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Abstract

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The central nervous system (CNS) controls crucial functions in mammals ranging from sensory processing and memory to hormonal regulation and motor function. Thus many diseases and injuries afflicting CNS cells, such as Alzheimer's and Parkinson's Diseases or stroke and traumatic injuries, result in devastating consequences. Stem cells serve as potential cell sources for cell transplantation therapies, but also model systems to study neural development. In both cases, it is crucial to understand how the hallmark properties of stem cells, self-renewal and potency, are regulated by their microenvironment. An important body of work has identified many biochemical factors, such as small molecules, growth factors, morphogens, and adhesive ligands that regulate stem cell behavior. However, more recently biophysical effects, such as microenvironmental stiffness, cell and tissue shape, and dynamic shear flow or cyclic strain, have been shown to affect diverse cellular processes, including proliferation, differentiation, and apoptosis. These dissertation studies aim to elucidate microenvironmental stiffness effects on stem cell systems, and how they can be harnessed to improve derivation of neural cell types.

A wide temporal range of development was studied using model systems with adult and embryonic origins. First, adult neural stem cells (aNSCs) from the rat hippocampus, in soluble conditions permissive of differentiation into neurons, astrocytes, and oligodendrocytes, were found to differentiate preferentially into neurons on soft substrates with stiffness similar to brain tissue (100-1000 Pa), while stiffer substrates promoted increased astrocytic differentiation. This bias in lineage commitment due to microenvironmental stiffness cues was transduced by a cellular mechanoadaptation mechanism in which aNSCs stiffened and increased cellular contractility in response to increasing substrate stiffness. This mechanotransduction was dependent on RhoA and Cdc42 activity and occurred within only 2 days after induction of differentiation. Downstream neuronal maturation and subtype specification was also investigated. In soluble conditions inducing primarily neuronal differentiation, intermediate microenvironmental stiffnesses around 700 Pa promoted neuronal maturation and subtype specification of GABA and glutamatergic neurons. RhoA and Cdc42 activity increased neuronal maturation on softer substrates while inhibiting RhoA and Cdc42 activity abolished the stiffness-dependent differences in neuronal maturation.

Earlier periods of stem cell development were studied with human embryonic and induced pluripotent stem cells (hESCs and hiPSCs). While pluripotency marker expression and self-renewal were not affected by microenvironmental stiffness, neuronal differentiation was enhanced on softer substrates. Furthermore, softer substrates increased the percentage of

dopaminergic neurons, the cell type lost in Parkinson's Disease. Interestingly, this increase in neuronal differentiation was due to the early increase in PAX6 and SOX1 positive neural ectoderm prior to neural patterning, demonstrating that microenvironmental stiffness may also be important at early periods of development and impact downstream lineage compositions.

Finally, with the discovery of cellular reprogramming, not only can the developmental timeline can be reversed but trans-cell type reprogramming can be studied as well. Preliminary studies showed that microenvironmental stiffness has potentially interesting effects on both reprogramming somatic cells to hiPSCs, but also translineage reprogramming of somatic cells to neurons. Intermediate substrate stiffnesses around 700 Pa promoted hiPSC reprogramming. Interestingly, cell migration was greater on softer substrates but persistence or directionality was greater on stiffer substrates, suggesting that substrate stiffness effects on hiPSC colony formation is not likely due to significant differences in cell migration and collision events. Substrate stiffness also biased reprogramming of somatic cells directly into neurons with stiffer substrates promoting neuronal reprogramming.

The studies comprising this dissertation demonstrate that microenvironmental stiffness is important throughout a wide temporal range of neural development modeled by adult and embryonic/pluripotent stem cells, as well in neuronal reprogramming processes. They motivate the consideration of microenvironmental stiffness in fundamental biological studies but also as a design parameter for stem cell cultures and bioreactor systems. These studies motivate the future study of additional biophysical factors as potential regulatory cues for stem cells, especially neural stem cells.

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Chapter 1
Introduction
Mechanical and material regulation of stem cell biology

Abstract

Stem cells reside in adult and embryonic tissues in a broad spectrum of developmental stages and lineages, and they are thus naturally exposed to diverse microenvironments or niches that modulate their hallmark behaviors of self-renewal and differentiation into one or more mature lineages. Within each such microenvironment, stem cells sense and process multiple biochemical and biophysical cues, which can exert redundant, competing, or orthogonal influences to collectively regulate cell fate and function. The proper presentation of these myriad regulatory signals is required for tissue development and homeostasis, and their improper appearance can potentially lead to disease. Whereas these complex regulatory cues can be challenging to dissect using traditional cell culture paradigms, recently developed engineered material systems offer advantages for investigating biochemical and biophysical cues, both static and dynamic, in a controlled, modular, and quantitative fashion. Advances in the development and use of such systems have helped elucidate novel regulatory mechanisms controlling stem cell behavior, particularly the importance of "solid phase" mechanical and immobilized biochemical microenvironmental signals, with implications for basic stem cell biology, disease, and therapeutics.

Introduction

In the early twentieth century, scientists observed that some but not all cells could give rise to multiple specialized cell types in blood [1], and that cell proliferation and lineage specification were required for embryonic development. These observations have supported the concept that stemness – the capacity for extended self-renewal and multilineage differentiation – is attributed to individual cellular entities. The idea of the stem cell was further supported by the first bone marrow transplant in 1956 [2], in which the proliferation and differentiation of cells from the grafted marrow repopulated the hematopoietic system of a cancer patient following radiation and chemotherapy. In the 1960s, McCulloch and Till provided the first definitive and quantitative evidence for the existence of stem cells by demonstrating that bone marrow cells injected into irradiated mice formed colonies in the spleen that were clonal in nature yet gave rise to cells from three different hematopoietic lineages [3, 4].

Although stem cell behavior was initially thought by some to be determined in a purely stochastic fashion [5-8], a wealth of research has established that numerous exogenous factors – including growth factors, morphogens, cytokines, small molecules, ECM proteins, and ligands presented from adjacent cells – can strongly affect stem cell self-renewal and differentiation. This regulatory influence of the extracellular microenvironment was formally conceptualized by Schofield as the "stem cell niche" [9]. Taken to an extreme, because the cell's behavior cannot be fully realized without exogenous cues, stemness can be regarded as a collective function of the stem cell and its microenvironment, a view supported by several lines of evidence. The loss of key regulatory proteins or supporting cells in the niche can lead to the depletion of stem cells in multiple tissues [10-12]. In addition, the natural niche can actively convert non-stem cells into stem cells [13], and remarkably, this dedifferentiation may be the primary source of germline stem cells in the *Drosophila* testes [14]. Furthermore, exogenous soluble factors can help induce multipotency in specialized progenitors [15] and pluripotency in non stem cells [16]. Finally, misregulation of niche

properties may lead to tumorigenesis [17]. These examples illustrate the importance of efforts to learn more about the stem cell microenvironment.

Toward this goal, there has been major progress in elucidating the roles of small, often soluble protein factors in stem cell systems, such as Wnt proteins [18-20], insulin and fibroblast growth factors [21], and cytokines [22, 23]. This important work has been extensively reviewed elsewhere [24-27]. In addition to soluble signals, however, it is becoming increasingly clear that biology encodes and conveys regulatory information in other ways. Specifically, there are numerous aspects of the solid-state microenvironment – in particular extracellular matrix (ECM) factors, proteins immobilized to the ECM, and neighboring cells – that may play a role in regulating stem cell behavior; however, these components are comparably difficult to study, due to experimental challenges in recapitulating complex cell-matrix and cell-cell interactions *in vitro*. To address this challenge, engineered material systems in combination with analytical methods developed over the past half century have provided platforms to perform reductionist biology on solid-state biochemical and biophysical aspects of the niche. This work has initially been phenomenological, conceptually akin to cloning a new growth factor without yet knowing its receptor or downstream signaling pathways, but it has benefitted from parallel progress in the fields of signal transduction and mechanobiology. As a result of these efforts, it is becoming increasingly apparent that numerous solid-state biochemical aspects of the stem cell microenvironment are important regulators of cell behavior, including the conformational, spatial, and temporal presentation of immobilized signaling factors and adhesive ligands, as well as the biophysical context in which these factors are presented, such as the stiffness, topography, stresses, strains, and dimensionality of the system. This chapter will therefore discuss the manners and in some cases the mechanisms by which biophysical and solid-state biochemical signals can regulate stem cell function and fate.

Engineered Stem Cell Culture Systems

The microenvironments surrounding stem cells are structurally complex, rendering experiments to explore the effects of this structure on cell function difficult. For example, biophysical characteristics of a tissue are the aggregate properties of numerous ECM macromolecules and resident cells. Thus, it is not trivial to independently control and vary the biochemical and biophysical properties of this amalgam, making it challenging to study the specific effects of, for example, various microenvironmental mechanical properties on cell function. Likewise, a number of regulatory proteins are presented in a complex manner that is difficult to control and emulate *in vitro*, for instance due to complex post-translational modifications [28, 29], presentation as transmembrane proteins from adjacent cells [30], or spatially structured presentation in three dimensions (3D).

To meet the need to conduct reductionist biology on such complex environments, engineered material systems have recently been developed with the capacity to quantitatively tune one or more regulatory properties in a modular manner, enabling detailed mechanistic studies. These systems have a number of capabilities that enable them to emulate natural microenvironments. For example, biological tissues are hydrogels, or networks of insoluble, natural biopolymers that absorb sufficiently large quantities of water that the majority of the resulting material is aqueous. Accordingly, a number of natural (e.g. collagen, fibrin, and hyaluronan gels) and synthetic (e.g. polyacrylamide, alginate, poly-ethylene glycol, and self-assembling synthetic peptides) gels have been utilized as ECM scaffolds. In addition, many of these hydrogels can be used to study stem cells in both 2D and 3D. Furthermore, synthetic

materials provide several advantages over natural ones, including the ability to generate a wide range of possible stiffnesses (in 2D: 10 – 10^6 Pascals, Pa), potential inclusion of degradable crosslinks (e.g. peptide substrates for matrix metalloproteinases or photo-labile linkages), the capacity to form complex geometrical structures such as ridges and microposts by polymer-casting techniques, and protein adsorption-resistant surfaces (e.g. polyacrylamide, poly-ethylene glycol) to avoid "fouling" by soluble or secreted proteins in culture over time.

Synthetic systems can also be engineered to independently modulate biochemical properties. Adhesive ligands and/or regulatory proteins can be grafted onto hydrogels at controlled densities, while the material's mechanical properties can be independently adjusted by tuning the crosslinking density of the hydrogel's inert polymer skeleton. The bioactive ligands typically used to functionalize synthetic hydrogels include natural proteins like laminin, fibronectin, collagen, and fibrinogen [31]. Also, more specific interactions can be studied by conjugating small biomimetic peptides containing sequences such as arginine-glycine-aspartic acid (RGD), an integrin-engaging motif in ECM proteins such as fibronectin and collagen [32], onto hard surfaces or synthetic hydrogels [33, 34]. RGD and other ligands can also be spatially patterned onto synthetic surfaces using microcontact [35] or inkjet printing [36] to study the effects of ligand patterning on stem cell function.

In addition to presenting constitutive cues, materials systems can be engineered for dynamic variation in properties or application of external mechanical forces. For example, hydrogels or flexible membranes can be compressed or stretched to assess stress and strain effects on cells, for example to simulate the effects of pulsatile blood flow. Fluid can also be flowed over cells at defined velocities and shear stresses. In sum, such engineered systems have been applied to present a variety of static or dynamic biochemical and biophysical cues in a modular and quantitative fashion to explore new mechanisms through which the niche can instruct stem cell biology (Figure 1).

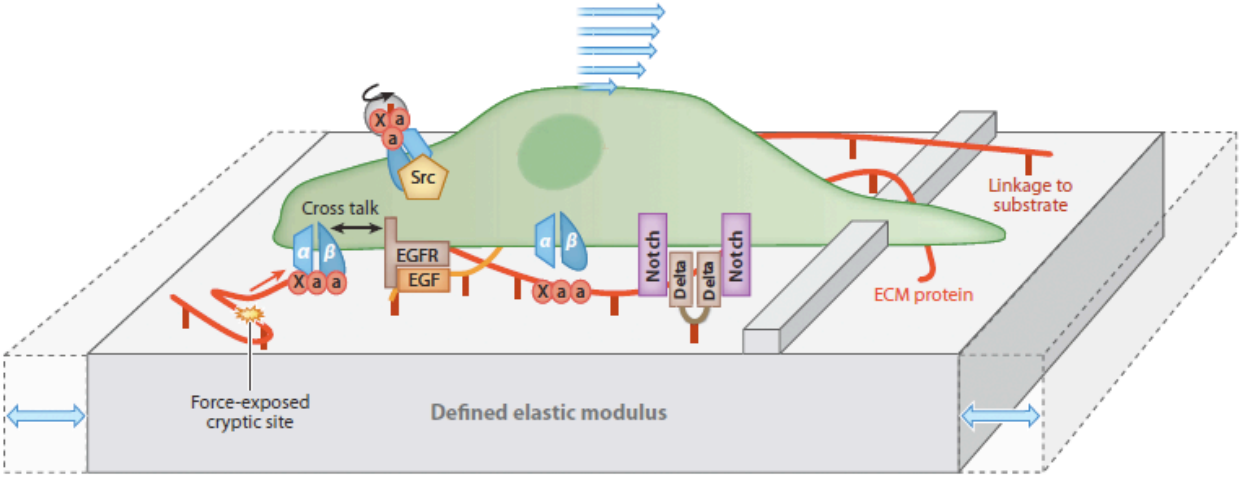

Figure 1. Numerous solid-state biochemical and biophysical microenvironmental cues regulate stem cell behavior. These include immobilized adhesive (i.e., Xaa amino acid/peptide sequence), growth [e.g., epidermal growth factor (EGF)], and morphogenic (e.g., Delta) biochemical factors interacting with cell surface receptors, for example integrins (α,β), EGF receptors (EGFRs), and Notch receptors. In addition, steric availability of receptor-ligand binding (e.g., Xaa on the free end of a protein versus in the middle of a protein), cryptic sites exposed by cell-exerted contractile forces (*red arrow*), and ligand clustering (e.g., Delta) may be necessary for or enhance biochemical signaling. Biophysical regulators include extracellular matrix (ECM) elastic modulus, topography such as ridges, and strains and stresses imposed by stretching the ECM, flowing fluid over cells, and locally twisting magnetic microbeads on cell surfaces [*gray sphere* functionalized with arginine-glycine-aspartic acid (RGD)

peptide]. Blue arrows signify external applications of force; Src is a mechanotransductive tyrosine kinase associated with focal adhesions.

Influence of Solid Phase Biochemical Properties

Specificity of interactions in biological systems is crucial for developing and maintaining the structure and function of organisms, tissues, and cells. For stem cells, this specificity is largely determined by the biochemical nature of the surrounding microenvironment, i.e. the molecular identities of soluble factors, ECM components, or factors on the surfaces of other cells. Much work has focused on the specific identities of these factors and their important effects on different stem cell types; however, the contextual manner in which these moieties are presented is also highly important, including potential immobilization on scaffolds or particles, molecular conformation and clustering, and temporal presentation. Here we acknowledge the importance of biochemical specificity in stem cell-microenvironment interactions, but emphasize the effects of the *contextual presentation* of solid-state biochemical factors on stemness.

Adhesive Ligands

Specific ECM-cell and cell-cell interactions are important in providing spatial anchors as well as signals that regulate stem cell maintenance, survival, and differentiation. Furthermore, cell adhesion is also required for a cell's ability to sense other contextual information, such as the mechanical properties of the microenvironment. Therefore, we begin by reviewing the importance of the specific identities of biochemical ligands in the solid phase of natural systems, as well as ways in which engineered systems have been utilized to both identify functional adhesive peptides sequences and to investigate their interactions with stem cells.

Anchoring or localization to proper niches is important for stem cell viability and function, since without proper localization, stem cells may not be exposed to proper survival and differentiation signals. The earliest known example of adhesive ligands regulating stem cell localization and maintenance is in the reconstitution of the hematopoietic system of cancer patients, where transplanted hematopoietic stem cells (HSCs) were found to relocate to bone marrow niches following chemotherapy or radiation [2, 37]. This clinical observation has motivated subsequent mechanistic research. In non-human primates, injection of antibodies against α4β1 integrin – which is expressed on HSCs and binds to fibronectin [38] and to the cell-surface sialoglycoprotein vascular cell adhesion molecule 4 (VCAM-4) [39] – mobilizes CD34+ hematopoietic progenitors and granulocyte/macrophage-colony forming cells to the bloodstream [40]. Furthermore, conditional ablation of β1 integrin yields HSCs that are unable to engraft in irradiated recipient mice [41]. The concept that key adhesive interactions are necessary for niche localization has been extended to other systems. In mice, ablation of β1 integrin but not the cell-cell adhesion protein E-cadherin impairs the ability of mouse spermatogonial stem cells to repopulate recipient testes, likely through a decreased ability to associate with the adhesive protein laminin [11]. Interestingly, in *Drosophila* testes the anchoring interactions of germline stem cells appears not to be integrin-based but instead to rely on DE-cadherins presented by adjacent "hub" cells [42]; however, integrins, specifically those containing the βPS subunit, do regulate the localization of the hub cells to the niche.

In addition to anchoring and maintaining stem cells within their niche, adhesive ECM and cell surface proteins also activate signals well known to regulate maintenance and differentiation. For example, the RGD sequence known to bind β1 integrins increases expression of integrin-linked kinase, whose subsequent activation of Akt supports human mesenchymal stem cell (hMSC) survival [43]. Similarly, survival of erythroid progenitors is enhanced by their binding to

fibronectin via the α4β1 integrin, which upregulates anti-apoptotic Bcl-xL [44]. Stem cell differentiation can also be regulated by adhesion to ECM proteins. hMSCs can be induced towards an osteogenic lineage by culturing them on laminin-5, which ligates α3β1 integrin, activates ERK, and leads to phosphorylation of the osteogenic transcription factor Runx2/CBFA-1 [45]. These studies demonstrate the integral role of the adhesive microenvironment in activating canonical cell signaling pathways.

To date, many *in vitro* studies examining the role of the ECM in stem cell systems have involved adsorption of natural ECM proteins such as laminin and fibronectin to traditional cell culture surfaces; however, the use of intact proteins presents several challenges. These large macromolecules contain numerous receptor-binding motifs, rendering it difficult to determine which one or ones are functionally important in regulating a key cell function. In addition, recombinant production is difficult, and their isolation from tissues often results in biochemically heterogeneous mixtures. Therefore, engineered systems have often instead utilized synthetic, ECM-based motifs or peptides, singly or in combination, thereby in principle enabling a "dissection" of the relative importance of specific receptors in transducing an ECM signal.

For example, RGD-containing peptides, which engage a subset of integrins, have been increasingly used to functionalize synthetic matrices for stem cell culture [46] and were recently adapted to form self-assembling peptide hydrogels capable of encapsulating neural stem cells (NSCs) without the need for synthetic polymer matrices [47]. Another peptide sequence prevalent in synthetic matrices, the isoleucine-lysine-valine-alanine-valine (IKVAV) motif found naturally in laminin, enhances the neuronal differentiation of neuronal progenitor cells when incorporated into self-assembling peptide hydrogels [48]. In addition, some stem cell cultures, like human pluripotent stem cells, require culture on complex blends of proteins or feeder cells with multiple unknown binding motifs to maintain growth and pluripotency. For example, Matrigel, a complex mixture of hundreds of ECM and other proteins derived from Engelbreth-Holm-Swarm mouse sarcoma, [49] has emerged as the standard substrate for human embryonic stem cell (hESC) and human induced pluripotent stem cell culture. To investigate adhesive interactions involved in Matrigel maintenance of hESC pluripotency, Meng and colleagues used blocking antibodies to identify αvβ3, α6, β1, and α2β1 integrins as functionally contributing to hESC attachment to Matrigel [50]. Adhesive peptide sequences adopted from laminin-111 were then chosen based on their ability to bind those integrins, and the authors found that while three peptides individually are able to support hESC growth and pluripotency for short periods of time (4 days), their combination enhances both the quality of cultures (i.e., the number of colonies) and duration over which pluripotency was maintained (> 7 days). This strategy emphasizes the ability of engineered systems to parse out the synergistic contribution of individual motifs within full-length natural protein and may inspire future mechanistic studies.

However, one challenge for the field is that beyond RGD and several others, there are simply limited numbers of known ECM-based motifs that engage specific adhesion receptors. The existence of numerous families of ECM proteins and cell surface receptors (e.g. 24 known integrin heterodimers in mammals [51]) suggests that developing other peptidomimetic ligands will enable the investigation of a broader range of ECM-cell interactions. Rational identification of short adhesive motifs from ECM has yielded the peptides widely utilized to date; however, library approaches may lead to the identification of additional natural sequences, and it is not even necessarily clear that an optimal adhesive peptide must exactly correspond in sequence to an ECM protein. One recent study employed phage display of a library of random 12-mer peptides to "pan" for peptides that bind hESCs. Two novel sequences that did not align to any

known extracellular protein were found to support extended hESC proliferation and maintenance of pluripotency on a self-assembled monolayer (SAM) surface. Interestingly, these peptides do not bind via integrins or proteoglycans [52], suggesting that adhesive interactions used for *ex vivo* culture of stem cells need not be limited to those found *in vivo*, though it remains to be determined whether other proteins adsorb to the SAM surface over time. In all, the combined use of rational and library-based screening methods will provide an increasing number of ligands for functionalization of synthetic systems and may aid mechanistic investigation of specific receptors and signaling events involved in regulating stem cell responses to their microenvironments.

Immobilization of growth factors and morphogens

The ECM offers sites for cell adhesion, but it can also serve as a platform for the presentation of other biochemical factors and orchestrate cell-cell interactions. While the stem cell field has often investigated growth factors, morphogens, and cytokines as soluble factors, many of these proteins have matrix-binding domains such that they may be presented within the niche as "solid phase" ligands. For example, Sonic hedgehog (Shh) binds vitronectin [53] while Hedgehogs in general, fibroblast growth factors (FGF), platelet derived growth factors (PDGF), vascular endothelial growth factors (VEGF), and several cytokines have heparin-binding domains [54-58]. Furthermore, numerous important ligands are integral membrane proteins presented from the membrane of adjacent cells, such as the Notch ligand families Delta and Serrate/Jagged [59]. Immobilization of factors may have several consequences, including increasing their local concentration and establishing concentration gradients emanating from the source [60], promoting sustained signaling by inhibiting receptor-mediated endocytosis [61, 62], and modulating the spatial organization or molecular conformation of factors to enhance signaling. Several engineered systems have been utilized to study these effects.

One biomimetic strategy to immobilize factors harnesses the affinity of some for heparin. In one study, heparin-binding peptides were crosslinked to a fibrin gel to enable non-covalent attachment to heparin, and the material was then loaded with the heparin-binding factors neurotrophic factor 3 (NT-3) and PDGF. The resulting material was shown to induce neuronal and oligodendrocytic differentiation of mouse NSCs while inhibiting astrocytic differentiation [63]. The protein factors were released over 1-14 day ranges, a capability that could be utilized for studying kinetic effects of signaling, controlled delivery of factors in transplanted engineered tissues, or potential extensions in the active lifespan of factors *in vitro*. In addition to natural, non-covalent matrix binding, covalent linkage of factors is an effective means to biofunctionalize materials. For example, Shh covalently grafted to a polymer hydrogel surface was shown to promote the osteogenic differentiation of MSCs [64], while linkage of leukemia inhibitory factor (LIF) to thin film polymer coatings supported mouse ESC pluripotency for 2 weeks without the addition of soluble LIF [65]. In addition, covalent tethering of epidermal growth factor (EGF) was shown to sustain MAPKK-ERK signaling in hMSCs and to achieve greater cell spreading and survival over unfunctionalized substrates in the presence of saturating levels of soluble EGF [66]. Finally, in work that extended this concept beyond proteins, the small chemical groups phosphate, t-butyl, and carboxylic acid were tethered to synthetic scaffolds to mimic the functional moieties exposed in mineralized bone, hydrophobic lipids in adipose tissue, and glycosaminoglycans prevalent in native cartilage, respectively. Interestingly, these chemical groups were shown to induce hMSC differentiation into osteogenic, adipogenic, and

chondrogenic lineages, respectively, in the absence of traditional soluble or immobilized morphogenic factors [67].

