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Material control of stem cell differentiation: challenges in nano-characterization

PC Dave P Dingal and Dennis E Discher

Recent experiments have revealed that stem cells respond to biophysical cues as well as numerous biochemical factors. Nanoscale properties at the cell–matrix interface that appear to affect adherent stem cells range from matrix elasticity to porosity-dependent matrix tethering and geometry of adhesive linkages. Some stem cells can also remodel their immediate environment to influence phenotype, but this depends on matrix-material properties such as covalent bonding and soft versus hard materials. Efforts to combine both matrix instructions and active cell feedback are required to properly direct stem cell behavior. Comparisons to tissues will be increasingly key and have begun to reveal remodeling of nuclear factors that influence epigenetics.

Addresses
Biophysical Engineering Laboratory and Department of Chemical & Biomolecular Engineering, University of Pennsylvania, Philadelphia, PA 19104, USA

Corresponding authors: Discher, Dennis E (discher@seas.upenn.edu)

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Introduction

Within the last ten years, stem cell approaches have become conceivable or even realized for each of the roughly 200 differentiated cell types in humans. Since most tissue cells are anchorage dependent — meaning that adhesion to a solid is necessary for cell survival, it is only sensible that the physicochemical nature of the support can influence stem cell fate. Substrate stiffness, geometry, porosity, and topography are now understood to influence stem cells, perhaps as much as biochemical factors [1]. Molecular pathways of cellular mechanotransduction that ultimately affect both cell phenotype and genotype are slowly becoming clear. Matrix stiffness-dependent lineage commitment of stem cell has been suggested to involve YAP/TAZ, which are transcription factors previously known to influence proliferation, whereas newer evidence suggests they are also nuclear mechano-transducers regulated by Rho GTPase and cytoskeletal tension independent of the canonical Hippo pathway [2•]. More recently, polymer physics type scaling between tissue stiffness and the expression levels in primary tissue of the nuclear envelope structural protein, Lamin-A, has been reported to co-regulate YAP, among other transcription and epigenetic factors [3]. The results suggested that increased matrix rigidity (due to greater collagen in high stress tissues) leads to nuclear stiffening and greater DNA protection as a homeostatic response in all tissue cell types, including stem cells [3•,4]. More pathways are likely to emerge as the field gains insight and control over micro/nano-environments as analyzed in vivo or applied to stem cells in culture.

Virtually every organ in the body contains resident stem cells or progenitors that contribute to organ homeostasis or repair. Exploiting stem cells for regeneration of damaged tissue has spurred research into their multipotentiality as well as immunocompatibility. Therapeutics is limited in part by *in vitro* cell expansion as well as materials issues that include the design of biocompatible scaffolds for co-transplantation. Since many stem cells are anchorage dependent, injection *in vivo* should work best if the cells adhere quickly and adequately, but it is clear in many trials with mesenchymal stem cells (MSCs) that the vast majority of injected cells die rather than contribute to tissue [5]. Recapitulating the various stem cell niches *ex vivo* is extremely challenging as it likely involves spatiotemporal regulation of biostimuli that extend to extracellular matrix architecture. Nonetheless, understanding the niche *in vitro* might help in translation to *in vivo* [6].

In this review, we describe recent advances as well as challenges in the material control of stem cell multipotency and lineage commitment. By 'stem cell', we refer to cited studies on MSCs (mesenchymal), NSCs (neural), ESCs (embryonic), iPSC (induced pluripotent), epidermal stem cells, etc., but we emphasize the generality merely with 'stem cell' and encourage the interested reader to seek the primary literature for specific stem cell types. We attempt to highlight in more detail how the field is beginning to formulate materials design rules for stem cell cultures down to the nanoscale in terms of fabrication and/or physical characterization. Approaches are crudely – split into soft materials such as hydrogels that are as soft as most tissues or else hard materials in which the softest thing in culture is the cell. Remarkably, there seem to be ways — i.e. rules — to manipulate 'boundary conditions' in order to fool cells into responding to a hard material in a manner similar to (but not exactly the same as) that on a much softer material, and *vice versa*.

