels allowed cells to exert traction to the matrices, and subsequently stem cell fate shifted to osteogenic differentiation (bone tissue) [33]. Despite the apparent difference on how the elasticity of the cell culture matrices would influence stem cell fate in 2-D and 3-D environments, cellular contractility remains the main driving force for differentiation of stem cell fate [26, 33, 35].

In this review, we aim to expand further how the cells incorporate the elasticity cues and traction-mediated deformation in final differentiation decisions. Initially, key papers establishing on how the stem cells directly perceived the elasticity of 2-D environment were reviewed. Next, we discussed the interplay of elasticity and dynamic physical properties of cell culture materials in influencing the differentiation of stem cell fate in 3-D cell culture environment. In the same section, the empirical model to explain how the traction-mediated deformation would regulate the lineage specification of stem cells was proposed. We concluded how the model can be stretched to the basic research in 2-D cell culture setting and the limitation of the empirical model.

2 The specific induction of stem cell fates by elasticity cues in 2-D environment

Elasticity of 2-D substrates or 3-D matrices refers to the resistance of materials to elastic deformation, as represented by elastic modulus or Young's modulus (E, Pa = N/m^2) [33, 35, 36]. Elasticity is the inherent property of materials and used interchangeably with 'stiffness' or 'rigidity'. The term 'stiffness' in cell biology is rarely assigned to the other definition of stiffness (N/m) which refer to the resistance of component to deformation. Elasticity is also defined by the viscoelasticity parameter (*e.g.*, storage or loss modulus) [37, 38]. Moreover, it is general practice to indicate materials with high and low elastic modulus as 'stiff' and 'soft', respectively. The elasticity of 2-D substrates or 3-D matrices can be measured by atomic force microscopy (AFM) (elastic modulus) [27], compression test (elastic modulus) [39], tensile test (elastic modulus) [8], or rheometer (viscoelastic modulus) [37, 38].

The influence of elasticity cues was initially focused on the motility and contractility of cells, such as cell locomotion [40], morphology [41], or spreading area [42], and not for the differentiation of stem cells. From these studies [40–42], it was found that the elastic modulus of substrates could regulate the intracellular formation of stress fiber (F-actin), which in turn lead to the modulation of cellular traction mode and magnitude. The strong correlation between cellular traction and substrate elasticity lead to the idea of 'mechanosensing', that is, cells were able to sense the elasticity of the surrounding environment by means of generating contractile force (Figure 1(A)) [36, 40].

The results of mechanosensing were communicated back-and-forth to the intracellular signaling pathway (focal adhesion kinase- (FAK), paxillin-, vinculin-dependent pathway) yielding the dynamic cellular responses (*e.g.*, low traction at soft substrate, migration to stiff substrate) [9, 40]. The transduction of mechanical signals into intracellular signal and subsequently into cellular responses is termed as "mechanotransduction". Inhibition of cellular contractility by addition of blebbistatin (non-muscle II myosin inhibitor) [36, 42] or Y-27632 (Rho-kinase (ROCK) inhibitor) [40] strongly hindered cellular migration or focal adhesion formation, emphasizing the important role of cellular contractility in mechanotransduction.

By the similar hypothesis, elasticity of cell culture substrates was shown to selectively induce differentiation of stem cells in contractility-dependent manner (*direct-sensing differentiation*) [26]. Engler*et al.* demonstrated that
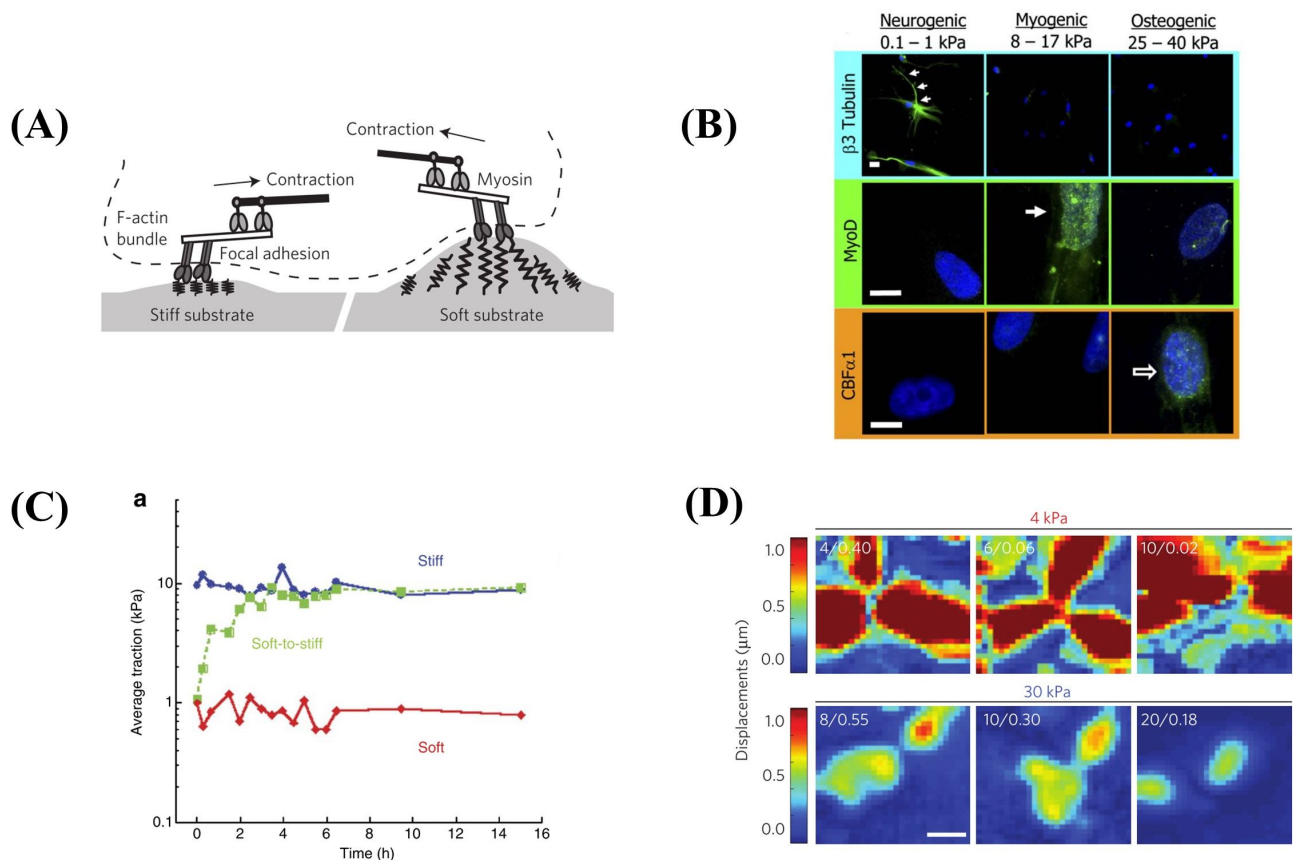
(A)