There is also evidence that immobilized growth factors, morphogens, and integral membrane protein ligands may act synergistically with one another or with ECM adhesive ligands. For example, culturing NSCs on immobilized NT-3 with fibronectin, but not laminin, enhances both neuronal and astrocytic differentiation [68]. Known crosstalk between growth factor receptor and integrin signaling through their intracellular domains may be responsible for this synergy [69, 70], and immobilization of ligands for both receptor classes may enhance this synergy by clustering their intracellular signaling domains. The above studies demonstrate that immobilization has important and sometimes necessary functional roles in stem cell systems, and the ability to immobilize factors in well-controlled and defined engineered cell culture systems may allow deeper mechanistic questions to be addressed in the future.

Ligand conformation

In addition to their manner of presentation, the molecular structure or conformation of these factors, as well as the accessibility or presentation of binding motifs within these factors, are important for their function. Altering the molecular conformation of ligands may form novel active sites or expose cryptic binding sites. For example, cell-generated forces have been found to unfold fibronectin, thereby exposing cryptic sites [71, 72] that have various biological activities, including self-assembling into fibronectin fibrils, binding tenascin, and cleaving collagen [73-75].

The molecular conformation of immobilized ligands is also dependent on the chemical nature of the surfaces to which they are absorbed. For example, fibronectin adsorbed to hydroxyl and amine-terminated surfaces promoted osteogenic differentiation more so than adsorption to carboxylic acid and methyl-terminated surfaces. These observations correlated with differences in the binding of antibodies to epitopes within fibronectin adsorbed to these different surfaces, a result attributed to different conformations of the fibronectin [76]. In addition to passive adsorption, covalent attachment chemistry and the steric availiability of ligands for binding can also regulate the activity of grafted synthetic peptides [77].

Spatial presentation of regulatory factors

In addition to the properties of individual ligands, collections of multiple ligands can exhibit higher degrees of spatial organization at the nanoscale as well as microscale. Nanoscale spatial clustering of ligands and receptors, such as at focal adhesions [78], can bring them into closer relative proximity, increase the local intracellular concentrations of signaling effectors (e.g., focal adhesion kinase, paxillin, and Src), and thereby enhance activation of downstream pathways such as the MAPK/ERK cascade [79, 80]. For example, clustered RGD ligands attached to the termini of star-shaped polymers promote motility in non-stem cells [81], likely via their clustering of integrins and subsequent enhancement of downstream signaling events such as focal adhesion kinase activation [82]. In addition, clustering of the Notch ligand Delta is necessary for Notch activation in numerous systems [83]. For example, in neural crest stem cell cultures, addition of antibody-clustered Delta inhibited neuronal and promoted glial differentiation [84]. Interestingly, in other stem cell systems, immobilization of Delta on a cell culture substrate or beads is necessary for downstream Notch signaling [85], T cell differentiation from HSCs [86], and the activation of hematopoietic cord blood progenitor cells for subsequent engraftment in bone marrow [87]. Some evidence suggests that mechanical forces exerted by ligation to clustered or immobilized Delta may be necessary for exposure of the Notch cleavage site [88]

In addition to nanoscale features, micron-scale patterning of adhesive or regulatory factors may regulate subcellular localization of signaling proteins, thus affecting cytoskeletal organization and organelle localization. In stem cells, asymmetric spatial presentation, in which only one side of the stem cell is exposed to specific adhesive ligands, has been shown to regulate cell behavior in natural niches, including asymmetric divisions of stem cells in hematopoietic [12], keratinocyte [89], hair follicle [90], esophageal epithelial [91], and germinal [92] stem cells. This effect can occur through orienting the centrosome and mitotic spindle perpendicular to the adhesive ligands [93].

The degree of asymmetric signal presentation can be finely controlled in culture through microcontact printing, which can be utilized to control ligand density and even cell shape. By patterning small and large islands of adhesive protein on a 2D surface, Chen and colleagues demonstrated that small and round MSCs preferentially differentiate into adipocytes, whereas spread cells differentiate into osteoblasts [94]. These shape-based effects are regulated by RhoA signaling and downstream actomyosin contractility, connecting cell shape changes induced by biochemical patterning of ligands to changes in cellular mechanics, properties that will be discussed in detail below. Another mechanism through which adhesive patterns, and therefore cell shape, may affect stem cell function is by directly altering nuclear shape, which has been suggested to modulate gene expression in osteogenic cells [95].

Micron-scale presentation of ligands can also regulate the multicellular organization of stem cells, as shown *in vivo* and in engineered systems. Early in development, the multicellular organization of stem cells is partially regulated by the spatial patterns of cell-cell and cell-ECM contacts during important processes such as germ layer segregation and neural tube formation [96]. ECM proteins have also been shown to differentially pattern epidermal stem cells and their progeny, transit amplifying cells, based upon the higher expression levels of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins on the stem cells [97]. The higher integrin expression levels anchor epidermal stem cells to collagen IV and the tips of the dermal papillae, while allowing for the migratory behavior of transit amplifying cells away from the stem cells toward the tips of the rete ridges nearer the dermis [98]. Finally, micron-scale patterning can also affect multicellular shape and mechanics as it can for individual MSCs. Patterned multicellular structures of hMSCs exhibit distinct differentiation patterns, as cells on the concave edges of structures experience high tension and differentiate into osteoblasts, while those on the convex or low tension edges generate adipocytes [99]. This study strongly emphasizes the intimate connection between the spatial organization of a material's biochemical properties and its control over the mechanical properties of stem cells. In addition, this example motivates the need to investigate how biophysical and biochemical properties of an environment can collaborate to regulate cell function.

Influence of Biophysical Properties

Just as the mammalian body exhibits incredible diversity in biochemical interactions and specificities, it also exhibits a wide range of biophysical properties defined not by the specific identities of interacting molecules but by their collective structural and mechanical characteristics. Examples of this diversity include the palpable differences in the stiffnesses of fat vs. bone tissue and the different topographies of layered 2D-like epithelial and intestinal sheets and bulk 3D liver and pancreatic parenchyma. In addition to differences in static biophysical properties, organisms are inherently dynamic, as is evident in bulk motions such as joint bending, muscle contraction, compressive impact and strains on tissues, and pulsatile flow of the circulatory system. There is even evidence for the generation of strong forces due to cell

adhesion and migration during embryonic development [100]. These large internal variations in the structure and mechanics of various tissues, and consequently in their resident stem cell niches, suggests that in addition to solid-state biochemical signals, stem cells may respond to biophysical properties of the microenvironment.

Elastic Modulus

Of all the many mechanical properties of biological systems, stiffness or rigidity is perhaps the most apparent and widely-studied. In general, the mechanical stiffness of a material can be determined by measuring its complex modulus, the ratio of stress (force per unit area) to strain (fractional deformation) applied to a material. This resulting value reflects the material's ability to both store and frictionally dissipate the applied mechanical energy, as reflected by a storage (elastic) modulus and loss (viscous) modulus, respectively. Tissues and cells are often viscoelastic in that they exhibit both fluid- and solid-like properties, but the viscous component has proven challenging to systematically measure and vary, and its investigation awaits the development of future material systems. However, the elastic modulus, the measure of the stress required to achieve a specific strain in a material without any *permanent* deformation, has been studied and emerged as an important regulator of stem cell function. The elastic moduli of various tissues range over four orders of magnitude from <1 kPa for fat [101], brain [102], and mammary tissue [17] to ~10 kPa for skeletal muscle [103] and 10 MPa for bone [104]. Individual tissues can also contain significant internal heterogeneities in stiffness, such as the nearly threefold variations in stiffness reported within the hippocampus of the brain [105]. In stark contrast, the typical surfaces used to culture cells (e.g. plastic and glass) have supraphysiological stiffnesses (> 1 GPa) [106], as much as 10 million fold stiffer than a natural stem cell microenvironment. This raises the question of whether stiffness can contribute to regulating stemness.

Mesenchymal Stem Cells

Because MSC-derived lineages are typically associated with "load-bearing" connective tissues that possess diverse mechanical properties (e.g., bone, muscle, and fat), MSCs are a particularly appropriate system for investigating mechanoregulation. In landmark work, Engler and colleagues found that hMSCs cultured on polyacrylamide gels (functionalized with type I collagen) that mimicked the stiffnesses of bone, muscle, and neural tissue preferentially differentiate into these corresponding specialized cell types [107]. This effect requires inclusion of a cocktail of soluble differentiation factors; however, culturing MSCs on different stiffness substrates in the absence of these soluble factors restricts their potency to the corresponding cell type upon later addition of soluble factors. This suggests that ECM stiffness on its own may have the capability to restrict potency, with subsequent differentiation requiring soluble factors.

In addition to modulating lineage commitment, there is also evidence that substrate stiffness can regulate MSC self-renewal. Similar to many specialized cell types that proliferate faster on stiffer substrates, hMSCs remain quiescent on soft substrates but proliferate on stiffer substrates functionalized with a mixture of type I collagen and fibronectin [108]. Likewise, partially committed osteoblastic cells proliferate at a higher rate on stiffer substrates [109]; however, multipotent mouse MSCs proliferate at similar rates on RGD-functionalized substrates of varying stiffnesses. Thus, while ECM stiffness is an important regulatory cue for MSC behavior, specific phenotypes may depend on details such as the adhesive ligand(s) and the species or tissue origin.

Neural Stem Cells

The brain is not exposed to exogenous mechanical forces in the same manner as bone and cartilage; however, brain function is exquisitely sensitive to altered intracranial pressure, and NSCs normally exist in mechanically heterogeneous niches. For example, the hippocampus varies in elastic modulus from 100 to 300 Pa in the CA1 and CA3 subregions, respectively. In addition, brain tumors can be delineated by ultrasound based on the density differences in tumor vs. normal tissue [110], and glial scars may in part prevent nerve regeneration by forming mechanical barriers, an effect interestingly attenuated by implantation of a soft hydrogel material [111, 112]. In this context, Saha and colleagues cultured adult hippocampal NSCs on RGD-functionalized, variable modulus hydrogels in the presence of soluble factors that promote either cell proliferation or differentiation. They found that NSCs optimally proliferate on an intermediate stiffness (~500 Pa) characteristic of brain tissue, and under conditions that strongly promote neuronal differentiation, optimally mature into neurons at the same intermediate stiffness. Furthermore, under conditions that promote mixed neuronal and astrocytic differentiation, NSCs differentiate predominantly into neurons on soft substrates (>90% neurons on 10 Pa gels) and into astrocytes on hard surfaces (>50% astrocytes on 10 kPa gels) [113]. A subsequent study in which NSCs were embedded in a 3D alginate gel of variable stiffness reported analogous findings [114], and collectively these results indicate that NSCs respond strongly to a combination of biochemical and mechanical cues.

For NSCs derived from the subventricular zone (SVZ) of the adult forebrain, a similar increase in neuronal differentiation is observed on soft, laminin-coated, methyacrylamide chitosan substrates [115]. However, astrocytic differentiation is low on all substrates (<2%) for these NSCs, and oligodendrocytic differentiation is favored on stiffer substrates (>7kPa). These differences in glial differentiation could be due to different anatomical origins of the adult NSCs. Likewise, NSCs derived from rat embryos and cultured on fibronectin rather than laminin exhibited increased astrocytic differentiation on softer substrates with low neuronal differentiation (<10%) on all substrates [116], indicating that both NSC origin and ECM can influence mechanoregulation of fate choice.

Potential mechanisms of modulus response

A rich mechanobiology literature suggests many possible mechanisms that may regulate ECM modulus effects on stem cell behavior. Several mechanotransductive proteins have been studied for their role in regulating cellular processes, including G-protein coupled receptors [117] and focal adhesion kinase [118] as well as integrins and Rho GTPases [119]. In addition, biophysical cellular responses such as changes in cell shape, contractility, stiffness, or cytoskeletal architecture may regulate stem cell responses by modulating nuclear architecture and/or transcription and transcription factors [95, 120], intracellular and cytosol-nuclear transport [121], or localization of signaling factors through cytoskeleton-mediated sequestration [122, 123].

Several studies indicate that a combination of such mechanisms may be important in stem cells, in particular changes in cellular contractility regulated through RhoA signaling and acto-myosin based forces. In hMSCs, inhibition of myosin II abrogates the effect of ECM stiffness on hMSC differentiation into all lineages [107]. Furthermore, decreasing ECM stiffness decreases RhoA activity and subsequently Ca^{2+} signaling in hMSCs [124], pathways known to regulate acto-myosin contractility. Interestingly, RhoA signaling may also regulate NSC differentiation, as suppression of Rho GDIγ decreases RhoA expression and increases the neuronal but not glial differentiation of immortalized murine neuronal precursors [125]. Although it is unclear if this is a

mechanical effect, recent work indicates Rho GTPase signaling transduces ECM modulus cues into biases in adult hippocampal NSC lineage commitment (our unpublished data). Interestingly, changes in cellular stiffness may also be intimately linked to cellular shape, as RhoA was also implicated in regulating the hMSC differentiation response to cell shape [94]. Future work may reveal additional mechanistic links between solid-state biochemical and biophysical cues.

Stress and Strain

In addition to intrinsic mechanical properties of the microenvironment such as modulus, extrinsic mechanical perturbations, specifically the application of forces or stresses that induce deformation or strain, are important characteristics of microenvironments surrounding stem cells. Tissue-scale examples of such dynamic, mechanical perturbations include stretching and contraction of tendons, ligaments, and musculature, as well as cyclic loading of vasculature. The mechanically dynamic nature of tissues suggests the potential importance of stress and strain in regulating stem cell behavior in native settings [126, 127]. In addition, the different modes of stress application (Figure 2) – including tensile, compressive, torsional, and shear forces – may influence stem cell behaviors in diverse ways.

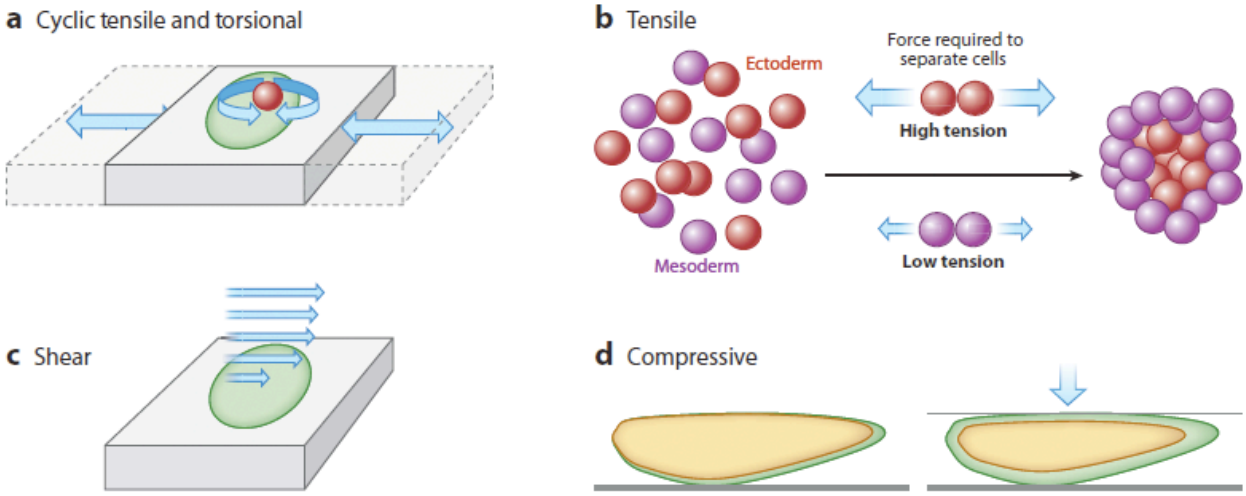

Figure 2. Mechanical forces have been applied to stem cell microenvironments and stem cells themselves in several distinct modes. (*a*) Cyclic tensile (*linear arrows*) and torsional (*rotational arrows*): Cyclic stretching of cell culture substrates regulates mesenchymal stem cell and embryonic stem cell differentiation. (*b*) Tensile: Distinct tensile forces between cells govern zebrafish germ layer organization (Krieg et al. 2008). Greater forces (*blue arrows*) are required to separate two ectodermal (*red*) compared with mesodermal (*purple*) cells.(*c*) Shear: Shear stress/strain regulate vascular and endothelial stem cell differentiation. (*d*) Compressive: Compression upregulates *twist* expression (*green region*) in the *Drosophila* blastoderm embryo (Farge 2003). In all panels, blue arrows signify applications of force.

Tensile and compressive strains

Tensile (stretching or elongating) and compressive strains have been observed at the cellular level in embryonic systems. In *Drosophila* embryos, artificial compression of cells induces expression of Twist, an important factor regulating germ layer specification and patterning [128]. Natural tissue dynamics during development, such as germ layer extension, may utilize this compressive mechanism to induce expression of patterning genes. Similarly, tensile strains may also be important in development and were recently shown to regulate zebrafish gastrulation, the first stage in vertebrate development where progenitors undergo sorting and

assembly into the distinct germ layers [129]. Contractile tension in the actin-myosin mesh comprising the cell-cortex, measured by atomic force microscopy (AFM) indentation, was found to vary almost two-fold within the embryo, with ectodermal progenitors exhibiting the highest tension and endodermal progenitors the lowest. When individual progenitors from different germ layers are mixed *in vitro*, ectodermal progenitors sort to the inside of heterotypic mixtures, as anticipated due to their high cell-cortex tension. Interestingly, this germ-layer sorting does not correlate with cell-cell adhesion strengths as determined by AFM, while genetic and pharmacological reduction of cellular contractility ablates the cell sorting behavior, supporting the hypothesis that cell-cortex tension is important in regulating germ layer patterning. Given the wealth of literature on the role of cell-cell adhesions in development and the requirement of cell adhesions to transmit tensional forces, it is likely that a combination of the differential cell-cortex tensions and adhesive forces between cell types may contribute to regulating germ layer specification, gastrulation, and other early developmental processes [96].

In addition to mechanical properties that vary on a developmental timescale, cyclic strains are an important feature of many natural microenvironments that can also influence stem cell behavior. Stretching lung embryonic MSCs stimulates expression and nuclear localization of tension induced/inhibited protein-1 (TIP-1) and inhibits expression of TIP-3, thereby promoting myogenesis and inhibiting adipogenesis, respectively. These proteins have been shown to act as transcriptional coactivators that enhance histone acetyltransferase activity at histones H3 and H4 within myogenic and adipogenic promoters [130]. Cyclic stretching also inhibits differentiation of hESCs through the upregulation of TGFβ1, Activin A, and Nodal and subsequent phosphorylation of Smad 2/3 [131]. By contrast, when a localized cyclic stress is applied by magnetically twisting a 4 μm diameter RGD-coated bead bound to the surface of mouse ESCs, expression of the pluripotency marker Oct3/4 is significantly reduced [132].

Shear flow

Another form of dynamic stress application is shear flow, which is most often associated *in vivo* with the circulatory system. While the effect of shear stress on vascular function and endothelial cell behavior has been appreciated for decades, shear stress more recently has been found to be important in regulating stem cell function as well. Early work demonstrated that shear flow promotes the maturation and capillary assembly of endothelial progenitor cells [133]. Subsequent studies have found that shear flow can induce differentiation of several stem cell types including murine MSCs [134] and ESCs [133, 135, 136] into specialized endothelial or cardiovascular cells. One study identified a potential epigenetic mechanism, as laminar shear stress enhanced total nuclear levels of acetylation at H3K14 and methylation at H3K79 while upregulating transcription from the VEGF-2 promoter as well as other vascular-related genes [136]. While this work demonstrates the importance of shear stress in vascular differentiation, two recent studies have specifically demonstrated the importance of shear stress in embryonic vascular development. North and colleagues demonstrated in zebrafish as well as mouse embryos that blood flow is necessary for the proper development of HSCs in the embryonic aorta-gonad-mesonephros (AGM) region. Activation of nitric oxide (NO) signaling was able to rescue hematopoiesis even in the absence of blood flow, implicating NO as a mechanotransductive signal [137]. Adamo and colleagues arrived at a similar result using a miniaturized *in vitro* flow chamber. Mouse ESCs cultured under shear flow expressed higher levels of CD31 and Runx1, proteins expressed in endothelial cells, and generated more

hematopoietic colony-forming units. Inhibition of NO production abrogated this shear flow effect [138].

Topography

Mechanical properties such as elastic modulus, stress, and strain play clear roles in regulating stemness. However, other biophysical properties include structural characteristics such as topography, a material's surface profile and shape. Topographical structures such as grooves, ridges, and pits are present in many natural systems at the nanoscale, such as the fibrous structure of collagen and other ECM proteins, as well as at the microscale, such as pores in bone marrow and undulating basement membranes in the epidermis. The presence of topographical information in natural systems motivates the use of technologies such as soft lithography, microfluidics, electrospinning, and deposition of nanostructures [139-141] to engineer a material's topography to study stem cell responses to both nano- and microtopography.

MSCs are likely to encounter and be influenced by these types of topographical cues in their tissues, and several studies using engineered ECM systems strongly support the concept that topography regulates cell function. Culture atop vertically oriented nanotubes of 70-100 nm in diameter (but not <30 nm), induces hMSCs to differentiate into osteoblasts in the absence of osteogenic media [142]. It was hypothesized that the larger-diameter nanotubes would place adhesion clusters farther apart, requiring the hMSCs to stretch and generate high internal tension, analogous to the use of a broad ECM island [94] or a stiff ECM [107]. Interestingly, culturing hMSCs on nanopits of the same length scale as the nanotubes, approximately 100 nm, also induces osteogenesis in the absence of osteogenic media. This study also identified anisotropic, or disordered, presentation of the nanopits as necessary for osteogenesis [143]. The disordered or asymmetrical nanopit presentation may be required for induction of cell polarity or of cellular heterogeneity within the monolayer culture, which could generate either intra- or extra-cellular gradients of soluble or cell-surface signaling molecules, respectively.

Fibrous proteins like collagen and laminin are also present in vascular basal lamina in the brain, suggesting NSCs could also be responsive to nanoscale topography. Indeed, culturing adult rat hippocampal NSCs on laminin-coated synthetic polyethersulfone fibers with 280 and 1500 nm diameters increases oligodendrocytic and neuronal differentiation, respectively, in differentiation-inducing media [144]. In the presence of growth factors, NSC proliferation increases with decreasing fiber diameter. Interestingly, NSCs spread extensively on smaller diameter fibers, raising the possibility of cell shape regulation of NSCs as previously observed for MSCs [94]. Collectively, these studies suggest nanoscale topography may act through regulating the spatial presentation of ligands and regulatory factors, or altering cellular morphology or mechanics, to modulate cell function, representing another example of the interplay between biochemical and biophysical cues.

At the microscale, NSCs are exposed to numerous topographical features in the brain, including many crevasses and undulations as well as intersections of layers of different cell types. Mimicking this topography, adult hippocampal NSCs have been co-cultured with astrocytes on micron-scale grooves etched into polystyrene substrates by photolithographic and reactive ion etching techniques. The NSCs aligned with the grooves and subsequently generated higher percentages of neurons on grooved compared to control flat substrates [145]. Several potential mechanisms may sense these topographical cues, including acto-myosin and RhoA signaling, which also have been implicated in regulating micropost inhibition of fibroblast

proliferation [146], in modulus sensing for NSCs and MSCs as discussed earlier [107], and in cell shape-mediated effects on MSCs [94].

A more specialized neural precursor, an oligodendrocytic progenitor cell (OPC), is also sensitive to topographical cues, indicating that progenitors can be topographically sensitive at multiple stages of specialization. Rat OPCs *in vivo* have been observed to differentiate at approximately postnatal day 8, a phenomenon traditionally thought to be regulated by an intrinsic timer. However, *in vitro*, OPCs differentiate at a rate dependent on cell density, not absolute time. Rosenberg and colleagues hypothesized this effect was not due to increased paracrine signaling or cell-cell contacts with increasing cell density but that it was a physical, steric effect. To test this hypothesis, rat OPCs were cultured with polystyrene beads that were biochemically non-interactive with OPCs. Interestingly, beads of intermediate size, 20 μm, were observed to induce oligodendrocytic differentiation whereas 5 and 100 μm beads were not, indicating that OPCs sense specific length scales of topographical cues on the size scale of the OPCs themselves (~20 μm) [147]. Despite the differences in length scales of topographies for this and the above examples, nano- and microscale topographies appear to induce some analogous changes in cell shape and morphology and thus may act through common signaling pathways, such as Rho GTPases, to regulate stem cell behaviors. Systems engineered to investigate the relative effects of different length scale topographies, as well as biochemical ligands patterned on different size scales, may help elucidate common mechanisms.