Soft matter control

Tissue stiffness or elasticity is dictated by the extracellular matrix (ECM). Even a few minutes of incubation with collagenase can soften a tissue dramatically [3•]. ECM is comprised a network of fibrous proteins, such as collagens, that are crosslinked in a homophilic or heterophilic manner. A hierarchical polymeric network of variable density allows for a broad range of characteristic microelasticities for tissues: brain [7] and fat [8] are hundreds of Pascals in stiffness whereas cartilage [9] and pre-calcified bone [10] are dozens of kiloPascals or even stiffer on larger length scales. Precise regulation of physical properties of the ECM seems to match and couple to the applied mechanical forces that contribute to specific cell differentiation programs in adult tissue and likely in the embryo. A differential cell response to both ECM elasticity and dimensionality (i.e. 2D vs 3D) — termed ECM mechanosensing — has been observed *in vitro* through various materials approaches, particularly with natural [11] and synthetic [12] hydrogels. Naturally derived polymers such as silk [13], collagen and hyaluronic acid matrices [14] are currently used as delivery vehicles for cell transplantation. Synthetic scaffolds are chosen based on properties that range from biostability or biocompatibility to biodegradability and porosity. Inert synthetic hydrogels are used *in vitro* for studying cell behavior such as migration, proliferation, and differentiation. Indeed, due to the chemistry that can sometimes be very simple, physical parameters such as elastic and viscous moduli can be precisely tuned to mimic biological tissues.

Synthesis, functionalization and characterization

The basic components for polymer hydrogel synthesis are a monomer, a crosslinker, and an initiator of polymerization. The ratio and concentration of monomer and crosslinker are varied to achieve desired viscoelastic properties, perhaps to mimic a normal or diseased tissue or perhaps to be distinct from a tissue. For example, a myocardial infarct stiffens twofold to threefold relative to normal heart tissue (~12–20 kPa) [5]. Rheological methods provide measures of a material's complex modulus or stiffness (G^*) composed of both an elastic modulus (G') and a viscous modulus (G''). These can be measured as a function of frequency of oscillatory shear with a rheometer, and one typically considers that the 1 Hz beating of the heart is close to the high frequency limit of cell biological relevance. Solid tissues are mostly elastic, with G' values ranging from 0.1 to 100 kiloPascals [10]. Material-dependent cell responses are thus strongly influenced by the elastic component of a hydrogel, at least when G'' is two orders of magnitude lower than G'. Viscous matrix effects on cell morphology are nonetheless interesting based on recent examples in the literature [15,16].

Control of hydrogel chemistry can extend to spatiotemporal control of polymerization [17] and micropatterning [18]. Non-uniform substrates might, for example, mimic a heterogeneous cell microenvironment, but in such a case, rheological measurements must be done at the cellular scale. One particularly attractive method is atomic force microscopy (AFM): a cantilever probe reflects a laser onto a photodiode detector that measures small variations in cantilever deflection as it indents a substrate. For such heterogeneous substrates, an AFM cantilever can probe and create a viscoelastic map along a preset path. For example, we recently examined 'durotaxis', which is a phenomenon in which a cell migrates toward increasing matrix stiffness [19,20,21], and so we and others made hydrogels with stiffness gradients and used AFM to measure the steepness of those gradients [20,21].

Most hydrogels require some form of functionalization to promote favorable cell–material interactions. This can be done by linking cell-adhesive moieties into the polymer backbone, via functional side group chemistry. The well-known integrin-binding tripeptide RGD can be incorporated into a methacrylated polymer backbone (e.g. methacrylated polyethylene glycol) via a Michael-type addition reaction between thiol (from a cysteine moiety in the cell-adhesive peptide) and methacrylate groups [18]. Large matrix proteins like fibronectin and collagen can also be covalently crosslinked into an amine-containing hydrogel backbone via a heterobifunctional crosslinker that contains a primary amine-reactive succinimidyl ester and a photoactivatable nucleophilic azide (e.g. sulfo-SANPAH) [22]. Conventional matrix functionalization of hydrogel systems involves copious coverage of the cell–material interface with cell-adhesive molecules, to ensure that cell attachment is not adhesion ligand-limited and that any differential cellular response is due to physical properties of the matrix.

Advances in soft matter research

Whereas past studies of the cell–material interface have focused on the effects of relatively homogeneous and weakly varying materials on stem cells, recent efforts have begun to address some aspects of matrix micro/nano-heterogeneity. Tools that allow non-invasive *in situ* measurements of cell–material interaction at small scales could ultimately clarify governing principles for cell–material interface design. Fabrication approaches are equally important as they should allow for systematic nanoscale control of substrate topography and functionalization. A great deal of effort needs to be spent in (1) understanding how matrix ligand is presented at the interface, (2) how a cell adheres and applies ligand-dependent and stiffness-dependent traction forces to a material, and (3) how a cell remodels or secretes adhesion-relevant molecules or other factors presented at its interface.