(B)

(C)

(D)

Figure 1: (A) Schematic illustration of mechanosensing by cells. **(B)** MSCs cultured on PAAm hydrogel of different stiffness (0.1-1 kPa, 8-17 kPa, 25-40 kPa) undergo differentiation correspondingly to neurogenic (nerve), myogenic (muscle), osteogenic (bone). **(C)** Magnitude of cellular traction exerted by MSCs at *soft* and *stiff* substrate. Cells gradually increase the traction when cell culture substrate is stiffened (*soft-to-stiff*) **(D)** Deformation of matrices as given by the displacement of fluorescent particles embedded in PA substrates of different stiffness (4 kPa and 30 kPa) by MSCs. Parts of figure are adapted with permission from [27] **(A)**, **(D)**, [26] **(B)**, [42] **(C)**

collagen-coated PA hydrogels with elasticity mimicking stiffness of brain (E~0.1-1 kPa), muscle (E~ 8-17 kPa), or bones (E~40 kPa) were able to selectively induce the differentiation of MSCs in the corresponding pathway; soft hydrogels at 0.1 and 1 kPa induced neurogenic differentiation, etc. (Figure 1(B)). Addition of blebbistatin blocked the elasticity-induced differentiation of MSCs, implying the important role of cellular contractility in the elasticity-driven fate specification of MSCs [26]. However, despite the strong correlation of elasticity and MSCs differentiation, the exact molecular mechanisms of mechanosensing and mechanotransduction are still elusive [43].

MSCs exert lower traction at soft substrates and higher traction at stiff substrates (Figure 1(C)) [36, 42, 44] and MSCs seemed to compensate the magnitude of these exerted traction by adjusting the internal stresses (prestress) and membrane stiffness (cortical stiffness) [26]. Based on these results, calculation of substrate deformation was conducted and it was suggested that larger defor-

mation occurred on matrices for soft substrates [27], while smaller deformation of matrices occurred for stiff substrates [26]. Follow-up experiments found that deformation of substrates by MSCs was maximized and minimized on soft and stiff substrates, respectively (Figure 1(D)) [27].

Despite the significant influence of substrate elasticity on initial commitment fate of MSCs, elasticity is unable to alter the fate of committed MSCs. Guvendiren*et al.* cultured MSCs on the soft substrates of methacrylated hyaluronic acid (MeHA) in the mixture of osteogenic and adipogenic mediums for 14 days [42]. As expected, the majority of MSCs, cultured on soft gels, differentiated into fat cells (adipogenic pathway). However, when the soft hydrogels were stiffened at 1 day, majority of MSCs differentiated into bone cells (osteogenic) and only small fraction differentiated into fat cells (adipogenic). The trend continued to increase when hydrogels were stiffened at 3 and 7 days later. These data suggested that, before the substrates stiffened, MSCs perceived the low elasticity cues of substrates

and correspondingly differentiated in the adipogenic pathway. However, as the substrate was stiffened, undifferentiated MSCs no longer 'feel' the soft substrates, instead MSCs sensed the high elastic substrates. As the results, MSCs committed to osteogenic pathway. The facts that adipogenic cells were still remained after the gels stiffened indicated that committed MSCs (adipogenic cells) would be insensitive for changes of elasticity. Another study employing iPSCs also confirmed the similar findings, that is, differentiation of iPSCs into myocardium was more sensitive to elasticity cues, while partially committed iPSCs were insensitive to elasticity cues [28].

2.1 The modulation of optimum substrate elasticity by the types of ECM molecules

Contrasting to Engler's finding, optimum elasticity to maximize the differentiation of stem cells seemed to be not only strictly dependent on the elasticity of the native tissues, but also on the types of ECM proteins used to coat the substrates [37, 39, 45]. Rowland *et al.* initially showed that the optimum elasticity to maximize the gene expression of *MyoD1* (myogenic marker) and *Runx2* (osteogenic marker) for MSCs was dependent on the types of ECMs used to coat the PA substrate [39]. Collagen I-coated PA showed the highest gene expression of *MyoD1* and *Runx2* for MSCs cultured on the hydrogels at 80 kPa of elasticity. In contrast, collagen IV-coated and fibronectin-coated PA showed the highest gene expression of *MyoD1* and *Runx2* for MSCs on the hydrogels at 25 kPa of elasticity. Theses observation suggested the possible interplay between elasticity and the types of ECMs in optimizing the specific differentiation of stem cells.

To conclude that the particular substrates are more osteogenic or myogenic than others, one need to demonstrate the upregulation of several transcription factors relevant with the differentiation pathway. Therefore, from the study of Rowland *et al.*, it was not possible to conclude that collagen I-coated PA (80 kPa) was more osteogenic than fibronectin-coated PA (80 kPa), since they investigated only one particular transcription factor (*MyoD1* or *Runx2*) for each differentiation pathway. To overcome this limitation, Hirata *et al.* investigated the expression of three different transcription factors-associated with cardiomyocte differentiation (heart muscle) of iPSCs cultured on PA substrates varying stiffness (9, 20, 180 kPa) and types of ECMs (collagen I, gelatin, and fibronectin) [45]. The elasticity of heart muscle tissue was found to be ~13 kPa [47]. The analyzed transcription factors were *GATA*-binding protein 4 (*GATA4*), T-box transcription factor 5 (*Tbx5*), and myocyte-specific enhancer factor 2C (*MEF2C*). The elasticity of collagen I-coated PA had no effects on the cardiogenic differentiation, as evidenced by the unchanged expression of *GATA4* and *Tbx5*. In contrast, gelatin and fibronectin-coated PA showed the relationship of elasticity and ECM proteins on the cardiogenic differentiation; the expression of G*ATA4*, T*bx5*, and M*EF2C* was maximized on PA with 20 kPa and minimized on PA with 9 kPa and 180 kPa. Taken together, these findings implied that elasticity of substrates would not be an overriding factor in specifying commitment of stem cells, since optimal elasticity would be dependent on the types of ECMs used.