The numerous examples above of stress and strain in stem cell systems have identified several signaling pathways that mediate mechanotransductive responses. However, as mentioned throughout this review, the observed effects of a particular cue may be partially or fully dependent on the presentation of other microenvironmental signals or conditions, and a recent study that investigated the interplay between topology and mechanics further illustrates this point. hMSCs were cultured on polydimethylsiloxane membranes micropatterned with grooves to align the hMSCs in one direction. When 5% and 1 Hz strains were applied parallel to the grooves, hMSCs upregulated the smooth muscle cell marker calponin 1 and downregulated chondrogenic and osteogenic markers, as well as increased their proliferation. However, when the topographical cue was altered so hMSCs were aligned perpendicular to the strain, the majority of these observed phenotypes were no longer induced [148].

Dimensionality

A wealth of cell biological knowledge has emerged from studying cells in 2D cell culture systems; however, topographical studies discussed above, while not fully 3D, hint at the importance of 3D features in regulating stem cell behavior. While there are 2D-like cellular structures *in vivo* – including epithelial sheets, endothelial layers, and epidermis – these as well as organs, tissues, and niches in general occur in a 3D context. 3D culture presents several important differences and considerations including slower diffusive transport of soluble factors, the natural or engineered formation of gradients of signaling factors, and spatial presentation of regulatory factors from all directions. Thus, studying stem cells in 3D is arguably one of the most important future directions for stem cell research.

Stem cell systems that are already traditionally grown in 3D include hESC colonies and embryoid bodies (EB). hESCs typically are cultured in cell clusters over 100 μm thick, adhered to feeder cells or Matrigel on 2D substrates, whereas EBs are aggregates of differentiating cells grown in suspension. 3D culture may in some ways recapitulate early stages of embryonic development, where the establishment of spatial gradients of factors, due to transport limitations

of soluble factors, functions to pattern early tissue structures. For example, mouse EBs cultured in serum-free media have been found to spontaneously form patterned and polarized neural tissue mimicking the temporal and spatial patterning in natural developmental corticogenesis [149]. In serum-containing conditions, mouse EBs exhibit gastrulation-like patterning dependent on Wnt signaling [150]. Interestingly, both studies found that controlling cluster size and generating relatively homogeneously sized EBs simply by aggregating single cells on low-adhesion plates or in hanging drops improves the efficiency of pattern formation and controls the rate of differentiation, respectively. Engineered systems have been developed to study the effects of cluster size more precisely. Microwells fabricated via lithography and polymer-casting techniques allowed for generation of distinct EB sizes of 150 and 450 μm [151]. Intriguingly, small EBs express higher levels of Wnt5a, and large EBs express higher levels of Wnt11. EB size control over Wnt signaling, in the context of Wnt signaling driving gastrulation-like patterning of EBs [150], suggests that EB size may result in differential gradients and molecular transport of signaling morphogenic molecules, thereby influencing patterning.

hESC colony sizes have also been controlled using microwells, which results in more homogeneous colony sizes compared to typical hESC cultures on 2D substrates [152]. Microcontact printing of adhesive islands also restricts hESC colony sizes as well as regulates differentiation, with smaller hESC colonies generating more endoderm over ectoderm [153]. Thus, for both ESCs and EBs the 3D size and shape of cellular assemblies likely regulate cell function through mechanisms relevant during organism development – spatial signaling gradients, changes in the spatial presentation and identities of cell-cell contacts, and potentially mechanical asymmetries – and the controlled investigation of these effects on cell function represents an interesting avenue for future research.

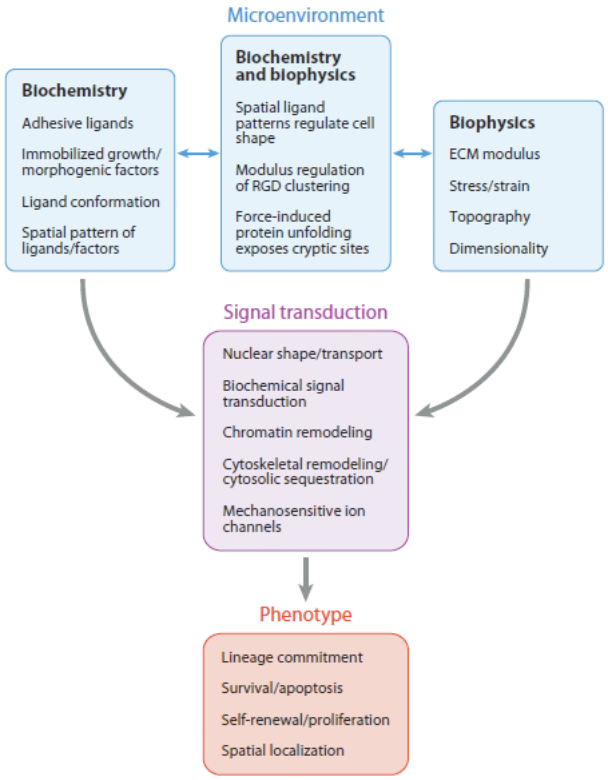

Figure 3. Solid-state biochemical and biophysical microenvironmental cues are highly interdependent (*blue*). Microenvironmental cues may be sensed and transduced by numerous cellular mechanisms (*purple*) resulting in

changes in stem cell phenotype (*red*). Engineered systems with the capability to regulate solid-state biochemical and biophysical properties, such as the spatial presentation of adhesive ligands and material modulus, will enhance research to identify interdependencies between regulatory cues, such as the regulation of cell shape by ligand patterning. Furthermore, these culture systems in combination with molecular cell biological techniques will elucidate signal transduction mechanisms, such as changes in nuclear shape or mechanosensitive ion channels, that sense microenvironmental cues and ultimately modulate stem cell fate choices such as lineage commitment, survival/apoptosis, self-renewal/proliferation, and spatial patterning/localization within tissues. RGD, arginine-glycine-aspartic acid; ECM, extracellular matrix.

Summary and Conclusions

The recent rapid development of novel cell culture systems has greatly expanded the possible regulatory cues researchers can explore. These engineered microenvironments have provided the tools needed to elucidate the importance of mechanical perturbations and solid-state biochemical and biophysical properties of materials in regulating stem cell behavior. Furthermore, pioneering studies are increasingly combining analytical cell biology techniques with these engineered systems to gain mechanistic insights. Future work will continue investigating novel mechanistic hypotheses, likely drawing upon mechanisms found in differentiated cells as well as some stem cells, such as signaling through focal adhesions [78], compartmental sequestration of transcription factors [154, 155], and force-induced conformational changes of biomacromolecules [71, 156] among others.

Reductionist biology using engineered cell culture systems not only provides the opportunity to explore new biophysical and solid-state biochemical parameters but also allows for quantitative, graded, and temporal control over these regulatory features. In addition, improving the ability to orthogonally vary microenvironmental parameters in engineered systems in the future will allow researchers to address complex mechanisms involving crosstalk between interdependent regulatory cues, to study the conversion and transduction between biochemical and biophysical signals, and develop a more complete systems-level view of stem cell processes. As alluded to throughout this chapter, no stem cell process is regulated in isolation from other elements of the microenvironment, and a systems-level perspective may shed light on novel regulatory interactions and networks beyond those traditionally studied through biochemical signal transduction (Figure 3). Developing this mechanistic and systems-level understanding of stem cell microenvironments promises to inform future stem cell-based therapies as well as our understanding of human homeostasis and disease states.

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Chapter 2

Rho GTPases Mediate the Mechanosensitive Lineage Commitment of Adult Neural Stem Cells

Abstract

Adult neural stem cells (NSCs) play important roles in learning and memory and are negatively impacted by neurological disease. It is known that biochemical and genetic factors regulate self-renewal and differentiation, and it has recently been suggested that mechanical and solid-state cues, such as extracellular-matrix (ECM) stiffness, can also regulate the functions of NSCs and other stem cell types. However, relatively little is known of the molecular mechanisms through which stem cells transduce mechanical inputs into fate decisions, the extent to which mechanical inputs instruct fate decisions versus select for or against lineage-committed blast populations, or the in vivo relevance of mechanotransductive signaling molecules in native stem cell niches. Here we demonstrate that ECM-derived mechanical signals act through Rho GTPases to activate the cellular contractility machinery in a key early window during differentiation to regulate NSC lineage commitment. Furthermore, culturing NSCs on increasingly stiff ECMs enhances RhoA and Cdc42 activation, increases NSC stiffness, and suppresses neurogenesis. Likewise, inhibiting RhoA and Cdc42 or downstream regulators of cellular contractility rescues NSCs from stiff matrix- and Rho GTPase-induced neurosuppression. Importantly, Rho GTPase expression and ECM stiffness do not alter proliferation or apoptosis rates indicating that an instructive rather than selective mechanism modulates lineage distributions. Finally, in the adult brain, RhoA activation in hippocampal progenitors suppresses neurogenesis, analogous to its effect in vitro. These results establish Rho GTPase-based mechanotransduction and cellular stiffness as biophysical regulators of NSC fate in vitro and RhoA as an important regulatory protein in the hippocampal stem cell niche.

Introduction

Neural stem cells (NSCs) in the adult mammalian brain generate new neurons, astrocytes, and oligodendrocytes throughout life. One population of NSCs resides in the subgranular zone of the hippocampus [1, 2], and NSC-mediated adult hippocampal neurogenesis has been specifically implicated in learning and memory, mood regulation, and neurological disorders [3-7]. Thus, a deeper cellular and molecular mechanistic understanding of the regulation of NSC self-renewal and differentiation may lend new insights into the roles of NSCs in these important biological processes.

In pursuit of these mechanisms, the field has focused primarily on the important roles of soluble cues and how biochemical signaling and epigenetics process these cues [8-15]. However, microenvironments also contain diverse biophysical inputs such as specific geometric and mechanical characteristics of both the cell and extracellular matrix (ECM) that have been shown previously to strongly regulate a variety of processes in non-stem cells including gene expression [16], cellular signaling [17], proliferation [18], and migration [19]. Biophysical cues may also be in a position to influence NSC behavior, as suggested by the findings that there are stiffness gradients in the hippocampus [20] and that brain tissue softens with increasing age [21]. Furthermore, the higher stiffnesses associated with glial scars and brain tumors compared to surrounding healthy tissue have been shown to modulate the behavior of cultured neurons [22] and glioblastoma cells [18] and may also affect NSC homeostasis.

Our initial study demonstrated that ECM stiffness does indeed modulate NSC behavior [23]. Although potential mechanisms have not been investigated, analogous studies with mesenchymal

stem cells (MSCs) would suggest that ECM stiffness modulates cellular tension, which in turn biases the composition of differentiated cultures [24]. However, many key questions remain to be explored, most pertinently: Might mechanotransductive proteins represent a new class of molecules that may regulate neural stem cells in vitro and in vivo? Do NSCs process ECM stiffness signals by adapting their own intrinsic mechanical properties? If so, is this mechanoadaptation necessary to bias differentiation, and which signaling pathways are responsible for transducing extracellular mechanical cues into intracellular biophysical responses (e.g. changes in cellular stiffness) and functional phenotypes (e.g. lineage commitment)? Furthermore, can biophysical signals impact stem cell differentiation in the complete absence of the strong soluble differentiation cues that have been included in previous NSC and MSC studies [23, 24]? Finally, does the effect of ECM stiffness on stem cell differentiation operate via a selective mechanism in which cells or precursors of one derivative lineage or another apoptose or proliferate preferentially as a function of stiffness, or an instructive mechanism in which ECM stiffness biases lineage commitment of multipotent stem cells? Here we have integrated biophysical, genetic, biomaterials, and animal model approaches to address these important questions.

Materials and Methods

Neural Stem Cell Culture

Neural stem cells were isolated from the hippocampus of adult female Fischer 344 rats as described here [25] and in Supplemental Data. For experiments, NSCs were seeded at a density of 15,000 cells/cm^2. For BrdU treatments, cells were cultured with 10 μM BrdU (5-Bromo-2'-deoxyuridine, Sigma) from hours 0-12, 12-96, or 96-144 prior to fixation. Finally, GLISA™ assays were performed according to manufacturer's instructions (Cytoskeleton, Inc., Denver, CO).

Rho GTPase Constructs

Dominant negative and constitutively active small Rho GTPase constructs (pcDNA3 myc CA RhoA Q63L, pcDNA3 myc DN RhoA T19N, pcDNA3 EGFP Cdc42 Q61L, pcDNA3 EGFP Cdc42 T17N, pcDNA3 EGFP Rac1 Q61L, pcDNA3 EGFP Rac1 T17N) were kind gifts of Dr. G.S. Martin, UC Berkeley. See Appendix A for details of viral production and in vitro and in vivo delivery.

Immunofluorescence Staining

Cells and tissue sections were immunostained as described previously [7, 23] and in Appendix A.

Polyacrylamide Substrate Preparation

Using a protocol similar to that described previously [19], polyacrylamide gels (70 μm nominal thickness) were synthesized on 12 mm glass coverslips using solutions composed of varying concentrations of acrylamide monomer and bisacrylamide crosslinker (Table 1). 100 μg/ml laminin was linked to the surface through sulfo-SANPAH (Thermo-Fisher, Waltham, MA) chemistry.

Table 1. Acrylamide and bisacrylamide concentrations used to make polyacrylamide gels with different Young's moduli as measured by AFM. Values are means and 95% confidence intervals, n = 14.

Polyacrylamide gel formulations and corresponding Young's Modulus		
Acrylamide v/v %	**Bisacrylamide v/v %**	**Young's Modulus (Pa)**
3	0.025	102 ± 2
3	0.04	207 ± 16
3	0.1	528 ± 11
4	0.05	692 ± 2
4	0.075	1498 ± 80
4	0.1	2123 ± 215
4	0.2	4018 ± 307
5	0.2	13365 ± 103
8	0.3	30567 ± 979
10	0.3	72904 ± 159
12	0.6	151002 ± 1498
15	1.2	292300 ± 528

Atomic Force Microscopy

An Asylum MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA) was used to probe single cells in contact mode. Silicon nitride pyramidal AFM tips (MLCT-ANUM, Veeco Metrology, Inc., Santa Barbara, CA) with spring constants of 10-30 pN/nm were calibrated by the thermal resonance method. All measurements were made at a constant velocity of 2 μm/s. Elastic moduli reported are Young's moduli calculated using the Hertz model [26] modified for a pyramidal tip geometry [27], assuming a Poisson ratio of 0.45. Cells were probed on the cell body and not on process extensions to minimize the impact of regional variations within cells. Cells were chosen randomly by rastering the sample stage blind to the location of cells and probing the nearest cell to the AFM tip after each location change. 50-100 cells were probed per culture. Force curves were fitted to the first 500 nm of indentation to minimize mechanical contributions from the underlying substrate or of the nucleus [26].

Results

ECM stiffness biases NSC differentiation

It was recently demonstrated that the lineage distributions of NSCs can be controlled by varying the stiffness (i.e. elastic modulus or modulus) of hydrogel scaffolds [23, 28-30]. In this work, we employed a polyacrylamide (PA) ECM system that is tunable over a broad range of stiffnesses that readily encompasses the stiffness of brain tissue. Furthermore, these surfaces can be covalently conjugated with full-length ECM proteins, as well as resist non-specific protein adsorption, yielding a well-defined substrate for cell adhesion. We conjugated full-length laminin protein, which is abundant in native NSC niches and supports NSC self-renewal and differentiation in vitro [31, 32], to PA hydrogel surfaces ranging from 100 – 75,000 Pa in stiffness. We then tested the capacity of ECM stiffness to drive cell differentiation under soluble conditions that induce differentiation into mixtures of neurons, astrocytes, and, to a small extent, oligodendrocytes (1 μM retinoic acid and 1 v/v% fetal bovine serum, or "mixed conditions") [23, 33], as well as under minimal growth factor conditions that promote cell survival but not proliferation (0.1 ng/ml fibroblast growth factor-2, or "survival conditions").

After 6 days in culture under mixed conditions, immunostaining for lineage markers

(neuronal β-tubulin III, astrocytic glial fibrillary acidic protein-GFAP, oligodendrocytic myelin basic protein-MBP) showed that relatively compliant ECM substrates (100 – 700 Pa) biased lineage distributions towards neuron-rich populations (60% neurons, 10% astrocytes, 5% oligodendrocytes), whereas stiffer substrates (1,500 – 75,000 Pa) yielded cultures with roughly equal proportions of neurons and astrocytes (~30% neurons, 20% astrocytes, 0% oligodendrocytes) (Figure 1A and 1C). The proportion of oligodendrocytes was generally very low but increased on softer substrates. It should be noted that the NSCs used in this study correspond to Type IIa neural progenitors that are GFAP negative as seen previously, and GFAP specifically labels astrocytes in this system [1, 25, 33-36]. Also, the remaining marker negative cells were largely undifferentiated, Nestin-positive cells [34] and 10-20% were partially differentiated Nestin-negative cells (Appendix A, Figure 1). Interestingly, stiffer substrates yielded higher levels of undifferentiated cells than softer substrates.

In parallel, under survival conditions, neuronal, astrocytic, and oligodendrocytic differentiation were observed at lower levels and with less mature morphologies than under mixed conditions, consistent with the absence of strong soluble factors to induce maturation following lineage commitment. However, ECM stiffness again strikingly biased lineage distributions towards neurons on soft ECMs and towards equal proportions of neurons and astrocytes on stiff ECMs (Figure 1B and 1D). We observed few oligodendrocytes, but as in mixed conditions their percentage slightly increased on the softest ECMs. These data demonstrate that ECM stiffness can strongly influence and drive NSC lineage commitment even in the absence of exogenous soluble differentiation cues.

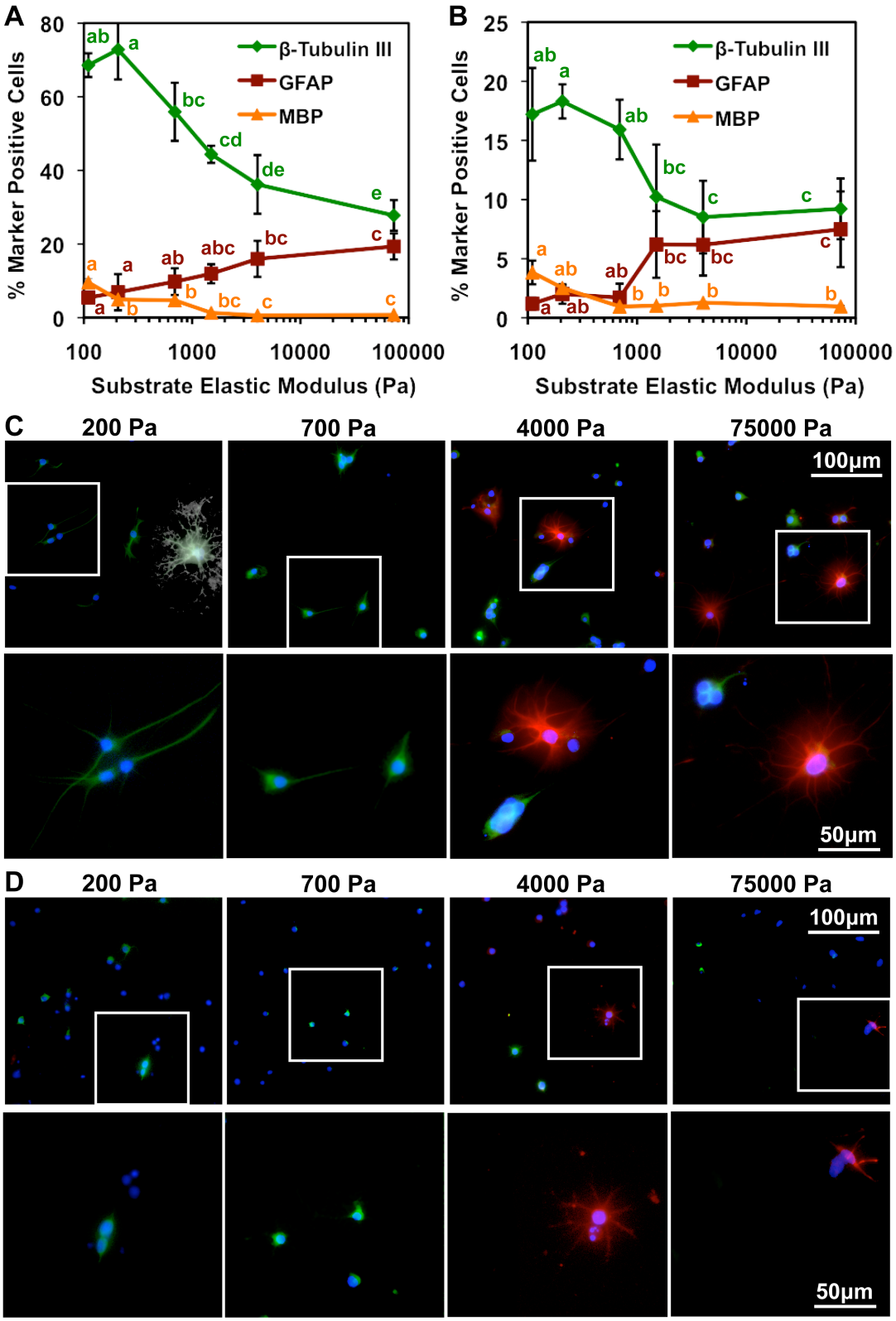

Figure 1. ECM elastic modulus biases relative proportions of neurons versus astrocytes. (A)(C) mixed and (B)(D) survival conditions. Error bars are 95% confidence intervals, n = 6. Means compared by analysis of variance, Tukey-Kramer post hoc (ANOVA-TK), $p < 0.05$. (C)(D) Neurons, β-tubulin III *(green)*; astrocytes, GFAP *(red)*; nuclei, DAPI *(blue)*; oligodendrocytes, MBP *(white)*. Insets (white boxes) are shown in bottom rows.

NSCs sense and respond biomechanically to ECM stiffness through RhoA and Cdc42

NSCs can sense mechanical information encoded within the ECM; however, it is unclear how they process these cues to modulate differentiation. One may anticipate that the most direct response to changes in ECM stiffness is for NSCs to adapt their own intrinsic mechanical properties. To test this hypothesis, we used atomic force microscopy (AFM) [37] to measure the elastic modulus of individual NSCs cultured on ECMs of defined stiffness. We seeded NSCs in mixed conditions, then probed them with AFM after 12 hours, a duration sufficient for cells to maximally adhere and spread but likely not for lineage commitment to occur [38]. Interestingly, by this early time point, cellular elastic modulus varied strongly and monotonically with increasing ECM stiffness, such that cells on the stiffest matrices exhibited elastic moduli nearly 8-fold greater than those cultured on the most compliant ECMs (Figure 2A). Prior work has indicated that ECM ligand density and presentation remain constant over this range of PA formulations [39]. However, to preclude the possibility that such surface biochemical differences may contribute to stem cell stiffness differences, we repeated these experiments on highly compliant and highly rigid gel formulations cast as very thin layers (<7 μm) on top of glass, such that the stiffness of the cell-ECM interface is dictated by the underlying hard substrate rather than the intrinsic properties of the gel [40] (Figure 2A). Both thin gels yielded NSC stiffnesses in the range of 700-800 Pa, similar to that observed on stiff gels, confirming that ECM stiffness modulates NSC stiffness.

The observation that cells stiffen in response to increasing ECM modulus indicates that cellular mechanotransductive signaling pathways may sense and process extracellular mechanical information into intracellular mechanical responses. The Rho family of GTPases – including RhoA, Cdc42, and Rac1 – have been extensively studied in somatic cells and are known to regulate the assembly and activity of cytoskeletal processes needed for the establishment of cell shape and the generation of contractile forces [41]. These proteins cycle between active GTP-bound and inactive GDP-bound states, and levels of the active, GTP-bound form can be measured using enzyme-linked immunosorbent assays (ELISA) [42]. We cultured NSCs on two ECM stiffnesses (700 and 75,000 Pa) and found that the cellular activities of RhoA and Cdc42, measured at an early time point (12 hours post seeding) in mixed conditions, were nearly two-fold higher on the stiff vs. soft ECMs (Figure 2B), whereas Rac1 activity remained unchanged, indicating that ECM stiffness preferentially activates specific Rho GTPases. Together with the AFM results, these experiments confirm the hypotheses that NSCs respond to increasing ECM stiffness both by altering their intrinsic mechanical properties (stiffness) and by activating mechanotransductive signals (RhoA and Cdc42).

To determine whether RhoA and Cdc42 activity mediate the effect of ECM modulus on NSC mechanoadaptation, we retrovirally transduced NSCs to stably express dominant negative (DN) and constitutively active (CA) mutants of RhoA and Cdc42 [43] (Appendix A, Figure 2), cultured them on a range of ECM moduli in mixed conditions, and measured NSC stiffnesses by AFM 12 hours after seeding. Compared to control cells transduced with an empty retroviral vector, the stiffnesses of NSCs expressing DN RhoA and DN Cdc42 were lower and less sensitive to changes in ECM modulus, whereas CA RhoA and CA Cdc42 retained normal mechanoadaptation and even increased cell stiffness for some ECM moduli (Figures 2C and D, respectively). These differences in stiffness, readily apparent from representative force-indentation curves (Figures 2C and D, right) and analysis of covariance in trends (Appendix A, Figures 2A and B), demonstrate that RhoA and Cdc42 activation are necessary for NSC

stiffening in response to increasing ECM modulus.