Insight into the first two issues above has been obtained from integrin clustering that occurs when a cell exerts traction forces in response to stiff matrix. Huebsch *et al.* [23••] found that increasing matrix resistance to adhesion

ligand displacement leads to a greater ability of a cell to exert traction forces that allow for more stable integrin–ligand bonds [24] and ultimately, greater propensity toward osteogenic commitment. They encapsulated stem cells in 3D non-degradable, RGD-modified alginate gels for which elastic modulus (2.5–110 kPa) was varied by the extent of ionic crosslinking. In contrast to 2D studies where cells spread more in response to increased matrix rigidity, encapsulation in 3D nanoporous hydrogels maintained a rounded shape. The ability of encapsulated cells to rearrange integrin–RGD linkages in the ionically crosslinked alginates is estimated by fluorescence resonance energy transfer (FRET) of rhodamine-labelled and fluorescein-labelled RGD peptides clustering near the cell membrane [24] and appeared to depend on matrix stiffness and seemed optimal at an intermediate stiffness (~20–30 kPa), where integrin receptors were estimated to probe 50 nm into the surrounding environment. In contrast, Khetan *et al.* [25•] made 3D covalently crosslinked methacrylated hyaluronic acid (MeHA) hydrogels (G' = 4–95 kPa) and found a lack of matrix elasticity dependence, with most cells driven toward adipogenic commitment. Differences between the two systems start with the nature of crosslinking and could extend to how growth factors in serum differentially adsorb to or bind the gels or permeate gel pores; differentiation-relevant factors in serum include TGFβ, which is in a large latent complex that must immobilize near a cell in order for traction forces to release the active growth factor [26]. Determination of traction-dependent stiffness sensing involves many assumptions about matrix stiffness at the cell interface and is difficult to assess when cells are embedded in 3D. Yet stiffness sensing seems essential for osteogenic commitment and appears dysregulated in cells entrapped within overly restrictive microenvironments. Moreover, dynamically fluctuating tractions in and around focal adhesions are necessary for matrix rigidity sensing [27].

The third issue mentioned above is how stem cells release their own matrix and other factors or else modify pre-existing factors. Minimizing cell-derived or serum-derived matrix deposition has been investigated to some extent on ultra-low fouling substrates that are zwitterionic in nature [28]. In all such studies, proteomic scale analysis of substrates is increasingly needed to define cell and serum responses to manufactured microenvironments; using antibodies to assess whether serum fibronectin or vitronectin adsorbs or not is just a start. Once measurements are made, however, efforts to prevent adsorption of proteins either from cells or serum can also point the way toward preventing foreign body reactions if materials are to be taken *in vivo* [29••]. In a study of 3D PEG hydrogels tethered with small-molecule functional groups, stem cell fate had been found to be directed toward adipogenic or osteogenic differentiation with respective functionalization of the gels by *t*-butyl or phosphate [30]. The effects of such small functional moieties have been speculated to reflect differences in cell-derived, lineage-specific matrix molecules which physisorb to matrix and thus accumulate at the cell–matrix interface to ultimately direct differentiation. In the RGD-modified MeHA hydrogels cited above [25•], local matrix changes due to cell-derived matrix did not seem to impede RGD-integrin signaling, but no measurements of matrix were pursued — 3D microenvironments require far more careful characterization than 2D, at least because of the complexity of protein entrapment through the depth of a gel. When the ability of a cell to probe its surrounding matrix is affected by complexities of weak or strong adsorption or encapsulation within covalently crosslinked gels, matrix rigidity (or other physical property) effects on stem cell fate are also likely to be affected.