Expansion of hematopoietic stem cells (HSCs) was previously shown to be influenced by the elasticity of substrates [12]. Choi *et al.* employed collagen I-coated PA and found that expansion of HSCs was maximized on the soft PA with 0.0442 kPa of elastic modulus, but not on stiff PA with 3.48 kPa and stiffer glass-coated with collagen I [48]. Contrary to these findings, Kumar *et al.* demonstrated that the substrates with the elasticity ranging from 12.2 kPa to 30.4 kPa (storage modulus) of poly(vinyl alcohol-co-vinyl acetate-co-itaconic acid) gels were required to maximize the expansion of hematopoietic stem and progenitor cells (HSPCs) on fibronectin or its oligopeptide (CS1, EILDVPST) coated substrates [37].

Several hypotheses have been raised to explain why different combinations of substrates and ECMs show different optimum elasticity to specifically differentiate stem cells. In one hypothesis, some ECM molecules have been known to bind different sites of integrin, resulting in possible different activations of mechanotransduction pathways [39]. In the other hypothesis, cells are argued to consider the mechanical feedback from ECMs, instead of directly sensing the elasticity of substrates (Figure 2(B)) [10, 37]. Each ECM molecule possesses a different mechanical property (*e.g.*, elasticity, molecule weight and length), meanwhile the modification of hydrogel elasticity is associated with the changes of pore size [46] or ECM anchoring density on the substrates [13]. Therefore, different combinations of ECM molecules and hydrogels with different elasticities yield the varying optimum values to activate stem cells [13]. The variations of optimum elasticity and ECMs molecule to support the stem cell differentiation and expansion are summarized in Table 1.

Table 1: Variation of optimum elasticity-type of ECM to support specific differentiation or expansion of stem cells

| Type of Lineage Commitment	Elasticity of Original Tissue	Type of ECMs	Optimum Substrate Elasticity
1. Myogenic (muscle tissue)	~8-17 kPa (muscle tissue) [26]	Collagen I-coated PA	11 kPa (Elastic modulus) [26]
			80 kPa (Elastic modulus) [39]
		Fibrinogen-coated PA	25 kPa (Elastic modulus) [39]
2. Osteogenic (bone tissue)	~40 kPa (bone osteoid) [26]	Collagen I- coated PA	40 kPa (Elastic modulus) [26]
			80 kPa (Elastic modulus) [39]
		Fibrinogen-coated PA	25 kPa (Elastic modulus) [39]
3. Cardiomyogenic (heart muscle)	~13 kPa (normal heart muscle tissue) [47]	Collagen I- coated	No effect of elasticity [45]
		Fibrinogen-coated PA	20 kPa (Elastic modulus) [45]
		Gelatin-coated PA	20 kPa (Elastic modulus) [45]
4. HSCs expansion (bone marrow)	~150 Pa (bone marrow niche) [37]	Collagen I-coated PA	0.0442 kPa (Elastic modulus) [48]
		Fibrinogen-coated PA	~12-30 kPa (Storage modulus) [37]
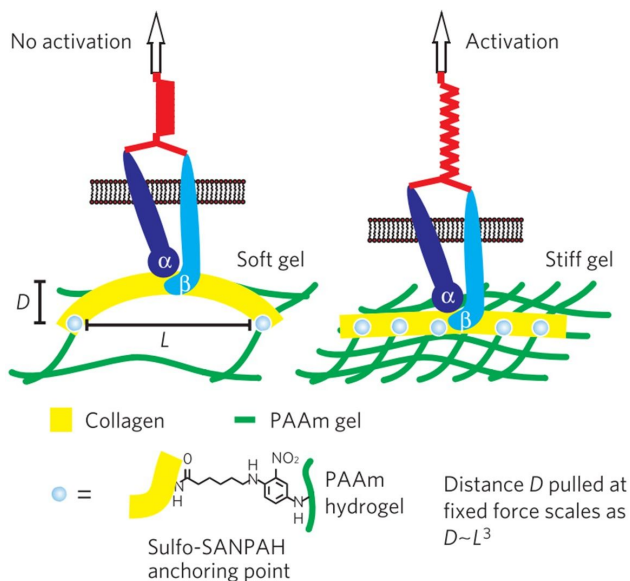
Figure 2: Schematic illustration of alternative mechanosensing mechanism. Instead of directly interacting with substrate, cells consider the mechanical feedback of ECMs molecules (collagen). Soft and stiff gel show different mesh size, thus different anchoring point of ECMs. As a result, cell sense loose mechanical feedback of collagen at soft gel and intense mechanical feedback at stiff gel. Sulfo-SANPAH is the chemical linker of collagen and poly(acrylamide) substrate (PAAm). Parts of figure are adapted with permission from [46]

2.2 The interplay of soluble factors and substrate elasticity for stem cell differentiation

Stem cells are conventionally induced by adding soluble factors (growth factors, small ECMs and chemical molecules) [49, 50]; however, elasticity of cell culture sub-

strates is capable to mimic the inductive effects of such soluble factors. In the initial study of Engler *et al.*, MSCs cultured on PA with different elasticities were induced to different types of lineage cells without the addition of differentiation-inducing factors [26]. Other investigators described that annulus fibrosis-derived stem cells would be differentiated into chondrogenic or osteogenic pathway, simply by adjusting the elastic modulus of substrates, such as poly(ether carbonate urethane)urea with the elasticity ranging from 2.5 MPa to 13.4MPa [17].

Holst *et al.* showed that elasticity of thin films of tropoelastin with a cell binding sequence of VGVAPG on oxidized polystyrene was able to selectively expand the fraction of Lin⁻ Sca-1⁺ c-Kit⁺(LSK)-expressing HSCs [15]. The selective expansion effect was absence on the tropoelastin-uncoated dishes, but it was achieved by subjecting LSK-expressing HSCs on the uncoated dishes with cocktail of cytokines (interleukine-3 (IL-3) and IL-6, and stem cell factor). Addition of blebbistatin in culture medium abrogated the effect of tropoelastin on LSK-expressing HSCs cells, indicating the correlation of selective expansion effect to the cellular mechanotransduction.