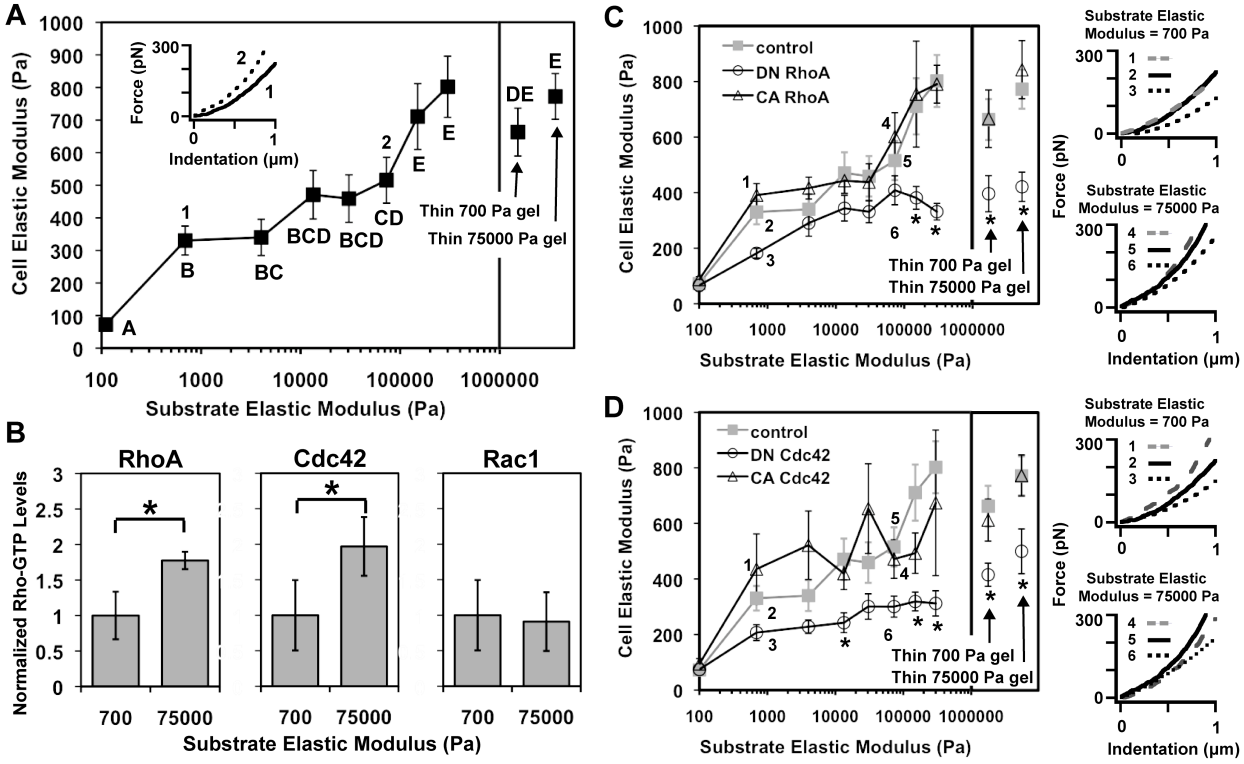

Figure 2. NSCs are mechanically and biochemically responsive to ECM stiffness through Rho GTPase activity. (A)(C)(D) NSC stiffnesses measured by AFM. Error bars are 95% confidence intervals for n = 14-50 cells. Means compared by ANOVA-TK, $p < 0.05$. (Insets) Representative AFM force-indentation curves for NSCs. (B) Rho-GTP levels of NSCs normalized to the soft gel (700 Pa) value. *$p < 0.05$, Student's unpaired two-tailed t-test.

RhoA and Cdc42 modulate the effect of ECM stiffness on NSC differentiation

Given that ECM stiffness modulates NSC lineage distributions (Fig. 1), cell stiffness (Fig. 2A), and RhoA and Cdc42 activities (Fig. 2B), and that direct manipulation of RhoA and Cdc42 activity modulates cell mechanoadaptation (Fig. 2C and D), we reasoned that RhoA and Cdc42 may be responsible for transducing the effects of variable ECM stiffness on NSC differentiation (Figure 1). To test this hypothesis, we cultured NSCs expressing DN and CA RhoA and Cdc42 on ECMs of different stiffnesses in mixed conditions and immunostained for lineage markers after 6 days. On soft (<1000 Pa) ECMs, expression of DN RhoA did not further increase the percentage of neurons observed compared to control cells (Figure 3A and C); however, on rigid (>4000 Pa) ECMs it rescued neuronal differentiation up to levels approaching 50%. In contrast, increasing RhoA activity had the opposite effect, reducing the fraction of neurons on compliant ECMs compared to control cells, but not appreciably changing the percentage of neurons on the stiffest substrate, thereby resulting in ~30% neurons on all ECM stiffnesses. Astrocytic differentiation followed complementary trends, with CA RhoA increasing astrocytic differentiation on soft substrates and DN RhoA decreasing astrocytic differentiation on stiffer substrates. Similar results were obtained with NSCs expressing DN or CA Cdc42 (Figure 3B and D). These results and further statistical analysis of covariance in trends (Appendix A, Figure 4) indicate that expression of DN RhoA or Cdc42 mimics phenotypes observed on soft gels, whereas expression of CA RhoA or Cdc42 mimics differentiation observed on stiff gels.

Interestingly, these trends persisted in survival conditions. DN RhoA and Cdc42 rescued neuronal differentiation (~ 20% neurons), while CA RhoA and Cdc42 slightly suppressed neuronal differentiation on all ECM stiffnesses (Appendix A, Figure 5). In addition, with the lone exception of DN Cdc42-expressing cells on soft ECMs, astrocytic differentiation was suppressed by DN RhoA and Cdc42, and largely unaffected by expression of CA RhoA and Cdc42.

The effects of RhoA and Cdc42 on differentiation in both mixed and survival conditions were also observed on traditional glass substrates (most similar to the stiffest hydrogel ECMs) by QRT-PCR (Appendix A, Figure 6) and immunostaining (Appendix A, Figure 7). It should be noted that cell populations expressing mutant Rho GTPases in mixed conditions all displayed classical neuronal, astrocytic, and oligodendrocytic morphologies, with no differences from differentiated control NSCs (Appendix A, Figure 7A) [33]. Furthermore, expression of DN and CA Rho GTPases as well as culture on soft and stiff polyacrylamide gels did not compromise later stages of neuronal maturation and subtype marker expression, with GABAergic and glutaminergic neurons detectable across all ECM stiffnesses and all RhoA/Cdc42 genotypes (Appendix A, Figure 8). Finally, expression of DN or CA Rac1 GTPase interestingly did not bias lineage distributions (Appendix A, Figure 7), consistent with our earlier finding that the activities of RhoA and Cdc42, but not Rac1, are regulated by ECM stiffness (Figure 2B). Collectively, these results under mixed and survival conditions show that RhoA and Cdc42 serve as important transducers of ECM stiffness into downstream cell fate decisions.

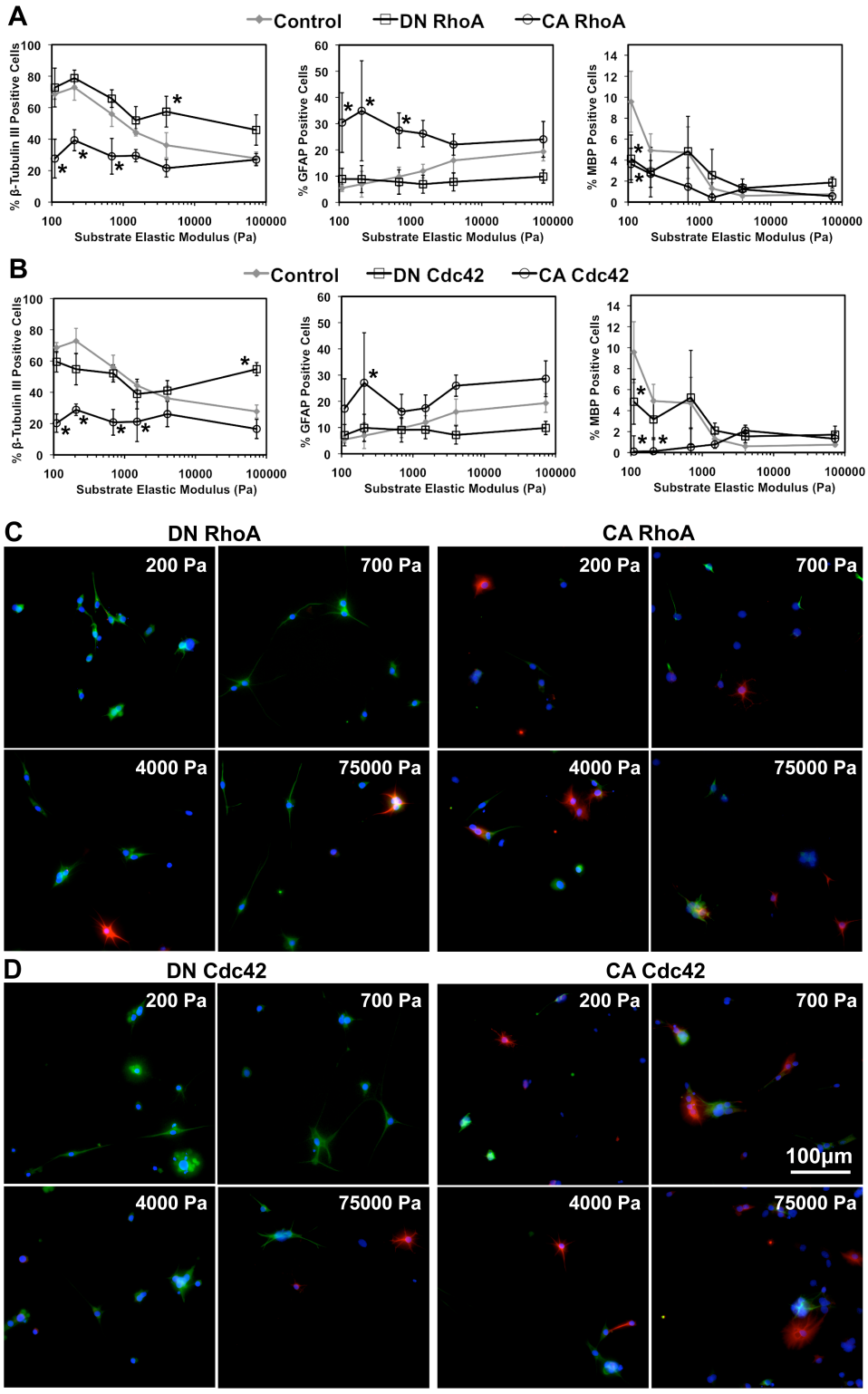

Figure 3. Rho GTPases modulate the effect of ECM elastic modulus on the proportions of neurons and astrocytes in mixed conditions. Error bars are 95% confidence intervals, n = 5-6. *p < 0.05 for comparisons to control for each substrate elastic modulus (control data previously shown in Figure 1A) (ANOVA-TK). β-tubulin III *(green)*, GFAP *(red)*, DAPI *(blue)*, MBP *(white)*. See Figure S4 for higher power images of (C) and (D).

Comparative longitudinal apoptosis and proliferation measurements strongly support an instructive mechanism

While RhoA and Cdc42 modulated the ECM stiffness effect on lineage distributions, it was unclear whether these changes in lineage distributions were due to instructive biasing of NSC lineage commitment or selection for specific populations via modulation of proliferation and/or apoptosis of lineage-committed cells. The initial homogeneity of the culture was assessed by single-cell sorting clonal analysis, which revealed that ~82% of clonal populations were capable of giving rise to neurons, astrocytes, and oligodendrocytes (tripotent), indicating that cells seeded at the beginning of the experiment were predominantly multipotent NSCs. Our previous work has also shown that unipotent soluble conditions were capable of generating almost pure neuronal or astrocytic cultures, providing further evidence of the NSC culture homogeneity [23, 24]. We then measured proliferation (BrdU) and apoptosis (active caspase 3) at early (0-12 hours), middle (12-96 hours), and late (96-144 hours) time points for NSCs on soft (700 Pa) and stiff (75,000 Pa) ECMs under mixed conditions (Figure 4). For all cultures, proliferation was moderate during the first 12 hours and thereafter decreased to minimal levels (~5% for BrdU pulse durations), and active caspase 3 levels were low across all conditions throughout the experiment (<1.5%). Furthermore, cells expressing the mutant Rho GTPases exhibited similar proliferation rates to control cells on soft and stiff ECMs. Importantly, all experiments were conducted at low initial seeding densities (<20000 cells/cm^2) to minimize confounding effects of cell-cell adhesion (e.g. contact inhibition). Repeating the experiment at initial cell densities ranging from 5000 to 25000 cells/cm^2 did not affect lineage distributions (data not shown). The overall low proliferation and apoptosis levels throughout the experiment strongly indicate that ECM stiffness and Rho GTPase regulate NSC lineage distributions through an instructive rather than selective mechanism.

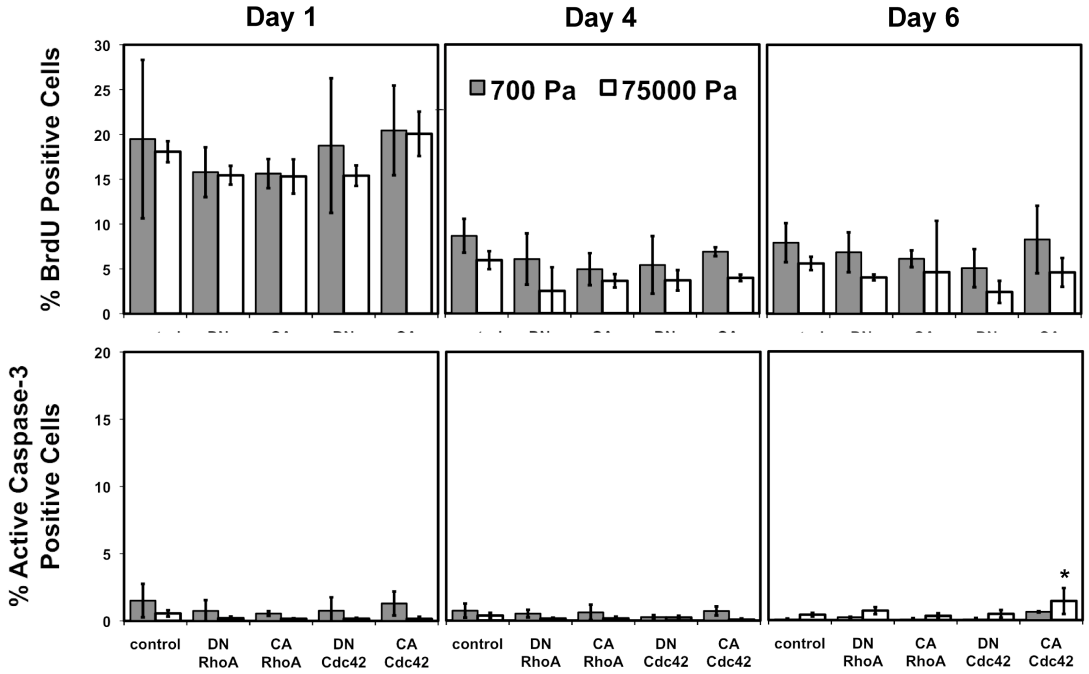

Figure 4. Rho GTPases and ECM stiffness do not affect proliferation and apoptosis rates during differentiation. Error bars are 95% confidence intervals, n = 3-6. *p < 0.05 for comparisons to control for each substrate elastic modulus for each day (ANOVA-TK).

Inhibition of contractile proteins rescues neuronal differentiation

While RhoA and Cdc42 can change NSC mechanoadaptation in response to extracellular stiffness (Figure 2) and instruct NSC differentiation decisions (Figure 3), it is not clear whether the former is necessary for the latter. While there are no clear means to directly manipulate cellular contractile properties in isolation, it is possible to inhibit the activity of cellular mechanotransducers and motors, such as the downstream Rho GTPase effectors Rho Kinase (ROCK) and myosin II whose activities directly underlie cellular stiffening, as well as other mechanotransductive proteins such as Myosin Light Chain Kinase (MLCK), Src, and Focal Adhesion Kinase (FAK). Furthermore, transient inhibition of these pathways can help address the question of whether they instruct cell fate during a critical, early time window. We therefore investigated whether inhibition of these contractility-related proteins early during lineage commitment could rescue neuronal differentiation under conditions that would otherwise instruct astrocytic fates.

NSCs expressing CA and DN RhoA and Cdc42 were cultured in mixed conditions on compliant (700 Pa) and stiff (75,000 Pa) ECMs and immunostained for lineage markers after 6 days. Inhibitors of ROCK (10 μM Y-27632), Myosin II (1 μM Blebbistatin), Src (0.5 μM PP2), FAK (0.5 μM PF-573228), RhoA/B/C (0.17 μg/mL C3), and MLCK (0.5 μM ML-7) were pulsed in the medium for the first 2 days, when lineage commitment decisions are most likely made, then washed out for the remaining 4 days to minimize any potential effects on later steps of cell differentiation. For CA RhoA- and Cdc42-expressing NSCs on soft ECMs, inhibition of RhoA/B/C and downstream effectors ROCK and Myosin II (for CA RhoA-expressing NSCs) strikingly rescued neuronal differentiation to control levels (Figure 5). By contrast, MLCK, Src, and FAK inhibition had no effect. Interestingly, all six inhibitors reduced astrocytic differentiation of CA RhoA-expressing cells (Appendix A, Figure 9), suggesting that this process may utilize additional Rho GTPase-independent mechanotransductive signaling machinery.

On stiff ECMs, all inhibitors restored neuronal differentiation for all NSC populations to levels found on compliant ECMs (~60% neurons) (Figure 5) and reduced astrocytic differentiation (Appendix A, Figure 9). Similar trends were observed in survival conditions, except that the rescue of neuronal differentiation on soft ECMs was not as pronounced (Appendix A, Figure 10A). Finally, under all conditions, oligodendrocytic differentiation was not appreciably affected (Appendix A, Figure 10B and 9C). These results strongly indicate that cellular contractility mediates the effects of instructive ECM stiffness cues on NSC lineage commitment.

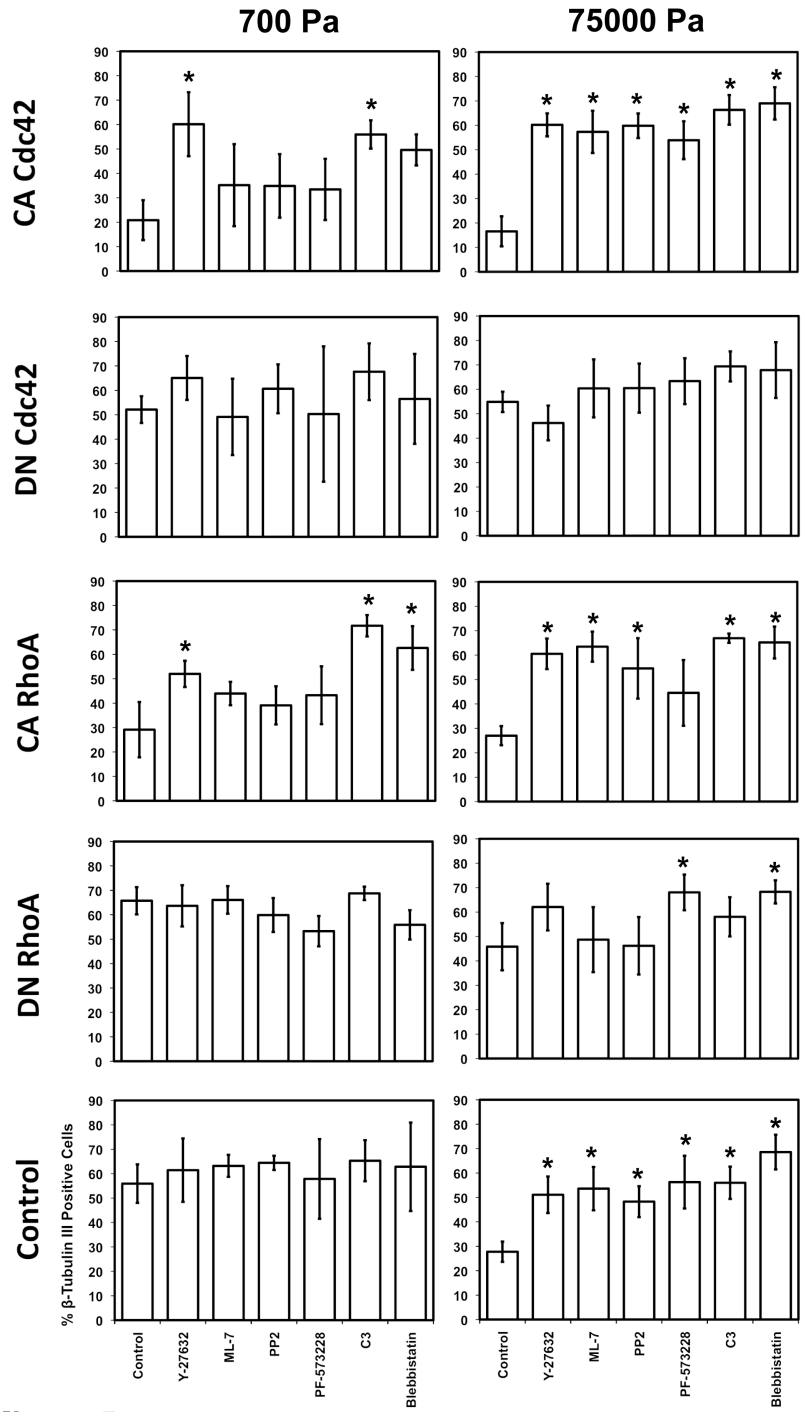

Figure 5. Inhibition of proteins that regulate cellular contractility rescues neuronal differentiation in mixed conditions on soft and stiff ECMs. Error bars are 95% confidence intervals, n = 5-6. *p < 0.05 for comparisons to NSCs in control media conditions expressing the same Rho GTPase mutant and on the same stiffness (control data previously shown in Figure 1A) (ANOVA-TK).

RhoA Activity Suppresses Neurogenesis In Vivo

We investigated whether RhoA also regulates NSC behavior in vivo. Retroviral vectors encoding either GFP only, DN RhoA and GFP, or CA RhoA and GFP (all driven by a CAG promoter) were stereotaxically injected into the hippocampal dentate gyrus of adult rats.

Retrovirus is known to infect dividing cells, specifically neural progenitors in the subgranular zone of the dentate gyrus [7], which we identified by GFP expression. At 1, 2, and 3 weeks post-injection, BrdU was administered to monitor potential differences in proliferation throughout the experiment. After 4 weeks, brain sections were immunostained for the neuronal marker neuronal nuclei (NeuN), GFAP, and BrdU (Figure 6). The percentages of mature, post-mitotic NeuN+ neurons [44] derived from neural progenitors infected with retroviral vectors (GFP+) (Figure 6A) strikingly followed the same trend as that on soft ECMs (Figure 3A), consistent with the fact that hippocampal tissue is also relatively soft at <1 kPa [20]. Specifically, CA RhoA reduced neuronal differentiation from ~50 to 30% of GFP+ cells, while DN RhoA produced a slight trend towards increased neuronal differentiation. Astrocytic differentiation was low as expected given that neural progenitors in the hippocampus are strongly biased towards neuronal differentiation (Figure 6B) [45]. Finally, proliferation was low and similar across all conditions, suggesting RhoA activity does not modulate NSC proliferation. These results show that RhoA regulates neurogenesis in vivo.