It must be noted that while promoting cell attachment with matrix-immobilized RGD peptides ensures direct matrix sensing, there are subtle, yet still confounding differences with tethering actual matrix proteins. For example, fibronectin contains an RGD motif, among other motifs, that seems to activate other signaling pathways within the cell, and at least affect cell migration differently from the minimal sequence [31]. Matrix tethering is an issue raised recently by Trappmann *et al.* [32•]. In contrast to utilizing RGD peptides, which do not involve the tethering issues in 3D, collagen fibrils were functionalized on 2D polyacrylamide (PA) gels or polydimethylsiloxane (PDMS) elastomers. While stem cells differentiated as expected on soft versus stiff PA gels, the apparent stiffness of PDMS had no effect. Unfortunately, soft PDMS is well-known to be difficult to make and requires careful nanoscale characterization; it is often accompanied by increased viscosity (or even fluid-solid heterogeneity), which was not characterized by Trappmann et al. but also affects stem cell differentiation [16] as well as epithelial cell sheet motion [15]. Additional studies by Trappmann *et al.* involved decreasing the collagen anchoring points on stiff gels by lowering sulfo-SANPAH crosslinker concentration with the effect of inducing a soft gel phenotype that prevented epidermal stem cell differentiation. Ligand density is likely decreased as sulfo-SANPAH is lowered, and cells simply do not spread on stiff PA gels when adhesive ligand is limiting [12]. Nonetheless, the idea of decoupling material stiffness and cell–matrix interactions may find application.

Hard matter approaches

The ligands, assembly, and overall architecture of ECM can all influence cell behavior. Parsing some aspects of cell–matrix interaction at the molecular level might be addressed with precision nano-fabrication of hard materials as used in the semiconductor industry. An array of nanotechnology-driven *in vitro* cell culture platforms has been reviewed recently [33], and so we only highlight here a couple of key advances in nanotopography design principles that are inspired from ECM [34] and/or inspire precise control of ECM [35,36] in directing cell fate.

Nanoscale patterning of cell adhesion ligands is problematic with soft materials because many cell types generate sufficient traction strain to rearrange any ligand pattern.

A minimal adhesive matrix unit required for cell attachment, spreading and migration is an important research question that is addressed with nanopatterned RGD surfaces. Schvartzman *et al.* [37••] varied RGD ligand spacing, density and cluster size, and found that a spacing of 60 nm in a cluster with a minimum of 4 RGD ligands is sufficient to support cell spreading. Although ligand surface density might have a role, they speculated that talin, an integrin-binding scaffolding protein that has 4 potential binding sites, is involved in the integrin clustering-derived cell response. Focal adhesion formation was indeed enhanced for a ligand spacing of ~50 nm, concomitant with increased cell attachment, migration [31] and stiffening [38]. In the alginate gel studies by Huebsch *et al.*, the highest RGD density is roughly ~1 RGD ligand per 70 nm × 70 nm surface patch on an entrapped stem cell of 10-μm radius. This might explain why matrix-rigidity dependent traction forces enhance clustering. In addition, highly ordered 120 nm ligand pits spaced 300 nm apart in a square lattice have been shown 'best' for maintaining adult stem cell multipotency for several weeks, whereas the exquisite sensitivity of stem cells to a pit placement offset of <50 nm leads to differentiation [35,39••]. Integrin clustering is thus likely to be a key to some pathways that signal intracellular changes from cytoskeleton to nucleus [3•,40]. Epigenetic state and cell reprogramming can also be affected by nanopatterns in recent studies by Downing *et al.* [41], which indicate how much more we can learn about the cell–matrix interface in vitro. However, the 'truth is in the tissue', and acutely controlled manipulations of matrix in fully functional, beating heart have only recently emerged to provide clear evidence – for example – that heart-specific stiffening in development parallels matrix and myosin expression to optimize beating – with additional evidence from cultures of cardiomyocytes derived from pluripotent stem cells [42].

Conclusion

In the heterogeneous microenvironments referred to as stem cell niches, various mechanical and biomolecular cues are integrated to maintain pluripotency or induce differentiation. Biological applications of both soft and hard matter systems to elucidate cell–material interactions have certainly increased our understanding of the stem cell–matrix interface, but there is much more to learn. Soft matter substrates are more tissue-mimetic than hard substrates, but precise nanoscale control of the cell–matrix interface provides powerful tools for understanding and directing a wide range of cell behaviors. Cell-derived molecules are generally overlooked when designing and using many of these materials, whether hard, soft, 2D, or 3D, although some stem cell types express lesser matrix than others. Controlling endogenous expression of such factors by methods such as siRNA knockdown is one approach. Ultimately, new design rules that are emerging for material control of stem cell fate could help with *in vitro* cultures as well as implantable scaffolds for more stem cell based therapies.

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