The inductive effects of soluble factors and elasticity cues are synergism. Jiang *et al.* reported higher expression of osteogenic markers for MSCs cultured on stiff gels than that for MSCs on soft gels [51]. Addition of alendronate (promoter of osteogenic differentiation) further enhanced the expression of osteogenic markers by MSCs on the stiff gels [51]. The synergistic effect of elasticity and soluble factors implies that elasticity and soluble factors would affect the cell differentiation through different mechanisms [12]. It is summarized that the elasticity of the cell culture substrates only guides the lineage specification at early stage
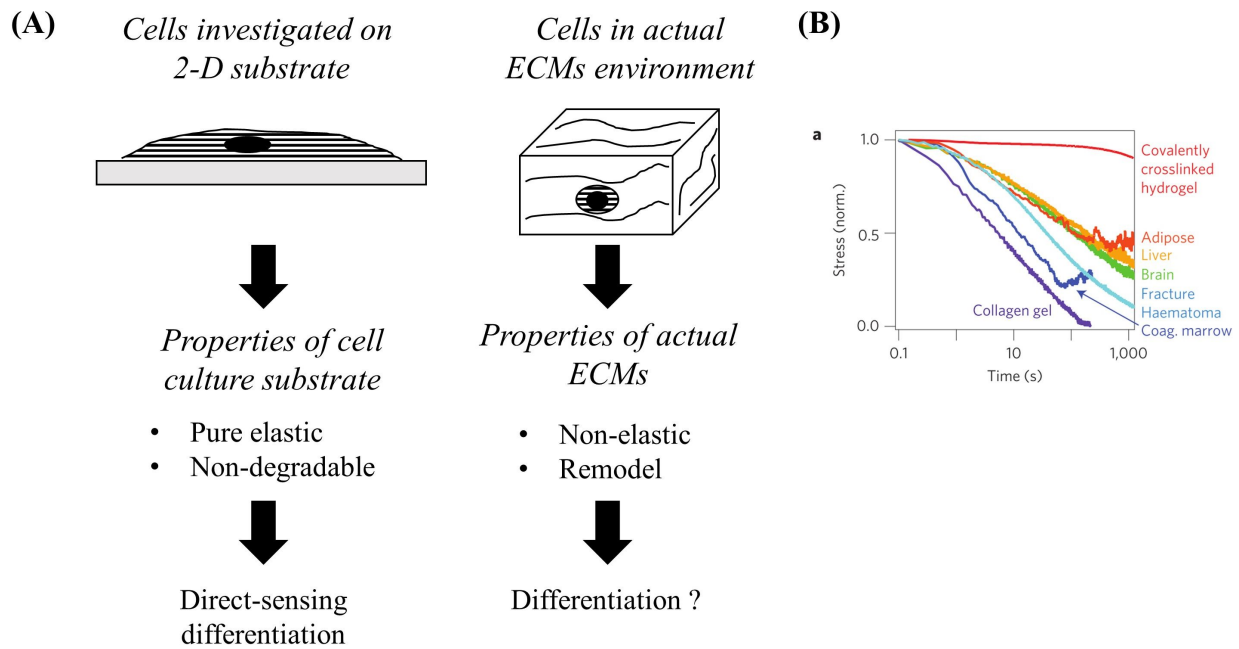
(A)

Cells investigated on 2-D substrate

Cells in actual ECMs environment

(B)

Properties of cell culture substrate

- Pure elastic
- Non-degradable

Properties of actual ECMs

- Non-elastic
- Remodel

Direct-sensing differentiation

Differentiation ?

Figure 3: (A) Schematic illustration of cells cultured on 2-D substrate and cells in actual ECMs environment. So far, responses of stem cells to elasticity when cultured in biologically relevant system are unknown. **(B)** Viscoelastic measurement of various living tissues. Covalently crosslinked hydrogel is poly(acrylamide). Parts of figure are adapted with permission from [31] **(B)**

of differentiation, and inductive media are still necessary to mature the differentiation of MSCs [10, 42].

3 Do the current studies of elasticity-driven commitment of stem cells reflect the true responses of stem cells in the actual ECMs microenvironment?

The most of studies of elasticity-driven fate commitment of stem cells are mainly conducted on 2-D cell culture substrates with different Young's modulus or storage modulus, based on the static experimental setting [8, 26, 37]. These researches are invaluable in revealing the fundamental insights on how cells response to the physical cues (*e.g.*, cells exert low traction and high deformation on soft hydrogels and high traction and low deformation on stiff hydrogels [40]), suggesting that stem cells would adapt its behavior and fate corresponding to the elasticity of substrates [26]. However, the 2-D culture system lack the dimensional and mechanical complexity of native 3-D ECMs environment, which exhibits viscoelasticity (stress-relaxing properties): that is, ECMs undergo continuous deformation under a constant stress (Figure 3) [31]. Moreover,

native ECMs are constantly remodeled throughout the life of individuals, which introduce the changes of mechanical properties inadvertently [31, 32, 52]. Thus, simple responses of stem cells to the stiffness of 2D substrates (tendency of stem cells to efficiently differentiate into specific tissue lineage when stem cells were cultured on the biomaterials with similar elasticity to the tissue) might not fully reflect the responses of stem cells in the actual ECMs microenvironment.

In this section, we discuss the interplay of elasticity and dynamic physical properties of cell culture materials in influencing the differentiation fates of stem cells. An empirical model to simplify the lineage specification of stem cells in dynamic cell culture environment is discussed and proposed. To the best of our knowledge, the responses of stem cells to the dynamic properties of cell culture materials are largely conducted in the 3-D cell culture environment. Therefore, the studies conducted in 3-D cell culture setting are mainly discussed.

3.1 Lineage specification of stem cell cultured in the material exhibiting dynamic physical properties

In the 3-D cell culture setting, adipogenic and osteogenic commitments of stem cells are commonly used to eluci-
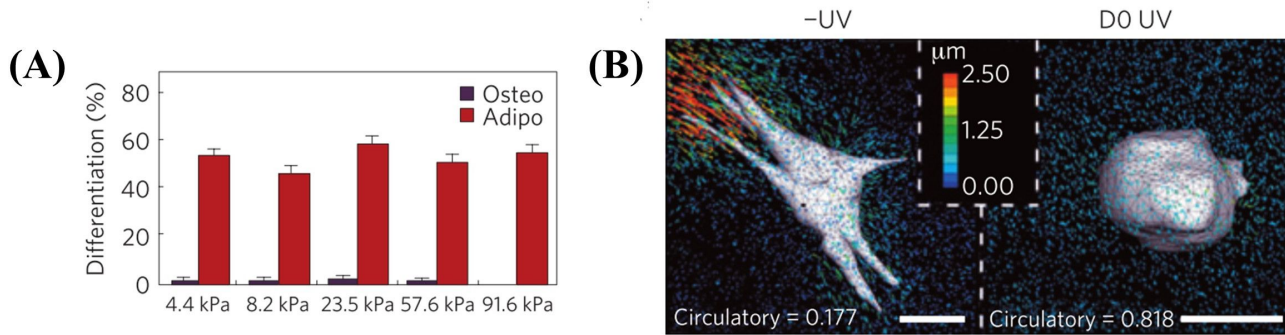
(A)