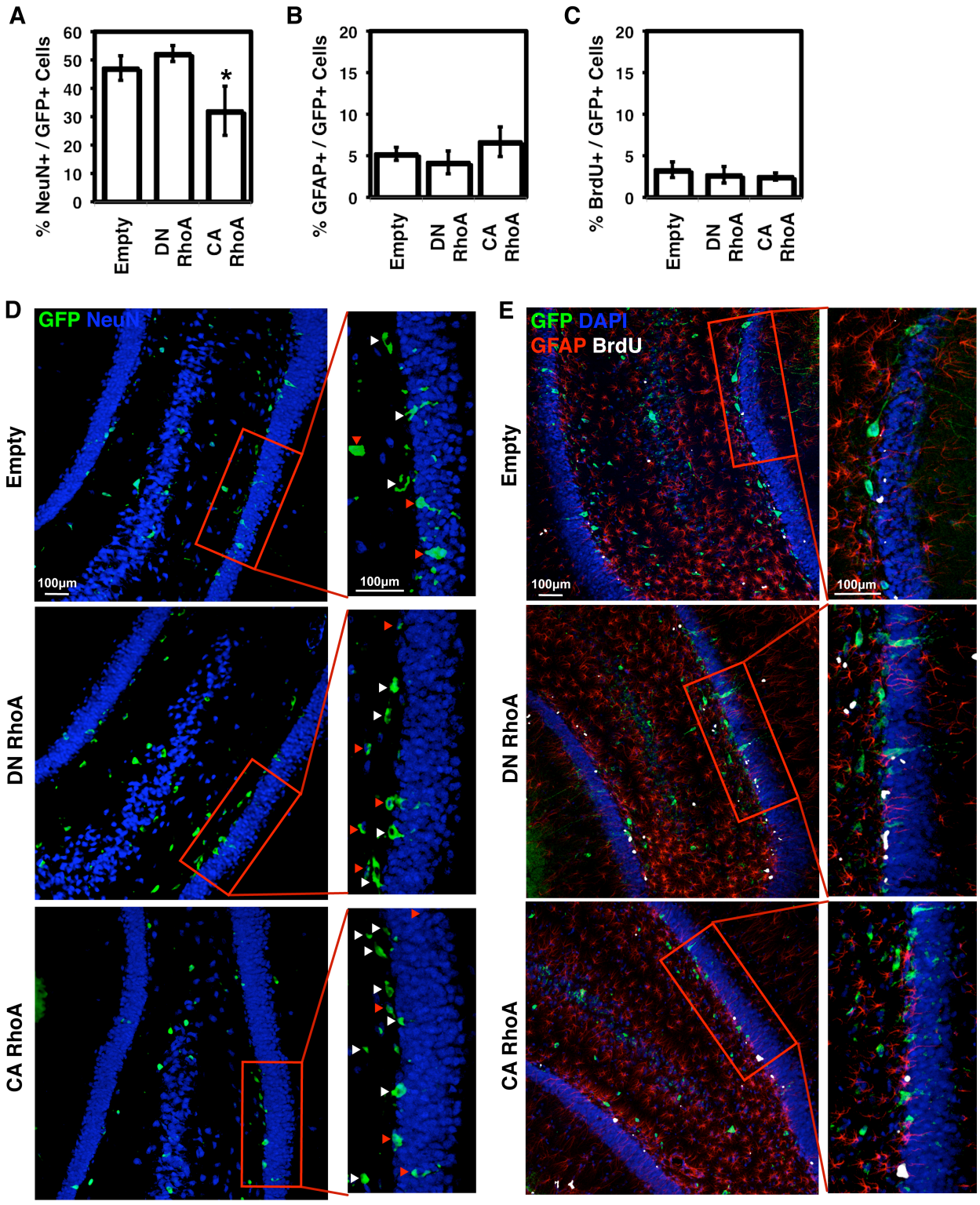

Figure 6. RhoA suppresses neurogenesis in vivo in the adult rat hippocampus. Error bars are 95% confidence intervals, n = 4 rats. *p < 0.05 for comparison to empty vector control (ANOVA-TK). Red arrows indicate cells double positive for GFP and NeuN. White arrows indicate cells positive for GFP only.

Discussion

We have demonstrated that stiffness cues encoded in the ECM directly bias NSC lineage commitment through RhoA- and Cdc42-regulated changes in actomyosin contractility and cellular stiffness. These results elucidate several novel features of NSC mechanotransduction that contribute to our understanding of stem cell biology, neuroscience, and mechanobiology: (1) Changes in cellular mechanics – which precede expression of lineage markers by several days – potently regulate ECM stiffness-dependent NSC differentiation. (2) This mode of regulation depends strongly on RhoA and Cdc42 activation, and other mechanotransductive pathways may be mobilized to control this response, including MLCK- and FAK-based signaling, depending on the mechanical properties of the ECM. To our knowledge, several of these molecules – including Cdc42, MLCK, and Src – have not previously been implicated in stem cell mechanosensitivity. (3) Transient inhibition of specific mechanotransducers during a critical developmental window is sufficient to profoundly alter lineage distributions that only declare themselves days after the inhibition is removed. (4) Along with early transient inhibition of mechanotransducers, similarly low proliferation and apoptosis levels for Rho-GTPase mutant-expressing cells on soft and stiff ECMs strongly indicate substrate mechanical properties can directly instruct, rather than select for, neural stem cell lineage commitment. In conjunction with clonal analysis of the stem cell population, this represents the most rigorous demonstration to date that ECM mechanics directly instructs a stem cell's fate. (5) RhoA activity suppresses neurogenesis in the native hippocampal niche of NSCs in a manner strikingly similar to an ex vivo niche with similar stiffness properties. (6) NSC differentiation is mechanosensitive even in the absence of strong morphogenic factors that have been used in previous studies of stem cell mechanosensitivity.

This finding that RhoA and Cdc42 are important regulators of NSC fate expands and casts a new light on their relevance in neurobiology, beyond their previously known role in the morphological maturation of committed neurons. For example, RhoA and Cdc42 are weakly expressed within the granule cell layer of the hippocampal dentate gyrus (with slightly higher expression in the hilus) but are strongly expressed in the molecular layer and other surrounding regions [46]. This spatial pattern of expression, in particular the low RhoA and Cdc42 levels surrounding the subgranular zone neurogenic niche, is consistent with our finding that suppression of RhoA or Cdc42 activation promotes neuronal differentiation in vitro and in vivo. Our findings may also yield additional mechanistic insights into recent observations that administration of ROCK inhibitors (Y-27632 and Fasudil) into mammalian brains can offer neuroprotection against ischemia [47] and epileptic seizures [48], promote spatial learning and working memory in mice [49], and increase neurogenesis and generation of neurons in response to hypoxic conditions [50]. We may also place our findings in the context of recent studies that directly connect Rho GTPases to neural development. Consistent with our study, RNAi-mediated downregulation of the Rho GDP dissociation inhibitor γ (RhoGDIγ) in v-myc immortalized, multipotent C17.2 neural cells has been shown to decrease RhoA and Cdc42 activity, but increase Rac1 activity, as well as promote neuronal but not glial differentiation [51]. In contrast, activation of Cdc42 has been shown to enhance neuronal differentiation in C17.2 and P19 cells [52] and neuroblastoma [53], while siRNA knockdown of Cdc42 in P19 cells almost completely inhibited astrocytic differentiation while modestly inhibiting neuronal differentiation [54]. These different results may arise from distinct culture conditions and cell type, which originate from different species and/or subregions of the central nervous system and may thus exhibit different mechanobiological properties. Furthermore, it is entirely possible that Rho GTPases may affect lineage specification very differently from how they affect maturation of already lineage-committed cells. It would be interesting to revisit these studies to determine

whether the observed effects of Rho GTPase signaling on neural differentiation have some mechanoregulatory component.

In principle, there are two distinct yet both physiologically relevant mechanisms by which ECM stiffness and Rho GTPases may impact lineage distributions: selection vs. instruction. In the hippocampus, some extracellular factors are known to instruct NSC commitment to specific lineages [55]. However, the majority of newborn cells in the hippocampus undergo apoptosis within 4 days [56], and ECM stiffness could alternatively affect lineage distributions through a selective mechanism, such as one that modulates cell survival. Our results show that Rho GTPase activity and ECM stiffness do not modulate NSC proliferation or apoptosis during differentiation in vitro, or NSC proliferation in vivo (Figures 4 and 6C), supporting an instructive mechanism in which ECM stiffness acts directly to perturb lineage commitment. While the mechanism of this instruction remains unclear, ECM mechanics may potentially function by modulating canonical signaling pathways including those downstream of Notch and Wnt/β-catenin, transcription factors like Sox2 and Tlx, or epigenetic regulators like RE-1 silencing transcription factor (REST) known to regulate NSC maintenance and differentiation [55, 57].

This study also adds new insight into the mechanobiology of stem cells. Previously with MSCs, pharmacological inhibition of myosin II reduced differentiation into all lineages on all ECM stiffnesses [24]. In NSCs, by contrast, we find that inhibition of contractility alters the distribution of differentiation trajectories (i.e. neuron vs. astrocyte) while actually modestly increasing differentiation. Two related innovations of our study are that we employed much lower effective dosages and included them only transiently during the period of lineage commitment, days before the appearance of lineage markers (e.g. 1 μM blebbistatin for 2 day pulse compared to 50 μM for full length of experiment). A similar early transient treatment of MSCs (12-24 hours) with Y-27632 in osteogenic differentiation media affected osteogenesis 7 days later, indicating the importance of early cytoskeletal contractility in stem cell differentiation [58]. By using transient exposures at a key early time window, we separated the regulatory contribution of early cellular mechanotransduction in multilineage stem cell differentiation from its longer-term contributions to maturation. Furthermore, the lower dosages of contractility inhibitors necessary for an observed phenotype, along with shifts in (rather than inhibition of) differentiation in NSCs compared to MSCs, suggests these two stem cell types may be sensitive to different ranges of ECM mechanical stiffnesses. This idea is further supported by the different ranges of cortical stiffnesses measured for the two cell types, with MSC stiffness generally exceeding 1 kPa [24] and NSC stiffness lying below 1 kPa even on the stiffest substrates (Figure 2). These findings are consistent with the notion that the differentiation of a specific stem cell population is most sensitive to ECM stiffness in a range that corresponds to its tissue(s) of residence, e.g., brain is softer than bone marrow and the connective tissues that MSCs chiefly populate.

Our study also reveals an interesting difference between NSCs and MSCs in the dynamic range of their stiffness-sensitive differentiation. The range of ECM stiffnesses required to alter differentiation is orders of magnitude smaller for NSCs compared to MSCs, here only 1 kPa (from 500-1500 Pa). This may reflect the fact that the NSCs used in this study arise from a single tissue with a well-defined, soft, anatomical niche, whereas MSCs arise from a broad range of tissues with niches that are comparatively poorly defined. This study also demonstrates that stem cells are capable of sensing much finer and subtler changes in microenvironmental stiffness than previously known. Future work should identify the mechanisms controlling the range of ECM

stiffnesses in which specific stem cells are most sensitive and whether these ranges correlate with in vivo niche properties.

It is important to note that the interplay between biophysical and biochemical signaling may be more complicated than currently appreciated and suggests specific future avenues of study. For example, our finding that the inhibition of FAK, MLCK, and Src rescued neuronal differentiation on stiff but not soft ECMs (Figure 5), implies that distinct mechanotransductive pathways may be mobilized by specific microenvironmental contexts. Basal levels of cellular contractility or perhaps basal flux through particular biochemical signaling pathways may be significantly different on soft versus stiff ECMs, resulting in differential regulation based on the biophysical context of the microenvironment. Interestingly, an earlier study found that constitutive activation of ROCK, but not RhoA, can induce MSC osteogenesis even when these cells are forced to adopt a rounded morphology and are presumably limited in their ability to stiffen [59] suggesting biochemical and biophysical signaling may intersect at distinct places depending on the microenvironmental context. Similarly, we found that while DN RhoA/Cdc42 compromised the ability of NSCs to adapt their intrinsic mechanical properties to those of the ECM, CA RhoA/Cdc42 did not strongly enhance this behavior (Figure 2) yet still biased differentiation by reversing the enhanced neurogenesis observed on soft ECMs. The fact that CA RhoA/Cdc42 does not produce dramatic "hyperstiffening" is not entirely surprising given that ECM compliance places fundamental limits on how hard cells can pull on the matrix without rupturing adhesions and contracting [39]. Furthermore, cortical stiffness as measured by AFM is an integrated readout of cell-matrix tensional homeostasis and may not detect particularly subtle changes in tensile forces localized to individual adhesions that are believed to directly modulate adhesion-based signaling [60]. Future work in both MSCs and NSCs will be needed to further investigate the intracellular and extracellular components of mechanotransductive signaling networks, their connectivity and regulatory logic, and their relative importance as a function of microenviromental context [61-67].

Finally, in addition to adding to our understanding of the molecular and biophysical mechanisms regulating NSCs and their roles in health and disease, this study also indicates that modulation or even manipulation of the mechanical microenvironment may have implications for human health. For example, the elasticity of the brain changes significantly with age in both rats [21] and humans [68], raising the prospect that these changes may potentially contribute to age-related dysregulation of neuronal differentiation and cognitive decline. In addition, the mechanical changes observed during the progression of brain tumors and neurodegenerative scarring [69-71] may induce cancer growth and metastasis by increasing cancer cell proliferation and motility [18, 72]. Furthermore, our results in vivo strongly suggest that such stiffness increases in the native hippocampal NSC niche may, through upregulation of RhoA activity, suppress neurogenesis. Future developments of methods to modulate tissue stiffness without altering other niche properties such as niche biochemistry will be of significant interest given these findings. Investigating these hypotheses should contribute to our understanding of CNS diseases and may offer new and unexpected biomedical avenues.

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Chapter 3
Mechanobiology of Neuronal Maturation

Introduction

Adult neural stem cells (aNSCs) can give rise to all three neural cell types, and this lineage commitment process is mechanosensitive. However, each of these three neural cell types can further mature into multiple subtypes with distinct functions. Neurons are especially diverse with over a hundred subtypes currently identified [1]. Because neuronal subtype specification dictates the neurotransmitter(s) a neuron secretes, and this in turn dictates synaptic connectivity, neuronal maturation is crucial for proper function. Thus, understanding neuronal maturation from aNSCs is important for our understanding of organismal and central nervous system development as well as the generation of therapeutic neurons that have proper functionality. aNSCs from the rat hippocampus have been shown to differentiate into mature GABAergic and glutamatergic neurons and are thus a good model system to continue our investigation of microenvironmental stiffness on neuronal maturation.

Here we show that in the presence of neuronal differentiation cues, an intermediate substrate stiffness close to that of native brain tissue (~700 Pa) promotes optimal neuronal gene expression and neuronal maturation after 11 days of differentiation. Inhibiting RhoA and Cdc42 activity abrogates stiffness dependent differences in neuronal gene expression to levels normally found on stiff substrates. Interestingly, increasing RhoA and Cdc42 activity enhances neuronal gene expression only on soft substrates. These effects are not preceded by differences in neural transcription factor gene expression, including NeuroD1, Sox2, and neurotrophin receptors TrkA/B/C suggesting that subtype specification through neurotrophic factors is unlikely to be biased by substrate stiffness. In addition, substrate stiffness effects likely exert themselves at intermediate or late time points during differentiation. Supporting these hypotheses, subtype specification is found to be unaffected by substrate stiffness and Rho GTPase activity, suggesting that microenvironmental stiffness promotes the quality or extent of neuronal maturation but not subtype specification.

Materials and Methods

Stem Cell Culture

Differentiation was induced on day 0 in DMEM:F12+N2 media by the addition of 1 uM retinoic acid and 5 uM forskolin. Half-media changes were performed daily. On day 7 20 ng/mL brain-derived neurotrophic factor (BDNF, Peprotech 450-02) was added. Cells were fixed and stained or isolated in Trizol on day 11.

Immunofluorescence Staining

Primary antibodies: rabbit anti-VGlut1 (1:2500, Synaptic Systems 135303), mouse anti-TUJ1 (1:1000, Sigma), guinea pig anti-GABA (1:2500, Sigma A2052). Secondary antibodies: FITC anti-rabbit, Cy5 anti-mouse, Cy3 anti-guinea pig (all 1:250, Jackson Immunoresearch). Immunostaining procedures were similar to those in Chapter 2.

Polyacrylamide Substrate Preparation

Substrates were prepared as in Chapter 2.

Quantitative Reverse Transcription Polymerase Chain Reaction

QRT-PCR was performed as in Chapter 2. Primer sequences were: rat trkA 5' (CACTGGGTGGCAGTTCTCTT), rat trkA 3' (CATGTACTCGAAGACCATGA), rat trkB 5' (GTTGCTGACCAAACCAATCG), rat trkB 3' (CATGTACTCAAAGACCATGA), rat trkC 5' (CTGAAGGATCCCACCTTGGC), rat trkC 3' (CATGTATTCAAAGACCATGA), rat p75NGFR 5' (CCCTGCCTGGACAATGTTAC), rat p75NGFR 3' (CTGGGCACTCTTCACAC), mouse neuroD 5' (GCATGCACGGGCTGAACGC), mouse neuroD 3' (GGGATGCACCGGGAAGGAAG), rat p21 5' (GCCCAAGATCTACCTGAG), p21 3' (GTGGGCACTTCAGGGCTTTC), rat c-Fos 5' (GGGTTTCAACGCGGACTAC), rat c-Fos 3' (GTTGGCACTAGAGACGGA), NeuroD1 5' (ATGACCAAATCATACAGCGAGAG), NeuroD1 3' (TCTGCCTCGTGTTCCTCGT), NeuroD2 5' (AAGCCAGTGTCTCTTCGTGG), NeuroD2 3' (GCCTTGGTCATCTTGCGTTT).

Results

Expression of neuronal and maturation genes is optimal on intermediate substrate stiffnesses

We previously found (Chapter 2) that aNSC lineage commitment into neurons at the expense of astrocytes is promoted on soft substrates in soluble conditions permissive of differentiation into a mixture of astrocytes and neurons. In the current chapter, we asked if neuronal maturation or the quality of neuronal differentiation could be enhanced by substrate stiffness if soluble conditions were selected to be completely neurogenic. aNSCs were cultured on laminin-coated polyacrylamide substrates with a range of elastic moduli (100, 700, and 75000 Pa). Soluble conditions were adapted from Takahashi et al. [2]. 1 μM all-trans retinoic acid and 5 μM forskolin were added to cells to initiate neuronal differentiation in the absence of factors from serum that would promote astrocytic and oligodendrocytic differentiation. After 6 days, in addition to retinoic acid and forskolin, 20 ng/mL brain derived neurotrophic factor (BDNF) was added as a neurotrophin/maturation signal. After a total of 11 days, TUJ1 gene expression was measured using QRT-PCR. TUJ1 expression was ~3-fold higher on 700 Pa compared to 100 and 75000 Pa substrates, demonstrating that neuronal differentiation was enhanced on this intermediate stiffness substrate (Figure 1A). Consistent with this finding, p21, a cyclin-dependent kinase inhibitor whose expression typically signifies an exit from the cell cycle [3-5] and correlates with the generation of post-mitotic neurons [6], was also upregulated on 700 Pa substrates, suggesting that neuronal maturation is enhanced on this intermediate stiffness (Figure 1B). Furthermore, p75 nerve growth factor receptor (NGFR) is also strongly upregulated on 700 Pa substrates (Figure 1C), suggesting that cells on this substrate are more highly sensitized to neurotrophic factors. NGF has also been shown to induce p21 activity to induce cell-cycle exit further implicating p75 NGFR as a marker of neuronal maturation [7]. These results indicate that not only is an intermediate substrate stiffness optimal for neuronal marker expression (TUJ1), but also for expression of genes important for neuronal development (p21) and neuronal maturation (p75 NGFR).

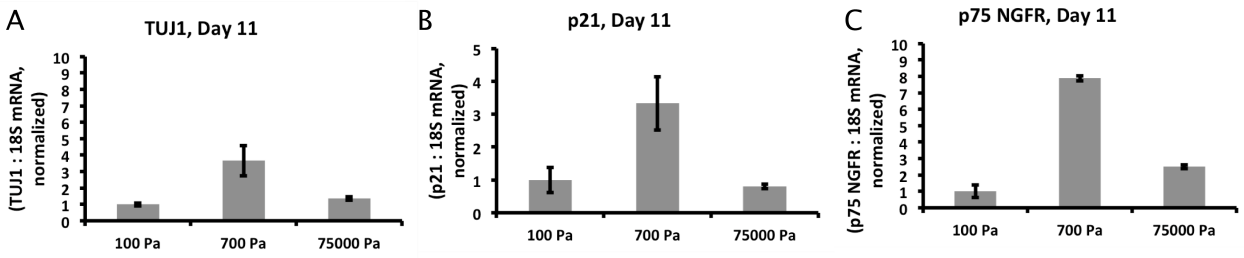

Figure 1. Neuronal marker and developmental genes are optimally expressed on an intermediate stiffness, 700 Pa, most similar to brain tissue. (A) TUJ1 is a marker of neurons. (B) p21 is a negative regulator of cyclin-dependent kinases and an indicator of a change in cell cycle status. (C) p75 NGFR (nerve growth factor receptor) is a neurotrophin receptor.

Intermediate substrate stiffness promotes optimal neuronal gene expression prior to neurotrophic factor addition

Substrate stiffness near that of brain tissue (~700 Pa) enhanced or accelerated neuronal maturation as measured after 11 days of culture. The expression of neurotrophin receptors that sensitize cells to neurotrophic factors can promote neuronal maturation. TrkA/B/C are responsive to neurotrophic factors like brain-derived neurotrophic factor (BDNF) and are expressed early (within the first 24 hours [2]) during neuronal differentiation. Therefore, TrkA/B/C mRNA expression was quantified by QPCR after 24 hours of differentiation. No differences in expression were observed as a function of substrate stiffness (Figure 2).

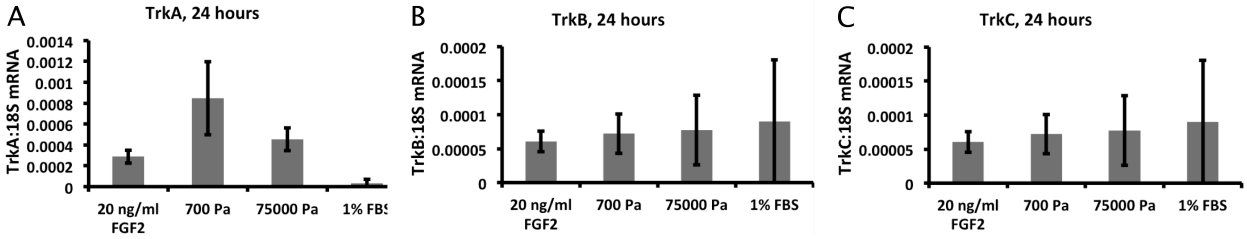

Figure 2. Substrate stiffness does not affect early, at 24 hours, expression of neurotrophin receptors (tropomyosin receptor kinase) (A) TrkA, (B) TrkB, and (C) TrkC.

Since TrkA/B/C expression was invariant with substrate stiffness, 700 Pa substrates could be promoting neuronal maturation without acting through neurotrophic factors. Optimal neuronal gene expression on 700 Pa substrates may therefore be observed prior to the addition of BDNF at Day 6. To test this hypothesis, aNSCs were seeded on polyacrylamide gels spanning a range of substrate stiffnesses. After 6 days of differentiation, TUJ1 gene expression was found to be optimal on 700 Pa substrates (Figure 3, control), similar to what was observed after 11 days of differentiation, supporting the hypothesis that substrate stiffness affects neuronal maturation independent of neurotrophic factor signaling.

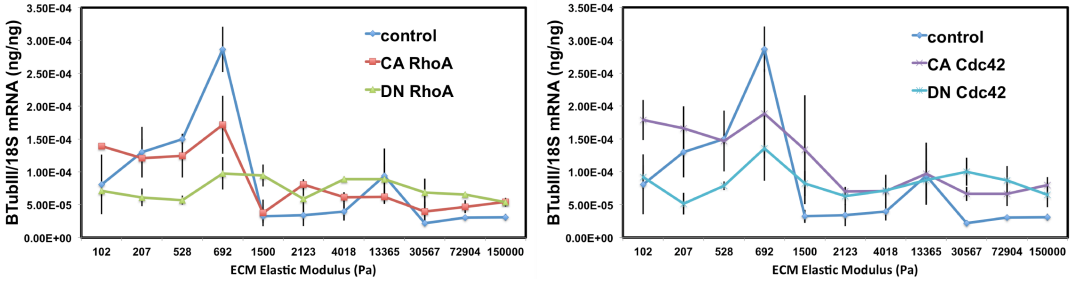

Figure 3. RhoA (left) and Cdc42 (right) activity increases TUJ1 expression on soft to intermediate stiffnesses while inhibition of RhoA and Cdc42 activity abolishes optimal TUJ1 expression at brain tissue stiffnesses.

RhoA and Cdc42 increase neuronal gene expression on soft substrates

We next asked what molecular mechanisms might be responsible for transducing ECM stiffness cues into modulation of neuronal maturation. Given that we had shown that RhoA and Cdc42 GTPases regulate mechanosensitive aNSC lineage commitment in mixed differentiation conditions (Chapter 2), we hypothesized these molecules may also regulate mechanosensitive neuronal maturation. Therefore, aNSCs expressing dominant negative or constitutively active RhoA or Cdc42 were similarly cultured for 6 days on polyacrylamide substrates. Strikingly, inhibiting RhoA or Cdc42 activity abrogated the enhancement of TUJ1 gene expression on 700 Pa substrates while expression of constitutively active RhoA or Cdc42 increased TUJ1 gene expression on substrates softer than 700 Pa while not affecting gene expression on stiffer substrates. These results implicate RhoA and Cdc42 in regulating the mechanosensitivity of neuronal maturation from aNSCs.

Rho GTPases and substrate stiffness do not affect the early expression of neural genes, NeuroD1, Sox2, and TUJ1

While neurotrophin receptor expression is insensitive to substrate stiffness, early modulation of genes regulating neuronal differentiation may be responsible for optimal neuronal gene expression at Day 6 (Figure 3). These include NeuroD1, known to drive neuronal differentiation [2], and Sox2, associated with the maintenance of neural progenitor states [8, 9]. QPCR analysis after 24 hours of differentiation of NSCs expressing DN or CA RhoA/Cdc42/Rac1 on 100, 700, and 75000 Pa substrates did not reveal robust regulation of neural gene expression by substrate stiffness (Figure 4). This suggests that substrate stiffness is operating at an intermediate and perhaps late time point to promote neuronal (TUJ1) gene expression (Figure 3).

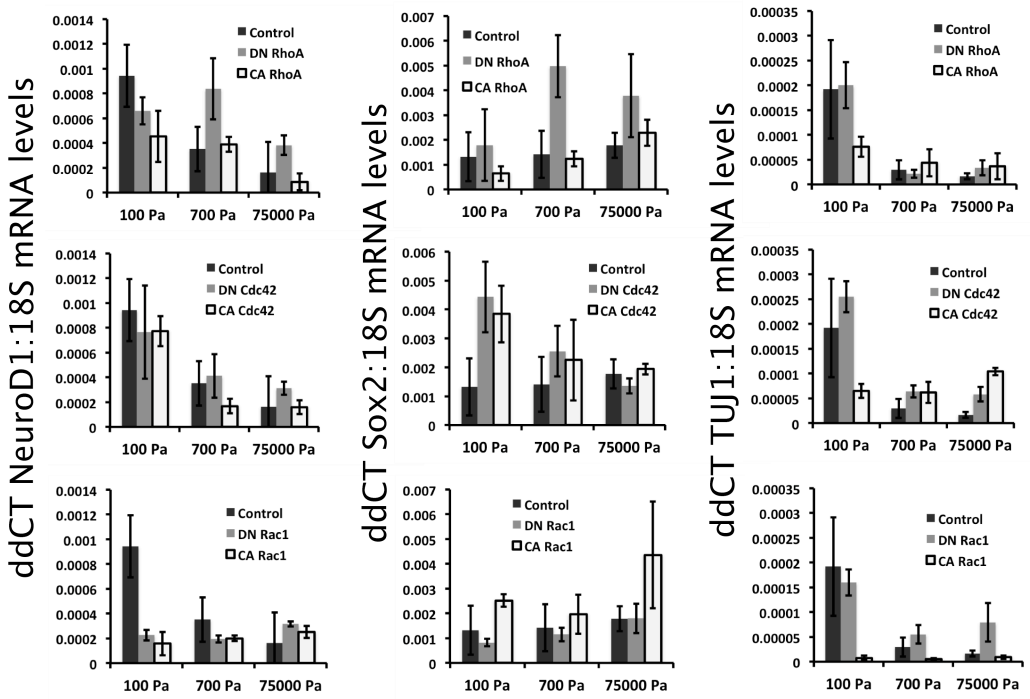

Figure 4. Rho GTPases and substrate stiffness do not affect the mRNA levels of NeuroD1, Sox2, and TUJ1 after 24 hours of differentiation.

Rho GTPases and substrate stiffness do not modulate neuronal subtype specification

Given the lack of substrate stiffness regulation of neurotrophin receptor and neural transcription factor expression (Figure 2 and 4), it is unclear if the optimal neuronal maturation observed on 700 Pa substrates at Day 6 and 11 (Figure 1 and 3) is also associated with or translated into an increase in neuronal subtype specification. Immunostaining against the neurotransmitter, gamma aminobutyric acid (GABA), and the vesicular glutamate transporter, VGlut1, assessed whether NSCs differentiated into GABAergic or glutamatergic neurons preferentially on different substrate stiffnesses and with different Rho GTPase activities. Neither substrate stiffness nor Rho GTPase activity biased subtype specification, suggesting that biophysical stiffness/contractility regulation primarily promotes the quality or extent of neuronal maturation/differentiation.

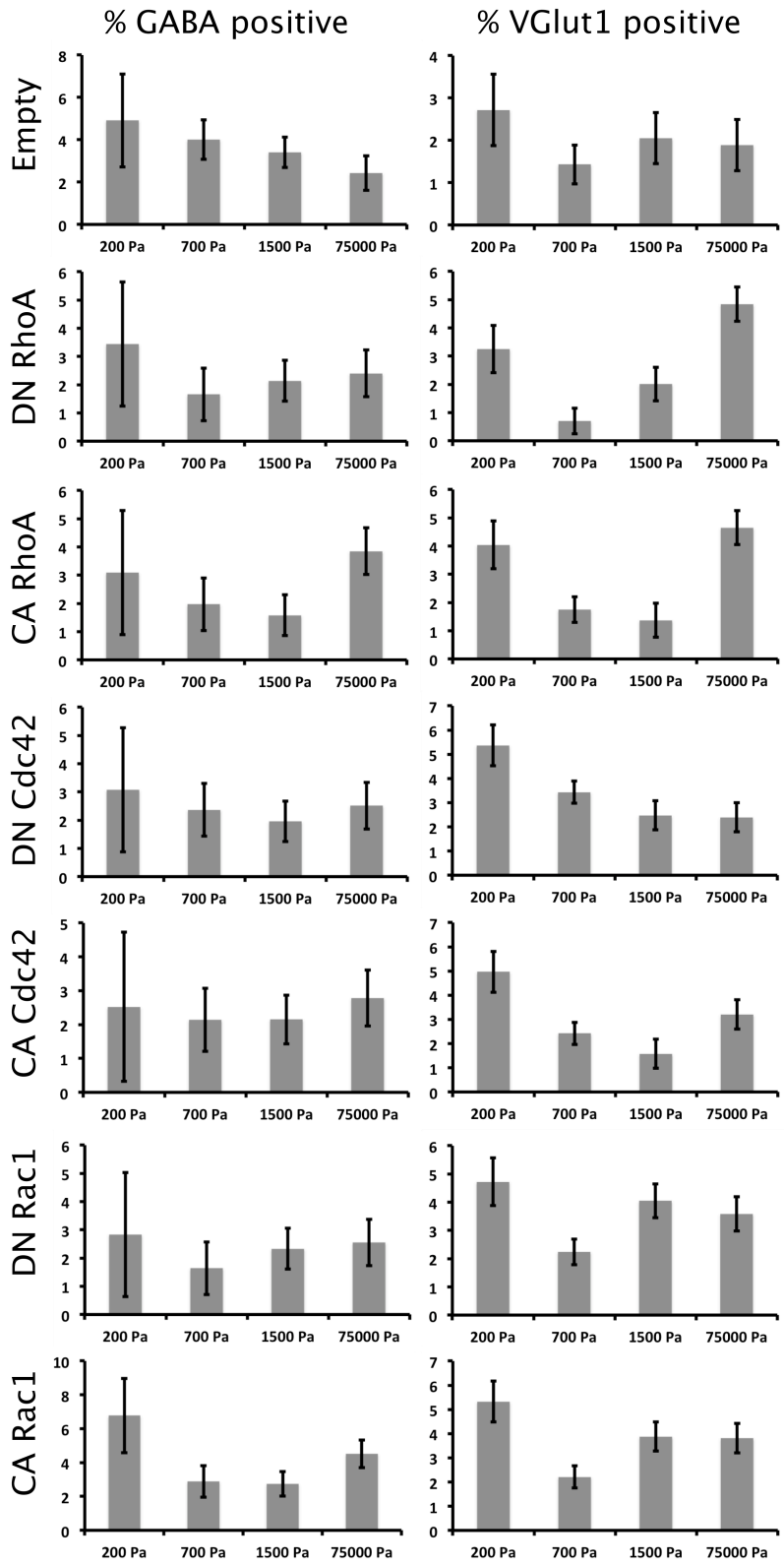

Figure 5. GABAergic and Glutamatergic neuronal subtype specification is not altered by substrate stiffness nor by Rho GTPase expression.

Discussion

We had previously found that in conditions permissive of aNSC differentiation into astrocytes, neurons, and oligodendrocytes, decreasing substrate stiffness gradually increased the percentage of neurons while decreasing the percentage of astrocytes generated. Interestingly, when soluble conditions are changed to induce primarily neuronal differentiation, substrate stiffness exerts a biphasic effect, with intermediate stiffnesses near 700 Pa promoting neuronal maturation. This may indicate the existence of multiple layers of biophysical control over stem cell differentiation, in which lineage commitment is determined by an initial set of biophysical regulatory rules, while differentiation along any given lineage is governed by a distinct set of biophysical rules. Here, biophysical regulation of neuronal differentiation appears to modulate the quality or extent of maturation but not subtype lineage specification, in contrast to its effect on lineage specification in neurons vs. astroctyes.

Furthermore, this distinction in substrate stiffness regulation of neuron vs. astrocyte specification (Chapter 2) and neuronal differentiation is also extended to the effect of Rho GTPases. In mixed differentiation conditions, aNSC mechanosensitivity was regulated by Rho GTPases; specifically, Rho and Cdc42 GTPase activation promoted astrocytic differentiation and inhibited neuronal differentiation. In contrast, in neuronal differentiation conditions, the activation of these GTPases promoted neuronal maturation on soft substrates, and inhibition of GTPase activity abrogated cellular mechanosensitivity. While the differentiation processes are clearly different in the two cases, the fact that Rho GTPase activity seems to inhibit neuron generation in the first case (Chapter 2) while promoting neuronal differentiation in the second (this chapter) is intriguing. This interesting difference suggests that, in addition to distinct differentiation processes occurring in each case, either distinct mechanotransductive mechanisms (either sensing or actuating) are regulating mixed and neuronal differentiation conditions, or there is some quantitative aspect of the signaling network regulating cellular response that switches mechanosensitivity from a monotonic response to a biphasic response. Future work may investigate in more detail the mechanotransductive mechanisms regulating neuronal maturation and lineage commitment in order to discern differences that may be responsible for the distinct behaviors.

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Chapter 4

Soft microenvironments promote the early neurogenic differentiation but not self-renewal of human pluripotent stem cells

Abstract

Human pluripotent stem cells (hPSCs) are of great interest in biology and medicine due to their ability to self-renew and differentiate into any adult or fetal cell type. Important efforts have identified biochemical factors, signaling pathways, and transcriptional networks that regulate hPSC biology. However, recent work investigating the effect of biophysical cues on mammalian cells and adult stem cells suggests that the mechanical properties of the microenvironment, such as stiffness, may also regulate hPSC behavior. While several studies have explored this mechanoregulation in mouse embryonic stem cells (mESCs), it has been challenging to extrapolate these findings and thereby explore their biomedical implications in hPSCs. For example, it remains unclear whether hPSCs can be driven down a given tissue lineage by providing tissue-mimetic stiffness cues. Here we address this open question by investigating the regulation of hPSC neurogenesis by microenvironmental stiffness. We find that increasing extracellular matrix (ECM) stiffness in vitro increases hPSC cell and colony spread area but does not alter self-renewal, in contrast to past studies with mESCs. However, softer ECMs with stiffnesses similar to that of neural tissue promote the generation of early neural ectoderm. This mechanosensitive increase in neural ectoderm requires only a short 5-day soft stiffness "pulse," which translates into downstream increases in both total neurons as well as therapeutically relevant dopaminergic neurons. These findings further highlight important differences between mESCs and hPSCs and have implications for both the design of future biomaterials as well as our understanding of early embryonic development.

Introduction

Human pluripotent stem cells (hPSCs) – including both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) – hold considerable promise as cell sources for biomedical therapies, disease models, and fundamental biological studies. Recent advances in culture systems [1,2] as well as in cellular reprogramming [3,4] have greatly accelerated progress towards many of these goals. For example, dopaminergic neurons, the predominant cell type lost in Parkinson's Disease, have been effectively generated from hPSCs [1,2,5] and functionally integrated into animal models [2], with promise for the development of cell replacement therapies. Clearly, improving our understanding of how the defining properties of self-renewal and differentiation are regulated in both hESCs and hiPSCs will improve the quantity and quality of hPSC-derived, therapeutically relevant cell populations as well as deepen our understanding of organismal development.

Over the past two decades, researchers have assembled considerable knowledge of how biochemical factors, signaling pathways, and transcriptional networks [6-8] regulate hPSC behaviors. At the same time, it has also become clear that the biophysical properties of the microenvironment, especially extracellular matrix (ECM) stiffness, can powerfully control a variety of cell behaviors, including the self-renewal and differentiation properties of adult stem cells [9-11]. However, our understanding of how these biophysical inputs may regulate hPSC biology and be leveraged to drive differentiation into therapeutically desirable cell types remains in its infancy. Previous work in mouse embryonic stem cells (mESCs) suggests that biophysical cues such as cyclic strain or stiffness [12,13] may be important in regulating cell behavior. However,

hPSCs exhibit starkly different behaviors from mESCs, including their colony-based growth, sensitivity to different growth factors [14], and response to the biophysical cues of cyclic strain [15]. These findings are consistent with early observations of differential marker expression [16] as well as recent sequencing efforts that have revealed substantial transcriptomic differences between mESCs and hESCs and only modest overlap between pathways critical for self-renewal in each species [17]. Thus, due to these overall differences between mESCs and hPSCs, phenomenology obtained with mESCs cannot be assumed *a priori* to hold for hPSCs. Furthermore, standard hPSC culture systems are more complex than those for mESCs. For example, hPSC culture requires either co-culture with feeder cells or matrix proteins, such as in the highly complex product Matrigel, instead of simple collagen coated surfaces. Moreover, hPSCs must be cultured as colonies due to the low survival of dissociated single hPSCs. This relative complexity of hPSC compared to mESC culture may explain why comparatively little is known about hPSC mechanobiology. Given the intense interest in biomaterials development for hPSC cultures [18, 19], it is important to understand how biophysical regulatory aspects of the microenvironment could be leveraged to create highly defined culture systems for hPSCs.

To study the effect of microenvironmental stiffness in isolation from other potentially confounding factors, hESC and hiPSC colonies were cultured on Matrigel-coated polyacrylamide gels and found to increase in cell and colony spread area with increasing extracellular matrix (ECM) stiffness. However, hPSC self-renewal was not affected, in contrast to previous findings with mESCs. Interestingly, using an adherent differentiation protocol [1] in which cells experience the mechanical properties of the biomaterial substrate, softer ECMs were found to promote both hESC and hiPSC differentiation into neurons and subsequently into therapeutically relevant dopaminergic neurons. Furthermore, by analyzing early neural ectodermal marker expression as well as by shortening the temporal exposure to soft ECM stiffnesses, hESCs and hiPSCs were found to be mechanically responsive at an early period during differentiation. These results expand our understanding of hPSC developmental biology and identify a new biophysical axis of control to improve the generation of therapeutically relevant dopaminergic neurons.

Materials and Methods

Stem Cell Culture

hPSCs were cultured on Matrigel-coated (BD Biosciences, Franklin Lakes, NJ) tissue culture-treated polystyrene plates and glass chamber wells. For growth or self-renewal conditions, H1 hESCs [16] were cultured in XVIVO media (Lonza, Basel, Switzerland) supplemented with 80 ng/mL human basic fibroblast growth factor (hbFGF, Peprotech, Rocky Hill, NJ) and 0.5 ng/mL transforming growth factor β1 (TGF-β1, R&D Systems, Minneapolis, MN). MSC-iPS hiPSCs [20] were cultured in mTeSR media with 1x final dilution of mTeSR 5x Supplement (Stemcell Technologies, Vancouver, BC).

Neuronal differentiation conditions were adapted from Chambers et. al. [1]. Briefly, ~25-50 cell clusters of hPSCs were seeded on ECMs of defined stiffnesses or polystyrene at a total cell density of 25000 cells/cm^2. hPSCs were cultured for 3 days in growth conditions. Differentiation was then induced on day 0 by changing media to DMEM:F12 (Invitrogen, Carlsbad, CA), 20 % knockout serum replacement (KSR, Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St Louis, MO), 1 μM LDN-193189 (Stemgent, San Diego, CA), 10 μM SB-451542 (Tocris Biosciences, Ellisville, MO). Half-media changes were made daily from day 1-4. On day 5, cells

were either maintained on their current ECM or passed *en bloc* to polystyrene. Media was changed to DMEM:F12, 15 % KSR, 0.25 % N2 supplement (Invitrogen), 0.1 mM β-mercaptoethanol, and 200 ng/mL recombinant N-terminal human sonic hedgehog (SHH) with a C24II Substitution (SHH, R&D Systems). On day 7, media was changed to DMEM:F12, 10 % KSR, 0.5 % N2 supplement, 0.1 mM β-mercaptoethanol, and 200 ng/mL SHH. On day 9, all cells were passed *en bloc* to polystyrene. Medium was changed to DMEM:F12, 5 % KSR, 0.75 % N2 supplement, 0.1 mM β-mercaptoethanol, 200 ng/mL SHH, 100 ng/mL recombinant human fibroblast growth factor-8b (FGF-8b, Peprotech), 20 ng/mL brain-derived neurotrophic factor (BDNF, Peprotech), and 0.2 mM ascorbic acid (Sigma). On day 12, medium was changed to DMEM:F12, 0.1 % N2 supplement, 0.1 mM β-mercaptoethanol, 20 ng/mL BDNF, 0.2 mM ascorbic acid, 20 ng/mL glial-derived neurotrophic factor (GDNF, Peprotech), 1 ng/mL transforming growth factor β3 (TGF-β3, Peprotech), and 1 μM cyclic adenosine monophosphate (Sigma). Medium was changed every two days until day 19.

Flow Cytometry Analysis

Cells were dissociated with 0.25% Trypsin/2.5mM EDTA and stained using the following primary antibodies: rabbit anti-Nanog (1:250 dilution; Abcam, Cambridge, UK), mouse anti-Stage-specific embryonic antigen-4 (SSEA-4, 10 μg/mL; Millipore, Billerica, MA), mouse anti-Tra-1-60 FITC conjugate (1:100 dilution; Millipore), mouse anti-Oct-3/4 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were FITC-conjugated goat anti-rabbit IgG and Cy5-conjugated goat anti-mouse IgG at a dilution of 1:250 (all from Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Samples were analyzed on a FC500 Analyzer (Beckman-Coulter, Brea, CA).

Immunofluorescence Staining and Microscopy

Cells were fixed with 4% paraformaldehyde. Samples were blocked and permeabilized in 2% goat serum (Sigma) and 0.3% Triton X-100 (Calbiochem, San Diego, CA) in pH 7.4 phosphate buffered solution at room temperature. Samples were incubated for 36 hours at 4°C with the following primary antibodies: mouse anti-β-tubulin III (TUJ1, 1:1000 dilution; Sigma-Aldrich), rabbit anti-tyrosine hydroxylase (TH, 1:1000 dilution; Pel-Freez, Rogers, AR), and rabbit anti-PAX6 (PAX6, 1:250 dilution; Covance, Emeryville, CA). The primary antibody solution was removed, and cells were rinsed and incubated for 2 hours with the secondary antibodies FITC-conjugated goat anti-rabbit IgG and Cy5-conjugated goat anti-mouse IgG at a dilution of 1:250 (all from Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Nuclei were stained with DAPI (Invitrogen) at 10 μg/ml. Cells were manually scored as positive or negative for lineage markers using the optical fractionator method in an unbiased stereological microscope (Zeiss Axio Imager, software by MicroBrightfield). 3-6 experiments were performed in parallel cultures for each study. 15-20 confocal images obtained on a LSM710 (Carl Zeiss Inc, Oberkochen, Germany) were z-stacked and flattened in ImageJ. Additional immunofluorescence and phase images were collected on a Nikon Eclipse TE2000-E microscope with a Photometrics Coolsnap HQ2 camera. Colony sizes were quantified in ImageJ using manual outlining.

Quantitative real time Polymerase Chain Reaction (QRT-PCR)

Cells were lysed and frozen in TRIZOL (Invitrogen), and mRNA was extracted and reverse transcribed to cDNA using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis (Invitrogen, Carlsbad, CA). Equivalent amounts of total RNA were transcribed into

cDNA, which was subsequently used as template for each QRT-PCR reaction (utilizing a Bio-Rad Laboratories iCycler 5, Hercules, CA). To normalize any remaining variations in starting cDNA amounts, each reaction was referenced to ribosomal 18S detected using Cal-dye TaqMan probes and the lineage marker was detected using FAM-dye TaqMan probes (Biosearch Technologies, Novato, CA) or Sybr Green (Invitrogen). QRT-PCR reactions were run for each biological sample with n=4-6 for each condition. The TaqMan probes used are listed as follows: (TUJ1, 5'-GCATGGACGAGATGGAGTTCACC-3', 5'-CGACTCCTCCTCGTCGTCTTCGTAC-3', 5'-FAM490-TGAACGACCTGGTGTCCGAG-BHQ-3'), and (18S, 5'-GTAACCCGTTGAACCCCATTC-3', 5'-CCATCCAATCGGTAGTAGCGA-3', 5'-CAL610-AAGTGCGGGTCATAAGCTTGCG-BHQ-3'). PAX6 and SOX1 gene expression was assayed using Qiagen Quantitect Primer Assays (Hs_PAX6_1_SG QT00071169, Hs_SOX1_2_SG QT01008714).

Polyacrylamide Substrate Preparation

Using a protocol similar to that described previously [11], polyacrylamide gels (70 μm nominal thickness) were synthesized on 12, 18, and 25 mm glass coverslips using solutions composed of varying concentrations of acrylamide monomer and bisacrylamide crosslinker. 100 μg/ml poly-D-lysine was linked to the surface through sulfo-SANPAH (Thermo-Fisher, Waltham, MA) chemistry and incubated for 3 hours at 37°C, and 250 μg/ml Matrigel was then absorbed for 3 hours at 37°C.

Results

hPSC cell and colony area increase with ECM stiffness

We first sought to ask whether microenvironmental stiffness altered hPSC morphology, self-renewal capacity, and pluripotency. Therefore, hESC and hiPSC colonies were dissociated to 25-50 cell clusters and seeded at equal cell density on 100, 700, and 75000 Pa polyacrylamide ECMs. After 3 days in growth conditions, 4X phase images were acquired (Figure 1, right), and hPSC colony area was quantified (Figure 1, left). Raising ECM stiffness from 100 to 700 Pa and from 100 to 75000 Pa dramatically increased colony area for both hESCs and hiPSCs by about 3-fold and 7-fold.

This rise in colony area may be due to either greater cell proliferation or a larger degree of cell spreading on stiff ECMs. To test these possibilities, cultures were dissociated after 3 days in growth conditions, and cell number was quantified. The overall cell density did not vary with ECM stiffness (Figure 2A), revealing that proliferation was not affected by ECM stiffness and instead indicating that cells on softer ECMs had smaller projected areas within colonies, also observed through phase images (Figure 1, right). In addition, flow cytometry analysis showed that regardless of stiffness, the expression of the pluripotency markers Nanog, Oct-3/4, and SSEA-4 expression were maintained at high levels (Figure 2B). Furthermore, while soft ECMs can maintain mouse embryonic stem cell pluripotency even in the absence of growth factors [12], hPSCs did not exhibit differences in Tra-1-60 marker expression as a function of ECM stiffness when the growth factors (FGF-2 and TGF-b for hESC, mTeSR supplement for hiPSC) were removed (Figure 2C). Therefore, while hPSC colony morphology was mechanosensitive, cell proliferation and pluripotency were apparently insensitive to ECM stiffness.