(B)

Figure 4: Degradation-mediated traction in chemically-crosslinked hydrogel. **(A)** Osteogenic and adipogenic differentiation of MSCs in chemically-crosslinked hydrogel of different stiffness in the mixed media. **(B)** Average drift-corrected bead displacement within 15 μm of cell surface as given by traction force microscopy. −UV is the degradable chemically-crosslinked hydrogel and D0UV is the non-degradable chemically crosslinked hydrogel. Parts of figure are adapted with permission from [33] **(A),(B)**

date the effect of elasticity matrices on the lineage specification of stem cells [31, 33, 34]. From the biological point of view, soft and hard tissues originated from fat (adipogenic) and bone (osteogenic)provide contrasting results on how elasticity of substrates would influence the commitment tendency of stem cells. Adipogenic and osteogenic pathways are previously shown to be highly sensitive to the changes of elasticity of cell culture materials [42]. Therefore, it is possible to identify non-elasticity factors that affect the elasticity-driven lineage specification of stem cells by keeping constant elasticity of matrices. From the technical point of view, the ratios of adipogenic to osteogenic differentiations are easily quantified by employing Oil Red O staining and alkaline phosphatase (ALP) staining [34].

Khetan *et al.* showed that if the stem cells are unable to exert traction to deform matrices, elasticity is not an overriding factor in determining lineage specification of stem cells [33]. They initially employed chemically-crosslinked hydrogels (restricted hydrogels) with stable bonds strong enough to prevent cells from reorganizing the matrices mechanically. As a result, MSCs cultured on this hydrogel with various elasticities (4-95 kPa) consistently adopted the adipogenic fate of differentiation (Figure 4(A)). Introducing degradable bonds to the structure of hydrogels (permissive hydrogels) allowed the stem cells to exert cellular traction on the matrices, which subsequently shifted the fate of stem cell differentiation into osteogenic pathway (Figure 4(B)). It is speculated that stem cells would attempt to modify the local microenvironment by degrading their surroundings and resulting in a deformable microenvironment. These observations suggest that traction-mediated deformation is necessary to promote the osteogenic differentiation, whereas the absence of such deformation is required for adipogenic differentiation of the stem cells.

Correlation of the degree of traction-mediated deformation and the commitment fate of stem cells was investigated by Huebsch *et al.* [34]. MSCs were cultured in the alginate hydrogels crosslinked physically with different elasticity at 2.5, 5, 12, 20, or 110 kPa (Figure 5(A) [34]; the hydrogels were mobile enough to allow cells to sense the matrices mechanically. It is thus possible to correlate the extent of matrix reorganization to the fates of stem cell differentiation. Adipogenic differentiation of MSCs predominated in soft microenvironments of the hydrogels at 2.5-5 kPa, whereas osteogenic differentiation occurred mainly in the intermediate hydrogels at 12-20 kPa. Interestingly, osteogenic differentiation of MSCs in the stiffest hydrogel at 110 kPa was apparently downregulated compared with that in the hydrogel at 20kPa, as evidenced by lower ALP staining. Further investigation revealed that traction-mediated deformation in the hydrogel at 20 kPa became maximum compared to those in the softest and stiffest hydrogels (Figure 5(B)). It would be explained by the fact that any cells could not assemble cytoskeleton-adhesion complexes in the extremely soft hydrogels and exert sufficient traction to deform extremely stiff hydrogels [44]. Taken together, these results are consistent with the study by Khetan *et al.*; the correlation of osteogenic/adipogenic differentiation of MSCs withthe degree of traction-mediated deformations.

Viscoelastic materials sometimes show non-elastic deformation under a constant stress, which is an ideal system to investigate the correlation of traction-mediated deformation and lineage specification of stem cells. Chaudhuri *et al.* investigated the hydrogel system with different viscoelasticity but a constant initial Young's modulus at 9 and 17 kPa [31]. The viscoelasticity of hydrogels is expressed by relaxation time ($\tau_{1/2}$); briefly, the hydrogels with the faster $\tau_{1/2}$ shows quick remodeling under
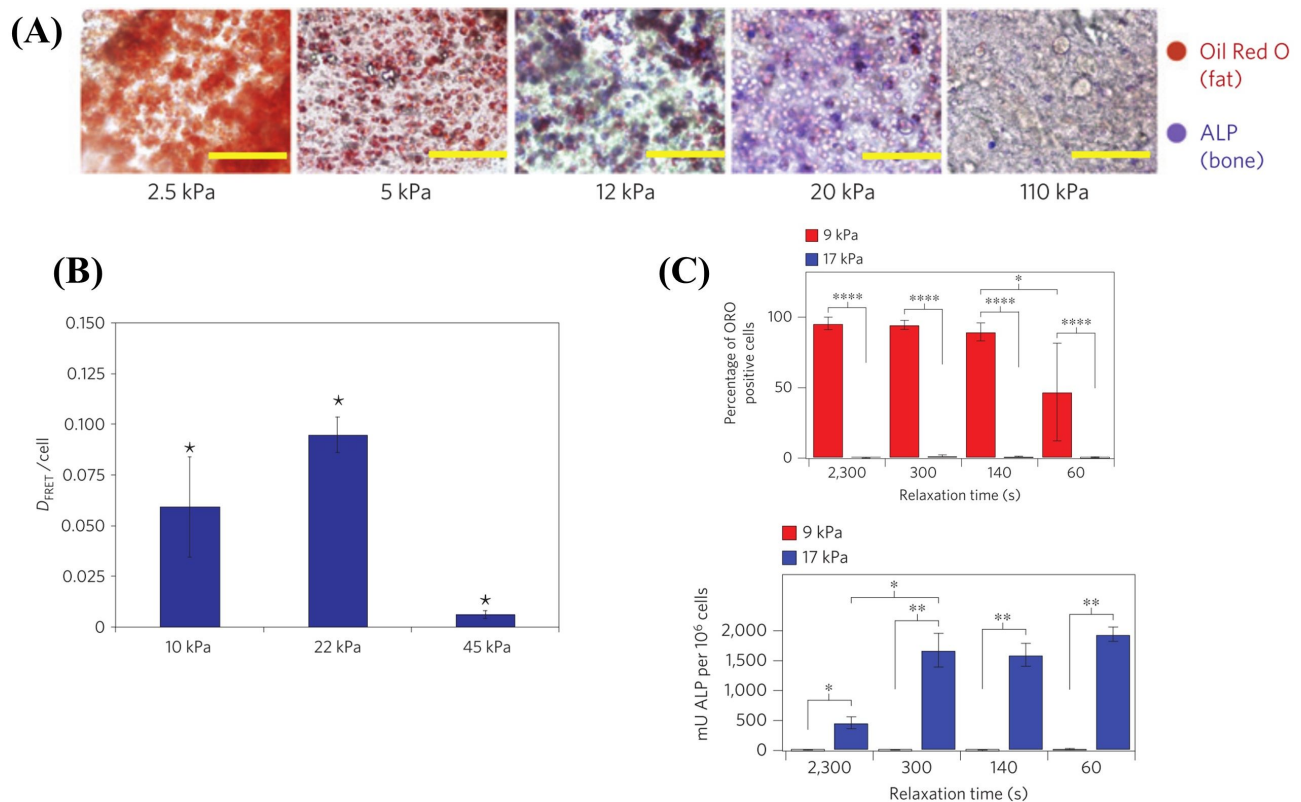
Figure 5: Elasticity-driven lineage of stem cells in 3-D matrix. **(A)** Osteogenic and adipogenic differentiations of MSCs in the hydrogel of different elasticity (2.5-110 kPa) in the presence of osteogenic-and adipogenic-inducing medium. **(B)** Measurement of ligand adhesion (RGD) displacement in hydrogels of different stiffness by fluorescence energy transfer (FRET). D_{FRET} is the degree of energy transfer. (*$p < 0.01$ compared with other conditions, Holm-Bonferroni test). Error bars represented standard deviation for clustering measurements ($n = 3$). **(C)** Quantification of adipogenic and osteogenic differentiations of MSCs when cultured on the hydrogel of constant elastic modulus (9 and 17 kPa), yet different viscoelasticity (expressed by relaxation time: 2300 – 60 s). Parts of figure are adapted with permission from [34] **(A),(B)**, and [31] **(C)**