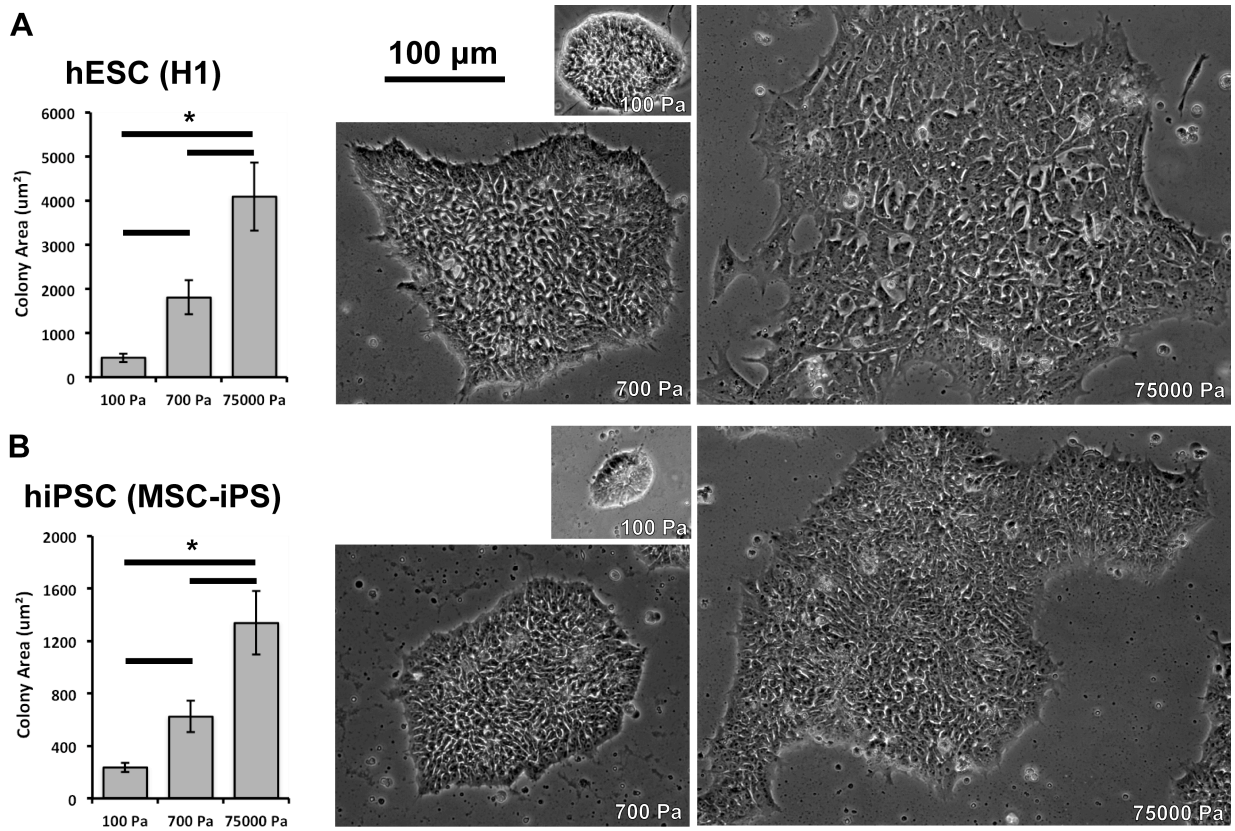

Figure 1. hPSC colony area increases with substrate stiffness. Quantification of colony area and phase contrast images of human (A) embryonic H1 and (B) induced pluripotent MSC-iPS stem cells cultured on 100, 700, and 75000 Pa polyacrylamide substrates after 3 days in self-renewal conditions. Error bars are 95% confidence intervals, n = 220-300 colonies. *$p < .05$ (ANOVA, Tukey-Kramer). Abbreviations: ANOVA, analysis of variance; MSC-iPS, mesencymal stem cell-induced pluripotent stem cell; hPSC, human pluripotent stem cell; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell.

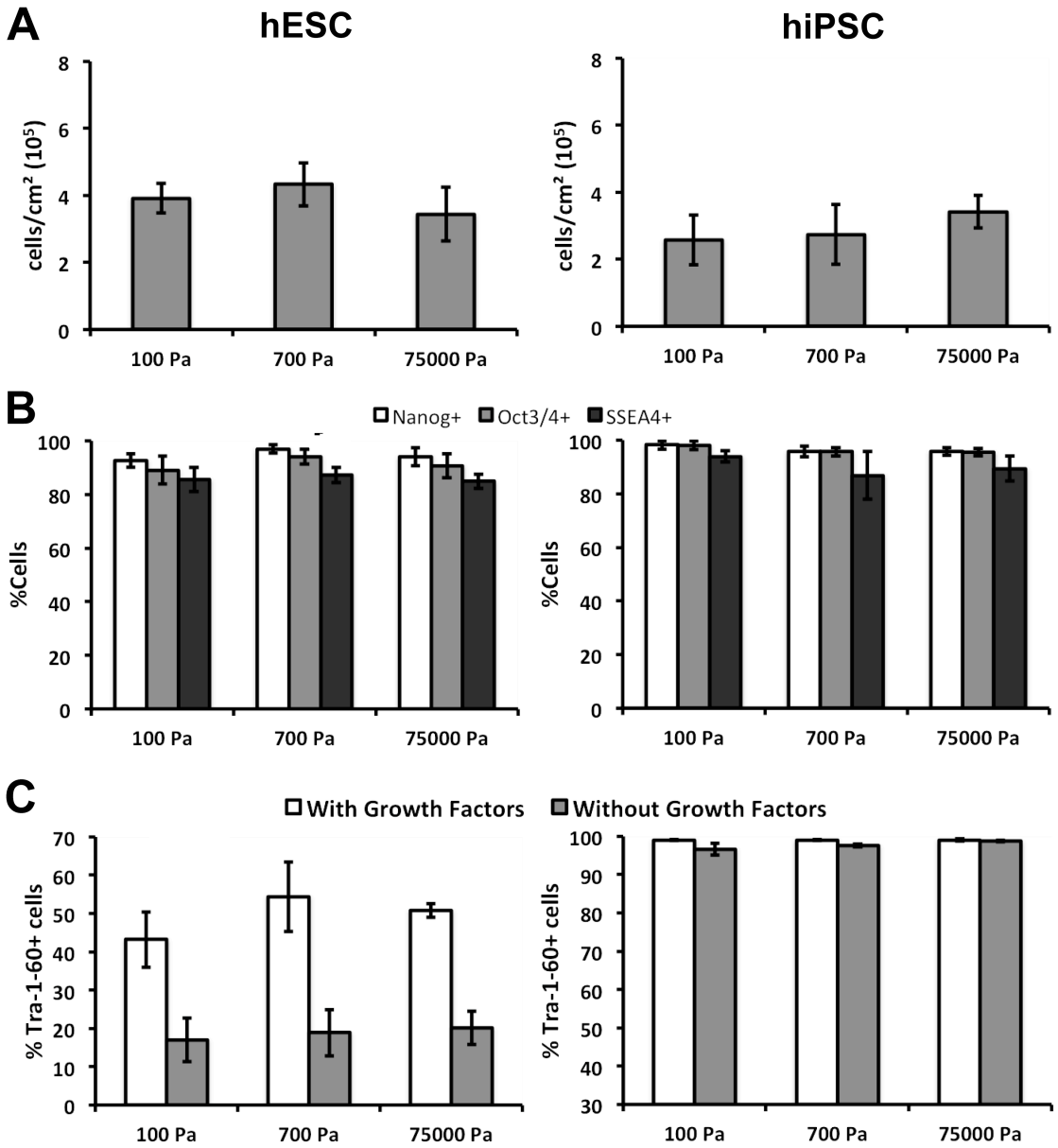

Figure 2. hPSC pluripotency marker expression is not altered by substrate stiffness. hESCs (left) and hiPSCs (right) show similar (A) cell numbers and (B) expression of pluripotency markers after 3 days in self-renewal conditions. (C) Substrate stiffness does not alter the expression of pluripotency marker Tra-1-60 even with the withdrawal of growth factors. Error bars are 95% confidence intervals, $n = 3$. *$p < .05$ (ANOVA, Tukey-Kramer). Abbreviations: ANOVA, analysis of variance; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell.

Soft ECMs promote early neural ectodermal and neuronal differentiation

Since ECM stiffness did not affect hPSC pluripotency and proliferation, we analyzed whether it modulated differentiation. Substrates with stiffnesses near that of brain tissue (such as 100 and 700 Pa used in this study) have been shown to regulate the behaviors of partially and fully differentiated cells, including adult neural stem cell (aNSC) differentiation [21] and neuronal morphology [22]. We hypothesized that ECM stiffnesses in the 100-700 Pa range may also regulate earlier processes in neural differentiation, such as the generation of neural ectoderm, and hPSCs offer a model to study such early human developmental processes. To address this question, a

recent adherent protocol for the generation of neural populations from hPSCs [1] was adapted by differentiating hESCs and hiPSCs for 9 days on ECMs of different stiffnesses (within the 12-day limit for the reliable stability of the polyacrylamide ECM coatings). 25-50 hPSC clusters were seeded on 100, 700, and 75000 Pa ECMs and cultured in growth conditions for 3 days. Next, to induce differentiation, cells were cultured in Smad inhibitors and KSR for 5 days, followed by the early initiation of dopaminergic neuronal patterning by Sonic hedgehog addition for a subsequent 4 days. Immunostaining (Figures 3C and 3F) showed that 100 Pa ECMs promoted PAX6 positive (neural ectoderm) cell generation at levels 15-20% greater than 700 and 75000 Pa ECMs (Figures 3A and 3D). In addition, consistent with this early stage of differentiation, PAX6 levels were high and comparatively fewer neurons were observed [1]. However, both 100 and 700 Pa ECMs interestingly promoted TUJ1 positive (neuron) cell generation at levels 2-3 fold greater than 75000 Pa ECMs (Figures 3B and 3E).

Quantitative reverse transcription polymerase chain reaction (QRT-PCR) analysis, conducted to complement the immunostaining, further demonstrated that 100 Pa substrates promoted PAX6 expression (Figure 4A), and both 100 and 700 Pa ECMs promoted TUJ1 induction over 75000 Pa ECMs and polystyrene (Figure 4B) after 9 days of differentiation. We next assessed whether the expression of another early ectodermal precursor marker, such as SOX1, may correlate with PAX6 and in particular TUJ1 positive cells on both 100 and 700 Pa ECMs. Interestingly, the early ectodermally expressed SOX1 tracked TUJ1 expression, with greater expression on both 100 and 700 Pa ECMs compared to 75000 Pa ECMs and polystyrene (Figure 4C). These results indicated that ECM stiffness modulates early neural differentiation events and therefore offers the potential to increase subsequent neuronal differentiation.

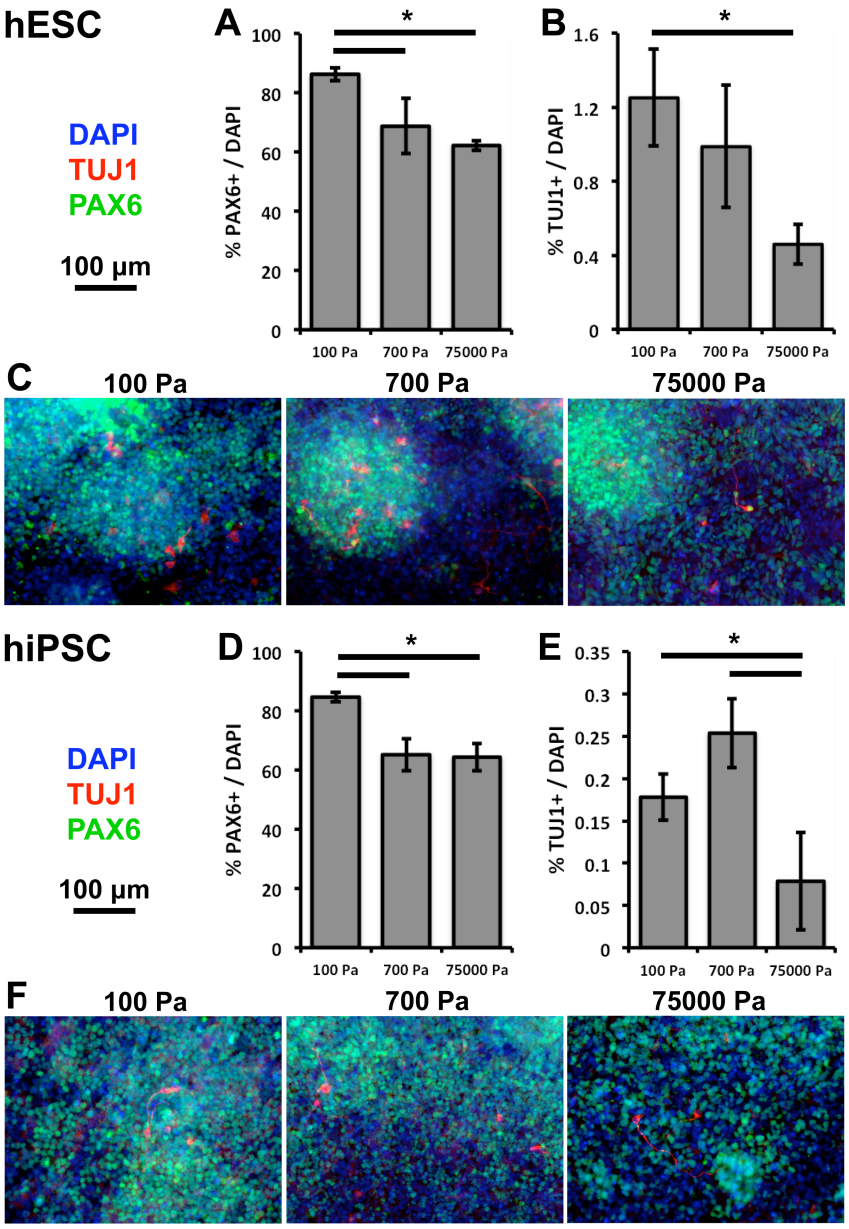

Figure 3. Softer substrates promote neural ectodermal and neuronal differentiation from hPSCs after 9 days of differentiation. Compared to 700 and 75000 Pa substrates, 100 Pa substrates promote PAX6 gene expression in cultures derived from (A) hESCs and (D) hiPSCs. Compared to 75000 Pa substrates, 100 and 700 Pa substrates promote neuronal marker TUJ1 gene expression from cultures derived from (B) hESCs and (E) hiPSCs. Representative immunofluorescence images of cultures derived from (C) hESCs and (F) hiPSCs. Red-TUJ1, Green-PAX6, Blue-DAPI. Error bars are 95% confidence intervals, $n = 3$. *$p < .05$ (ANOVA, Tukey-Kramer). Abbreviations: ANOVA, analysis of variance; hPSC, human pluripotent stem cell; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell.

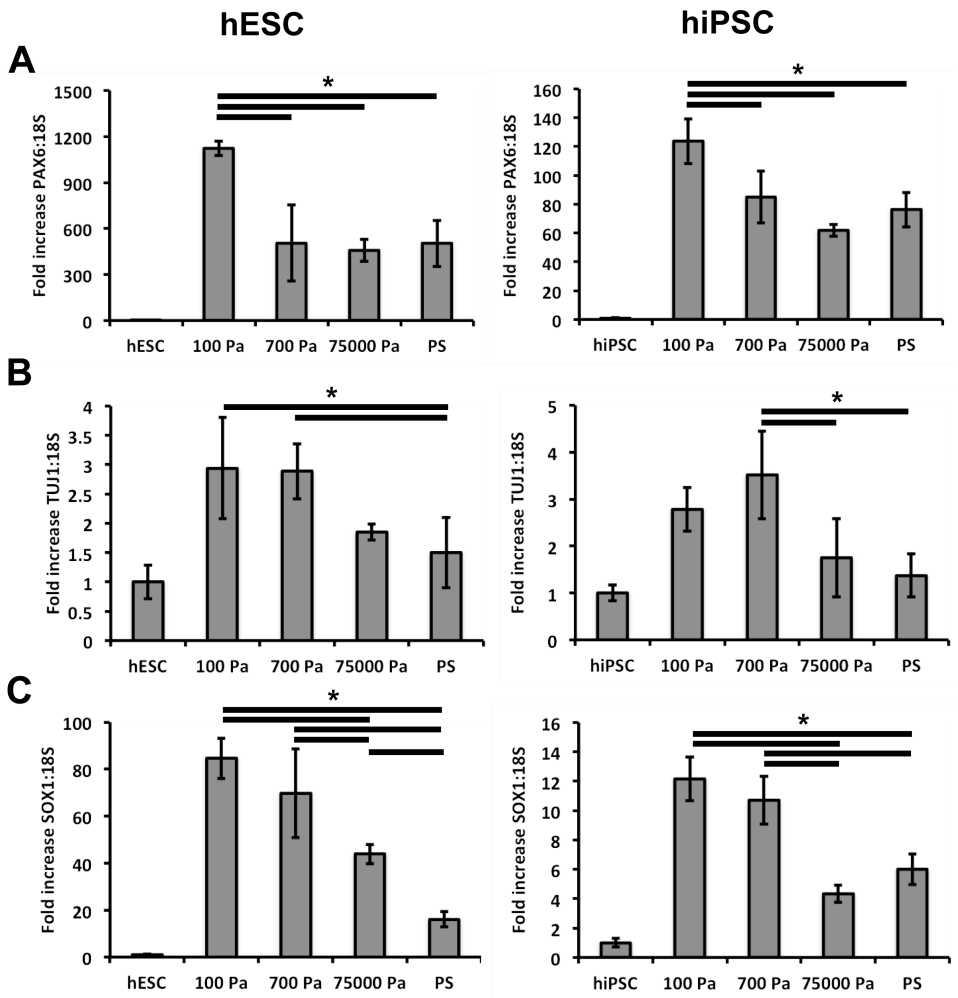

Figure 4. Softer substrates promote neuronal and early ectodermal differentiation from hPSCs after 9 days in culture. (A) 100 Pa substrates promote PAX6 gene expression in (left) hESCs and (right) hiPSCs. (B) 100 and 700 Pa substrates promote TUJ1 gene expression in hPSCs. (C) 100 and 700 Pa substrates promote SOX1 gene expression in hPSCs. Error bars are 95% confidence intervals, $n = 4$. *$p < .05$ (ANOVA, Tukey-Kramer). Abbreviations: ANOVA, analysis of variance; hPSC, human pluripotent stem cell; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; PS, polystyrene.

Soft ECMs promote dopaminergic and overall neuronal differentiation

We next determined whether the higher levels of neural differentiation observed on soft substrates after 9 days translate to downstream increases in the number of mature neurons, particularly dopaminergic neurons. hPSCs were cultured for 9 days on 100, 700, 75000 Pa ECMs or polystyrene as before, passaged *en bloc* to glass chamber wells for an additional 10 days to allow for neuronal maturation, and analyzed for the number of TUJ1 positive neurons and tyrosine hydroxylase (TH) positive dopaminergic neurons. The proportion of neurons within the culture progressively increased with decreasing ECM stiffness for both hESCs (Figures 5A, 5B, and 5D) and hiPSCs (Figures 5E, 5F, and 5H), with slightly lower numbers for hiPSCs. Furthermore, the fraction of these neurons that were TH+ did not change with stiffness, suggesting ECM stiffness may act primarily during early differentiation processes into early neural ectoderm/progenitors and not during neural patterning; however, the yield of TH positive cells increased with softer materials since the number of neurons increased. In sum, the

proportion of TUJ1 and TH positive cells are nearly 3-fold higher on soft ECMs compared to traditional polystyrene ECMs.

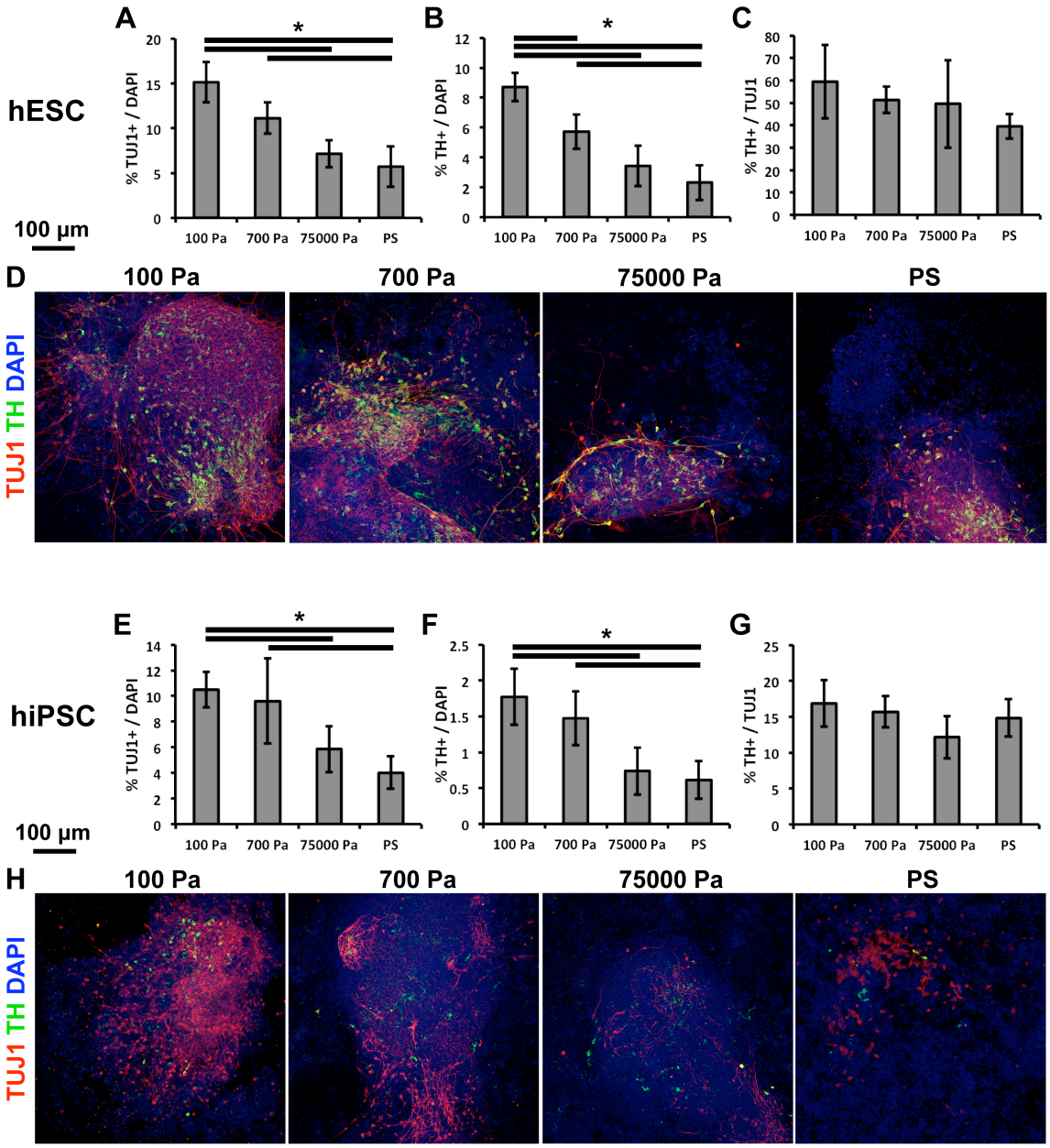

Figure 5. Softer substrates promote the differentiation of hPSCs into neurons and dopaminergic neurons but do not change the proportion of dopaminergic to total neurons after 19 days of differentiation. The percentage of cells (DAPI+) that are TUJ1+ (A-hESC, E-hiPSC) and TH+ (B-hESC, F-hiPSC) decreases with increasing substrate stiffness. The percentage of TUJ1+ cells that are also TH+ does not change with substrate stiffness (C-hESC, G-hiPSC). Representative immunofluorescence images of cultures derived from (D) hESCs and (H) hiPSCs. Red-TUJ1, Green-TH, Blue-DAPI. Error bars are 95% confidence intervals, $n = 4$. *$p < .05$ (ANOVA, Tukey-Kramer). Abbreviations: ANOVA, analysis of variance; hPSC, human pluripotent stem cell; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; PS, polystyrene.

Only a brief 5 day exposure to soft ECMs is required to increase dopaminergic differentiation

SOX1 expression has been shown to plateau after 5 days in Smad-inhibiting conditions [1]. Therefore, ECM stiffness may modulate SOX1 expression (Figure 4C) by regulating early differentiation processes prior to the addition of neural patterning factors on day 5 (Figure 6A). Furthermore, the percentage of dopaminergic over total neurons is invariant with ECM stiffness (Figure 5C and 5G), indicating that ECM stiffness exerts less influence from days 5-9, the first days of neural patterning when dopaminergic specification would begin to occur. We therefore asked if cells cultured on different ECM stiffnesses for less time (5 rather than 9 days) could still enjoy the full effects of ECM stiffness on the final dopaminergic neuron levels (Figure 6A). When this "stiffness pulse" window was decreased to the first 5 days of differentiation, 700 Pa ECMs still promoted neuronal and dopaminergic neuronal differentiation over 75000 Pa ECMs (Figure 6B, 6C, 6F, and 6G) and importantly yielded similar percentages of each cell type as with the longer 9 day pulse (Figure 5). Thus, ECM stiffness operated primarily during initial differentiation into neural ectoderm and exerted less influence during the subsequent neural patterning.