a constant value of stress and slow remodelling for hydrogels with slower $\tau_{1/2}$. If elasticity of materials solely governs the differentiation of stem cells, faster (lower) relaxation time would be a factor for downregulation (upregulation) of the osteogenic (adipogenic) differentiation of the cells. Interestingly, the experimental data showed the opposite results. Osteogenic (adipogenic) differentiation was upregulated (downregulated) in hydrogels with faster $\tau_{1/2}$ (slower $\tau_{1/2}$) (Figure 5(C)). It is suggested that the viscoelastic materials cause traction-mediated deformation, which in turn enhances (inhibits) the osteogenic (adipogenic) differentiation of MSCs.

Other studies also confirmed the importance of traction-mediated deformation in determining lineage specification of stem cells. Toda *et al.* described that osteogenic differentiation of MSCs was promoted in the decrease of elasticity (storage modulus) of hydrogels during the culture period [38]. Das *et al.* investigated the promotion of adipogenic differentiation of MSCs in the stress-stiffening hydrogels with lower critical stress (the threshold stress value to initiate the stiffening of hydrogels); however, the hydrogels with higher critical stress upregulated the osteogenic differentiation of MSCs [53]. These results do not conform with the idea that the stem cell differentiations of stiff/soft tissue lineage only occur at stiff/soft substrates. These observations can be only explained by taking into account the correlation of traction-mediated deformation and stem cell differentiation.

In summary, when the stem cells are allowed to exert traction to deform the matrices significantly, the cells commit to differentiate into the osteogenic fate (hard tissue). Meanwhile, when the stem cells are unable to deform the matrices significantly, the cells adopt the differentiation into adipogenic lineage (soft tissue).

3.2 Empirical model of traction-mediated deformation to explain the lineage specification of stem cells

An empirical model to explain how the traction-mediated deformation regulates the lineage specification of stem cells is proposed (Figure 6) based on four major assumptions:

1. Cells exert "*traction*" with the magnitude corresponding to elastic modulus (E) of materials. At the stiff materials with $E \propto E_X$, cells are unable to increase the magnitude of "*traction*" [40, 54]. Thus, the cellular traction is saturated beyond the point at E_X. The traction force is generally measured by a traction force microscopy, and subsequently expressed in the dimension, pN/μm^2, of force/surface area (normalized against the focal adhesion size) [55].

2. The tentative term '*stability*' should be introduced; it is defined as the compliancy of materials to deform under a stress applied. The magnitude is proportional to E value. Pure elastic materials exhibit time-independent deformation [56], therefore "*stability*" is unchanged with time (t); $stability_{(elastic)} \approx$ f(E). On the other hand, deformation in viscoelastic materials is time-dependent, therefore "*stability*" of viscoelastic materials gradually decreases over a period of time; $stability_{(viscoelastic)} \approx$ f(E,t) [56]. Low "*stability*" implies significant deformation under a stress applied and vice versa for high "*stability*". When the materials are deformable by cells, it is followed that the gradient of "*traction*" is larger than the "*stability*" at any points before E_X. "*Stability*" possesses same dimensional unit with elasticity that is force/surface area.

3. The "*net product*" is defined as subtraction of "*stability*" from "*traction*", of which physical interpretation is net cellular traction; it might also corresponds to the net matrix deformation. Due to the restriction of physical interpretation, the lowest value of "*net product*" is zero. The "*net product*" shows biphasic relationship with elasticity; it gradually increases from low elasticity, maximizes near E_X, and steadily decreases afterwards. Traction and stability lines meet at hypothetical point E_d, and consequently the *net product* is zero.

4. Lineage specification of stem cells depends on the "*net product*". The relationship of "*net product*" and differentiation fate is depicted in (Figure 7). Adipogenic differentiation of stem cells was maximized at small values of "*net product*" and gradually decreased as *net product* became larger. On the other hand, osteogenic differentiation of stem cells was minimized at small value of "*net product*" and gradually increased and maximized near the point E_X. Beyond the point E_X, the cellular traction was saturated, while the stability continues to increase. As a result, the value of *net product* gradually decreases, concurrently with the downregulation of osteogenic differentiation. However, beyond the E_X point, the changes of net product do not correlates with adipogenic differentiation as shown in previous experimental results [34].
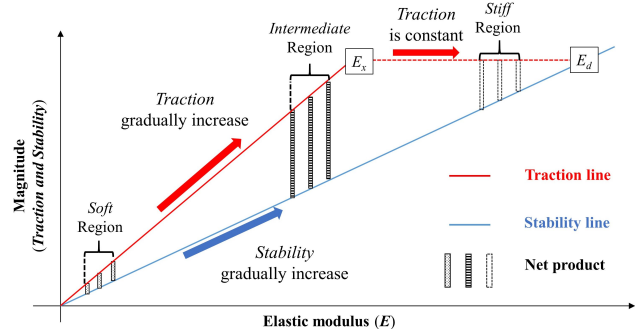

Figure 6: Model of traction-mediated deformation to explain the lineage specification of stem cells in dynamic microenvironment. Cellular *traction* and matrix *stability* are the function of elastic modulus. *Traction* gradually increases with E up to the E_X where the traction is saturated and incapable to increase further with elasticity. *Stability* gradually increases with elasticity and keep increasing even after the elasticity is beyond E_X. *Net product* is represented by rectangular with different fillers. *Net products* in soft, intermediate, and stiff regions of hydrogels are represented by dotted, lined, open rectangular, respectively
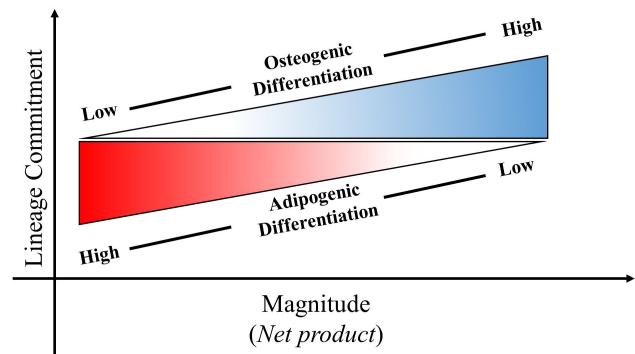

Figure 7: Relationship of *net product* (y-axis)and the extent of lineage specification of stem cells (x-axis). Osteogenic differentiation is optimized when the magnitude of net product is maximized. On the other hand, adipogenic differentiation is optimized when the magnitude of net product is minimized
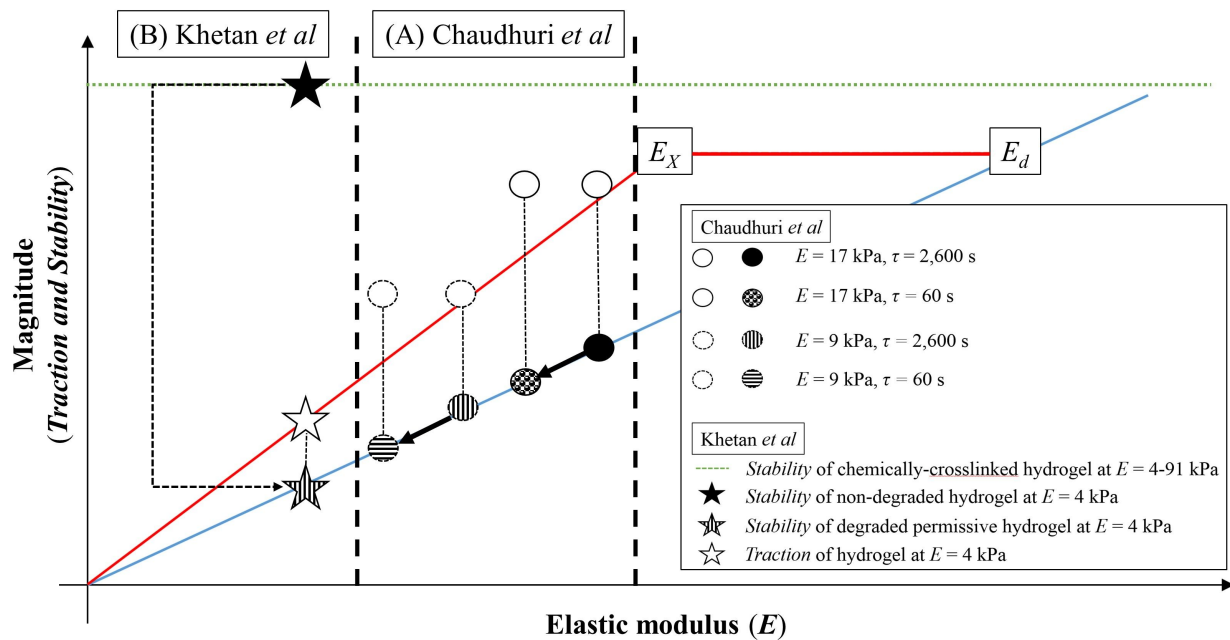
Figure 8: Model of traction-dependent differentiation used to explain the studies by (A) Chaudhuri *et al.* [31], (B) Khetan *et al.* [33]. There are two types of stability lines: (1) full-line indicates *stability* of matrix capable to be mechanically reorganized (*e.g.*, physically-crosslinked hydrogel). (2) dot-line indicates *stability* of matrix incapable to be mechanically reorganized (*e.g.*, chemically-crosslinked hydrogel). Vertical dot-line connecting *traction* and *stability* indicates *net product* with the length correspond to the magnitude of *net product*. Full-arrow indicates shift of *stability* of materials with different viscoelastic properties. Dot-arrow indicates shift of *stability* when local degradation occurs at the matrices incapable to be mechanically reorganized

The proposed empirical modelcan explain the prevalent results of *direct-sensing differentiation of stem cells*. Pure elastic materials exhibit time-independent deformation and no plastic deformation occurs under higher magnitude of stress [57, 58]. Therefore, the "*stability*" line strictly depends on the elasticity. As a result, "*net product*" and commitment of stem cells simply depends on the elasticity of substrates. As previously described, *direct-sensing differentiation* is insufficient to explain the lineage specification of stem cells cultured in mechanically dynamic materials [31, 33]. In the viscoelastic materials, plastic deformation of materials continuously occurs at a constant stress after enough time of stress application, thus *stability* value at a given elastic modulus should be shifted down.

Based on the data by Huebsch *et al.* [34], the *net product* is low for MSCs cultured in the hydrogels at 2.5 or 5 kPa (soft region). The *net product* is maximized for MSCs cultured at 12 or 20 kPa hydrogel (*intermediate region*), and gradually decreased for that cultured at 110 kPa (*stiff region*). In the similar manner, adipogenic and osteogenic differentiations are maximized at the lowest and highest *net products* in the *soft* and *intermediate regions* (Figure 7). It is interesting to note that the decrease of *net product* in the *stiff* only affects the osteogenic differentiation but not the adipogenic differentiation, suggesting the involvement

of other types of mechanosensing mechanism on the excessively stiff substrate (beyond Ex point).

We further expand the model to apply the results investigated by Chaudhuri *et al.* [31] and Khetan *et al.* [33] in Figure 8. Although the elastic modulus of the hydrogels was kept constant at 9 kPa and 17 kPa, each hydrogel exhibited different relaxation times at 60 - 2600 seconds [31]. The hydrogels with faster relaxation time were quickly deformed compared to those with slower relaxation time, therefore the soft hydrogels decreased stability. On the other hand, cells exerted the constant traction value. As a result, net product (osteogenic differentiation) was maximized for the hydrogels with faster relaxation time.

The chemically-crosslinked hydrogels used by Khetan *et al.* were stable enough to prevent matrix reorganization by cellular traction. Therefore, the stability magnitude is significantly greater than traction at all values of elasticity, resulting in the depreciation of net product at any ranges of elasticity of hydrogels (commitment to adipogenic lineage of differentiation). In the hydrogels with degradable bonds, the stem cells were capable of locally degrading the matrix, and the stability therefore would shift to the lower magnitude. As a result, the stem cells are able to exert trac-

tion todeform the matrices and differentiate in osteogenic pathway.

Two important points were summarized based on the empirical model proposed; 1) the net product determines the lineage differentiation of stem cells and 2) the time-dependent deformation of viscoelastic material changes the stability value, which in turn alters the net product (cell commitment) for a given initial elastic modulus.

3.3 Lineage specification of stem cells on 2-D substrates and 3-D materials based on the empirical model

As previously mentioned in section II, investigations of mechanotransduction of stem cells on 2-D substrates have been conducted by defining elasticity as the elastic and viscoelastic parameters [8, 27, 37, 38]. Based on our model, it is clear that viscoelasticity may influence the final fates of stem cell differentiations. Cameron *et al.* cultured MSCs on the substrate exhibiting similar storage modulus but different loss modulus [59]; the cell surface area on the substrate was increased with large loss modulus. Chaudhuri *et al.* further demonstrated that variation in storage modulus may alter the commitment fates of stem cells [31]. Failure in taking into account the viscoelasticity of material may explain the discrepancies observed in the elasticity-driven mechanotransduction literatures [27, 37, 46, 60, 61].

The influences of degradability of substrates to the mechanotransduction of stem cells were rarely investigated. Peng *et al.* cultured MSCs on the 2-D poly(ethyleneglycol) (PEG) substrates with different degradation rates [52] and the substrates with the highest degradation rate had the highest loss of elastic modulus (softening of substrate). Interestingly, the highly degraded substrates promoted the osteogenic differentiation of MSCs, which would be contradicted the conventional direct-sensing differentiation. Based on our model, MSCs cultured on PEG substrates with elastic modulus in the range of ~500 - 1,000 kPa saturated the cellular traction. The degradation of material would reduce the elastic modulus, resulting in the decrease of both traction and stability. As a result, the net product would be maximized and osteogenic differentiation was promoted.

It is worth to mention that the empirical model developed here was intended to augment the direct-sensing differentiation model widely used in 2-D cell culture study. In other words, both of these models are based on the cellular contractility as the main agent of mechanosensing. Several investigators have raised the possibilities of differ-

ent mechanisms of mechanosensing in 2-D and 3-D environments [62]. If such case was true, then the empirical model might not be fully applicable to explain the stem cell differentiation in mechanically dynamic 2-D environment.

4 Conclusions and future outlooks

Elasticity of materials for culturing cells is commonly characterized by the elastic modulus or viscoelastic modulus. Adult cells have been known to adjust their motility (cell locomotion) or contractility (*e.g.*, membrane stiffness, exerted traction) in response to elasticity of substrates. The responses of adult cells to substrate elasticity were speculated to be mediated by the mechanotransduction process. Not only adult cells but also stem cells were shown to be responsive to the elasticity. Stem cells efficiently differentiate into the particular tissue lineage of the cells when the cells are cultured on the materials of which elasticity mimic the stiffness of targeted tissue.

Sensitivity of stem cells to elastic cues depends on the differentiation stages of the cells, with the early differentiated stem cells being more sensitive to elasticity cues than the late differentiated stem cells. The optimum value to induce the specific differentiation of the cells depends on the type of ECMs used to connect materials and cells. Several explanations have been raised why different types of ECMs alter the optimum elasticity for the differentiation of stem cells into specific lineages of the cells. ECMs bind different receptor (integrin) of the cells, and thus activate the different mechanism of mechanotransduction. Some investigators proposed that the stem cells did not directly sense the elasticity of substrates, but instead interacted with the ECMs [10]. Future investigations are necessary to elucidate the factors underlying the variation of optimum elasticity.

Direct-sensing differentiation is insufficient to explain the fate commitment of stem cells cultured on materials with dynamic physical properties. It is found that stem cells exert cellular traction to deform the matrices and differentiate into hard or soft tissue lineage of the cells by considering the traction-mediated deformation. Viscoelastic materials accommodate the traction-mediated deformation. Therefore, highly viscoelastic materials would promote or inhibit the osteogenic (hard tissue) oradipogenic (soft tissue) differentiation of stem cells.

In this paper, the empirical model based on the traction-mediated deformation is proposed. There are three important components in this model: (1) traction, (2) stability, (3) net product. Differentiation of stem cells is largely determined by magnitude of net product. By chang-

ing the elasticity of materials, it is possible to shift the traction and stability points. For the pure elastic materials, it is not possible to shift the traction and stability points independently, resulting in the common responses observed for direct-sensing differentiation. However, the viscoelastic or degradable materials allow the shift of stability points independent of cellular traction, resulting in inconsistence of direct-sensing differentiation. Range of substrate elasticity seems to alter the responses of stem cells to changes of net products. It is necessary to further investigate the physiologically relevant elastic modulus of materials. Lastly, the empirical model of traction-mediated deformation can be applied as a framework to design future studies to investigate the differentiation of stem cells into various lineages in mechanically dynamic 2-D or 3-D cell culture materials.

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