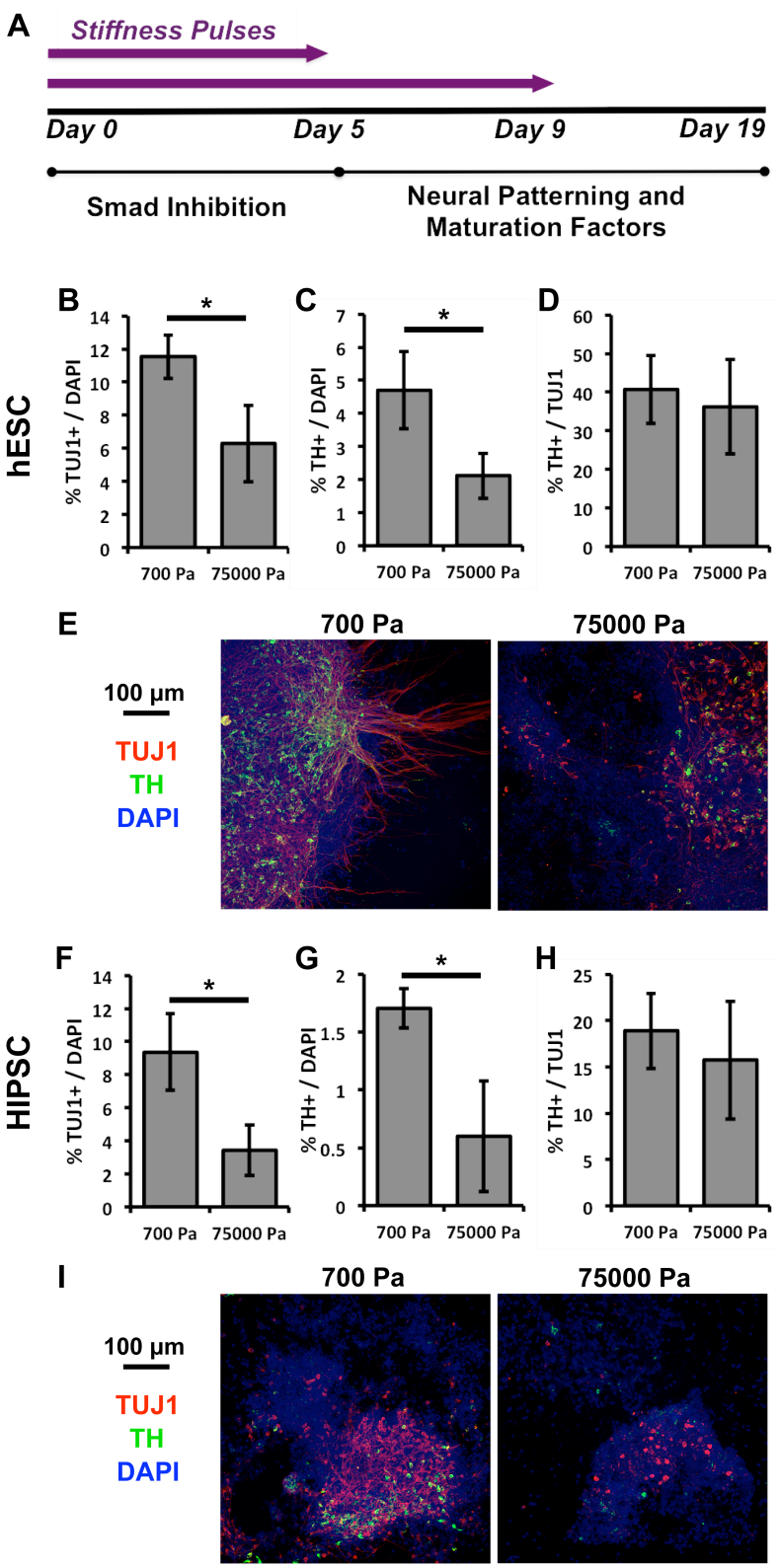

Figure 6. (A) A shortened substrate stiffness pulse (from 9 to 5 days) prior to neural patterning is sufficient to modulate neuronal and dopaminergic differentiation to the same extent as a 9 day pulse. The percentage of cells (DAPI+) that are TUJ1+ (B-hESC, F-hiPSC) and TH+ (C-hESC, G-hiPSC) decreases with increasing substrate stiffness. The ratio of TH+ to TUJ1+ cells does not change with substrate stiffness (D-hESC, H-hiPSC).

Representative immunofluorescence images of cultures derived from (E) hESCs and (I) hiPSCs. Red-TUJ1, Green-TH, Blue-DAPI. Error bars are 95% confidence intervals, $n = 4$. *$p < .05$ (ANOVA, Tukey-Kramer). Abbreviations: ANOVA, analysis of variance; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; PS, polystyrene.

Discussion

We have shown that ECM stiffness regulates early differentiation but not self-renewal of hPSCs. In particular, while hPSC morphology is mechanosensitive under growth conditions, since cell and colony areas increase with increasing ECM stiffness, pluripotency marker expression and cell proliferation are not affected by ECM stiffness. In contrast, early neurogenic differentiation into SOX1 positive neural ectoderm prior to neural patterning is strongly modulated by this biophysical input. After further neural patterning and maturation, this effect translates to higher percentages of total neurons as well as dopaminergic neurons.

The relative stiffness-insensitivity of hPSC pluripotency marker expression contrasts with the observed rescue of mESC pluripotency on soft ECMs [12] and suggests that continued investigations into ESCs derived from both species will be needed to develop a more complete picture of the nature of pluripotency. These species-dependent differences are consistent with past comparative observations between hPSCs and mESCs. For example, these two cell types have exhibited differing responses to another biophysical cue, cyclic strain, which was shown to inhibit human ESC [15] but promote mouse ESC [13] differentiation. These contrasting mechanosensitive phenotypes may arise from a growing list of observed differences in the fundamental cell biology of human and mouse ESCs, including in developmental stage [23, 24], transcription factor binding [25], pluripotency marker expression [26], nuclear receptor expression during differentiation [27], keratin expression [28], and growth factors and signaling pathways that maintain pluripotency [29, 30]. In the future it may be interesting to compare potential crosstalk between these numerous factors and candidate mechanotransductive signaling pathways [11, 31-33] to elucidate species-distinct mechanosensitive behaviors and to understand biomedically relevant, human-specific properties that could be harnessed for therapeutic application.

Our finding that hiPSCs, like adult NSCs [11, 21], increase neurogenesis on softer substrates offers important implications for the future development of biomedical therapies. hiPSCs in particular hold promise for patient-specific cell replacement therapies since they may more effectively evade immune responses than allogeneic hESC grafts, as well as bypass potential ethical concerns and corresponding supply limitations of embryo-derived hESCs in some countries. hiPSCs did exhibit lower overall levels of neurogenesis compared to hESCs, potentially due to epigenetic memory of their mesenchymal origins [20, 34, 35]. However, the fact that hiPSC neural differentiation was still mechanosensitive despite epigenetic and transcriptomic differences between hiPSCs and hESCs [36] and significantly different methods of derivation [3, 16] suggests the the observed mechanosensitivity of neuralization may generalize to many different types of hPSCs.

We and others have previously shown that ECM stiffness can modulate adult neural stem cell (aNSC) differentiation into neurons, astrocytes, and oligodendrocytes [21, 37, 38], and the use of hPSCs in this study allows investigation of progenitor cells representative of earlier developmental periods. Culturing hPSCs on soft ECMs that mimic the stiffness of neural tissue (~100-1500 Pa) promoted the generation of neurons as it did with aNSCs. However, in contrast to the alteration of neuronal lineage commitment observed for aNSCs, for hPSCs the stiffness effect was mediated by increasing the percentage of early (SOX1+) neural progenitors. Interestingly, exposure to soft ECMs for only 5 out of a total of 19 days was sufficient to observe

the downstream increase in neurons. Implementation of this "stiffness pulse" strategy thus reveals that *when* a signal is presented may be just as important as *what* signal is presented. Given that mechanical properties can function during multiple stages of differentiation, from neural conversion of hPSCs to neuronal differentiation and maturation of aNSCs, stem cell differentiation protocols that rely primarily on soluble media conditions [2] could be further improved by designing an optimal and temporally dynamic biophysical microenvironment. Our findings can therefore be applied to engineer biomaterials scaffolds and bioreactors for human pluripotent stem cell differentiation.

Future work could investigate extension of these observations to non-neural lineages, as well as address potential mechanisms responsible for our observations. In our system, softer ECMs resulted in lower extents of cell and colony spreading but did not affect hPSC proliferation. The resulting higher effective cell densities or packing may yield smaller and more condensed individual cells and nuclei, as well as impact both the quantity and quality of cell-cell contacts during subsequent cell differentiation. These factors may in turn invoke cell and nuclear size/shape mechanisms important in mesenchymal stem cell differentiation [33, 39] and/or cell packing/density effects found in hESC systems [40]. It is interesting to note that, while Chambers and colleagues observed a bias in downstream neuronal subtype specification and neural patterning due to cell density/packing [1], we instead observed an earlier bias in the generation of neural progenitors and early neural ectoderm, suggesting that different combinations of cell-cell contacts, cell density/packing, and/or perhaps cell/nuclear shape may play important roles throughout neurogenesis.

The shape of a cell has been shown to affect its mechanical properties [33], and ECM stiffness may also directly modulate cellular mechanics, either or both of which could in turn affect neural differentiation. Due to low survival of single hPSCs and inefficient clonal growth [41], it is difficult to study the effects of ECM stiffness directly on single cells. However, intracellular probes of force generation and mechanical properties [42 43], in conjunction with biochemical and genetic studies, may help elucidate mechanisms of mechanosensitive hPSC differentiation into neural lineages.

Conclusion/Summary

We have shown that hPSC self-renewal is insensitive to ECM stiffness, yet neural differentiation is mechanosensitive. Furthermore, only an early, short stiffness pulse is required to enhance downstream neuronal differentiation. In addition to providing potential mechanistic insights into the mechanosensitive neural differentiation of hPSCs, the short temporal window of exposure to soft ECMs required to improve neurogenesis is important from a technological perspective in the design of cell culture systems. The early mechanosensitivity of hPSCs shown here may thus not only influence the future design parameters of biomaterials to improve the generation of therapeutically relevant cell populations such as dopaminergic neurons, but also inform our understanding of the influence of biophysical cues in early embryonic development.

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Chapter 5
Mechanobiology of Cellular Reprogramming

Introduction

We have studied the mechanosensitivity of stem cell differentiation into neural lineages at various temporal or developmental stages, from embryonic stem cells to neural progenitors to mature neurons. However, processes that are the conceptual opposite of differentiation also exist, both in natural and engineered systems. For example, differentiated cells in tissue have been observed to revert to stem-like states and are important in repopulating germline niches. [1, 2] Future work may identify additional natural occurrences of reprogramming. Furthermore, recent advances in artificial reprogramming have provided scientists the opportunity to study how native niches induce reprogramming as well generate patient-specific therapeutic cell populations or disease-specific models.

Since somatic mouse [3] and human [4] cells were first reprogrammed to pluripotent states, an impressive body of work has elucidated biochemical signaling networks and small molecules regulating or enhancing cellular reprogramming. However, there is also evidence that cell-cell interactions [5] and epithelial-mesenchymal transitions [6] are required for reprogramming. These respective microenvironmental and shape-changing behaviors suggest that biophysical cues that can alter cellular mechanics may also affect cellular reprogramming. Furthermore, reprogramming has been extended to the direct conversion of one somatic cell type into another. Of particular interest to this dissertation is the conversion of fibroblasts into neurons, which has tremendous clinical appeal given that it raises the prospect of creating patient-specific neuronal populations from a simple skin biopsy [7]. Here we describe several interesting observations of the biophysical regulation of cellular reprogramming into induced pluripotent stem cells as well as neurons.

Materials and Methods

Cell Culture

Thy1+ mouse fibroblasts were obtained by retinoic acid differentiation of mouse induced pluripotent stem cells carrying a doxycycline-ON cassette expressing c-Myc, Klf4, Oct4, and Sox2 reprogramming transcription factors [8]. Fibroblasts were maintained in DMEM media with 10% fetal bovine serum and 1 % penicillin/streptomycin. Reprogramming was initiated by addition of 2 ug/mL doxycycline in mouse embryonic fibroblast-conditioned DMEM media.

IMR-90 fetal lung fibroblasts were cultured as described previously [9]. Tet-O-FUW plasmids driving expression of Brn2, Ascl1, Myt1l, and NeuroD1 (BAMN) were kind gifts of Marius Wernig. Lentiviral particles were packaged as described in Chapter 2. pSLIK-Neo [10] was similarly packaged. Cells were infected with pSLIK-Neo at MOI 3, cultured for 2 days, the infected with either Ascl1 alone or BAMN at MOI 3. 2 ug/mL doxycycline was added a day later to initiate reprogramming.

Immunofluorescence Staining

Cells were stained as described in Chapters 2 and 4.

Microscopy

Cells were imaged as described in Chapter 2. Live-cell imaging movies were analyzed using the Particle Tracker plugin in ImageJ.

Polyacrylamide Substrate Preparation

Substrates were prepared as described in Chapter 4.

Quantitative Reverse Transcription Polymerase Chain Reaction

QRT-PCR was performed as described in Chapter 2. Primers and Taqman Probes for QPCR were: Human TUJ1 5' (GCATGGACGAGATGGAGTTCACC), Human TUJ1 3' (CGACTCCTCCTCGTCGTCTTCGTAC), Human TUJ1 probe (FAM490-TGAACGACCTGGTGTCCGAG-BHQ), Human Sox2 5' (CGAGTGGAAACTTTTGTCGGAGAC), Human Sox2 3' (CGGGCAGCGTGTACTTATCCTTCTT), Human Sox2 probe (FAM490-CGCTGCACATGAAGGAGCACC-BHQ)

Results

Substrate stiffness effect on pluripotency induction of mouse fibroblasts

The generation of induced pluripotent stem cells (iPSCs) from somatic cells was the first experimental achievement of cellular reprogramming [3]. Therefore, we first asked if varying substrate stiffness could affect the number of pluripotent colonies formed from mouse fibroblasts derived from a secondary induced pluripotent system [8]. Six days after initiation of reprogramming, we found the greatest number of Nanog+ and SSEA1+ colonies on 1500 Pa substrates (Figure 1).

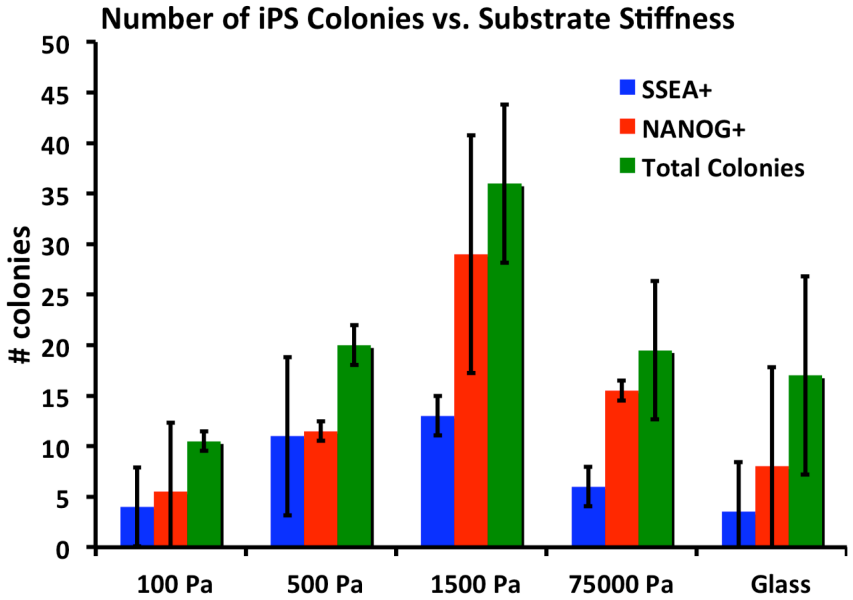

Figure 1. An intermediate stiffness of 1500 Pa increases the number of colonies and colonies expressing pluripotency markers SSEA1 and Nanog.

Softer substrates increase migration speed, decrease persistence, but do not affect cell clustering

Maximal colony formation at 1500 Pa may be due to increases in reprogramming efficiency on this stiffness, clustering of reprogrammed cells on softer and stiffer substrates thus reducing the number of colonies, or differences in total number of cells initially adhered. To

distinguish between these possibilities, we began by testing contributions of cell adhesion by counting the number of cells attached to the substrates 12 hours after cell seeding. No difference in cell adhesion was observed (Figure 2). Cell clustering could also explain colony number differences if cell migration rate or persistence was different on different stiffness substrates. Using live cell, time-lapse, phase microscopy cell speed, total path-length distance traveled, and persistence were measured between hour 12 and 24 post cell seeding. Cells migrated faster on soft substrates but migrated in a less persistent fashion than on stiff substrates, as evidenced by the finding that the linear distance between initial (12 hour) and end (24 hour) points of each cell were similar across matrix stiffnesses. The parameters of speed and persistence therefore may affect cell clustering in opposing ways. Therefore we measured the degree of cell clustering directly and found no difference across substrate stiffnesses.

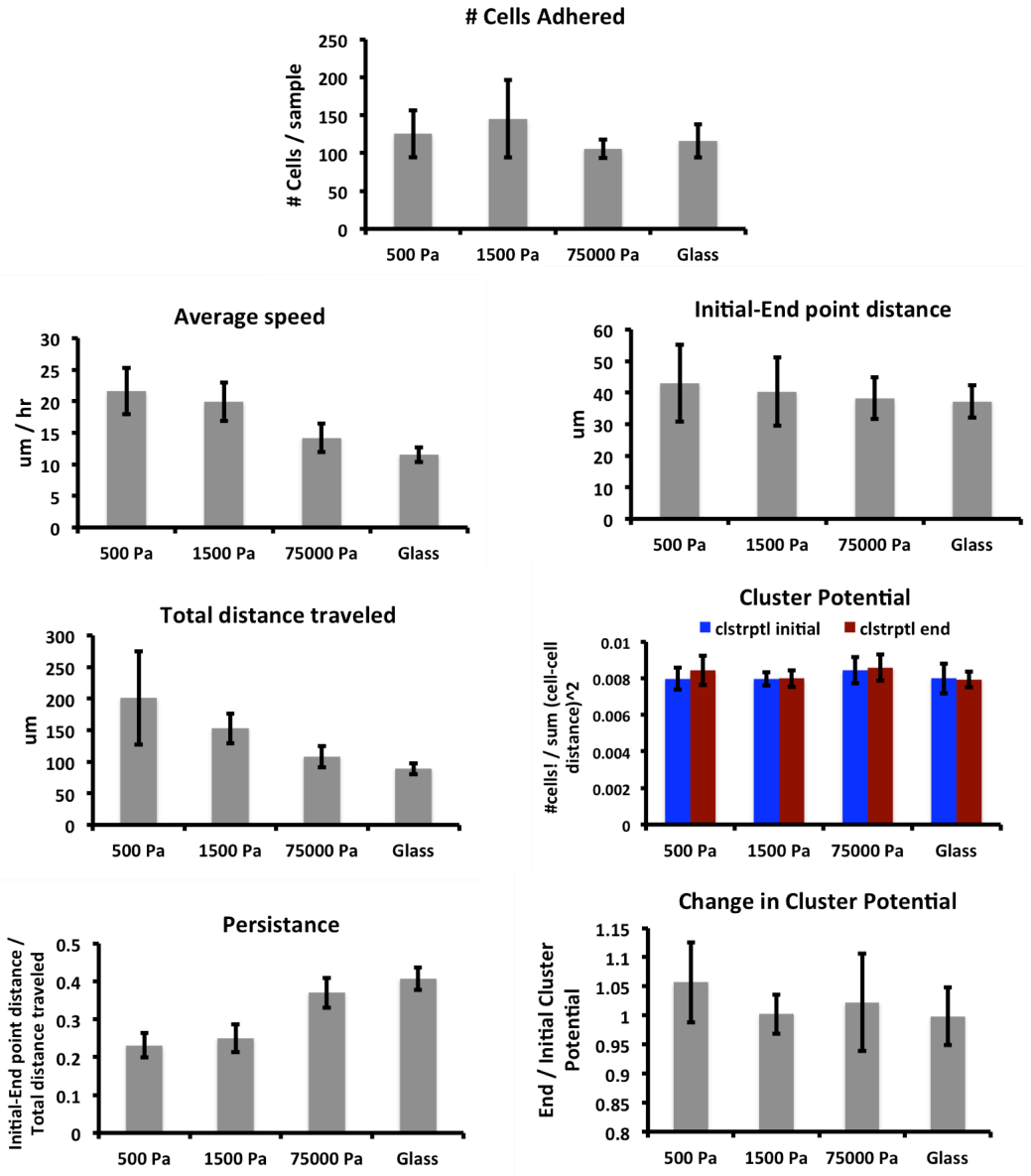

Figure 2. Cellular migration and coalescence of cells is unlikely to contribute significantly to miPS colony formation. Thy1+ cells differentiated from mouse induced pluripotent stem cells adhere to soft and stiff substrates similarly; however, they migrate faster on softer substrates while having higher persistence on stiffer substrates. The clustering potential of these cells does not vary with substrate stiffness.

Stiff substrates promote neuronal reprogramming

In addition to iPSC reprogramming as a strategy for generating patient-specific neurons from fibroblasts, it has also been shown that fibroblasts can be directly reprogrammed into therapeutically important cell types, such as neurons [7]. Following these protocols, we confirmed that reprogramming is effective in converting IMR90 fetal lung fibroblasts into TUJ1+ neurons either by expression of Ascl1 alone or by expression of Brn2, Ascl1, Myt1l, and NeuroD1 (BAMN) (Figure 3). Reprogramming with BAMN yielded morphologically more developed neurons than with Ascl1 alone, confirming a previous observation [7].

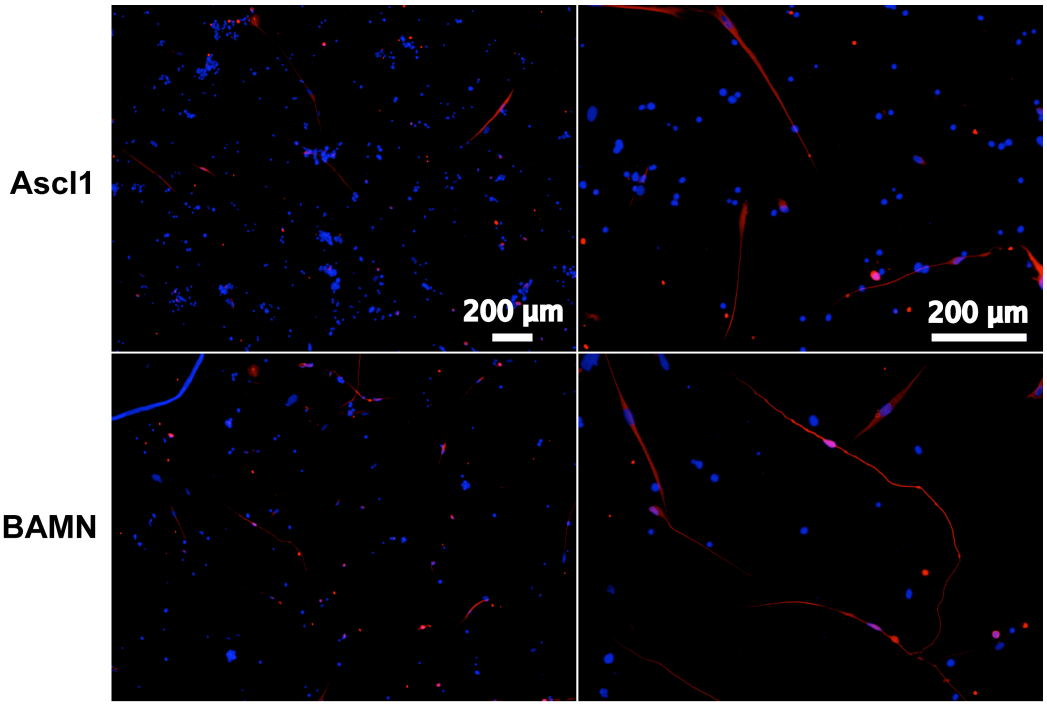

Figure 3. IMR-90 lung fibroblasts can be reprogrammed to TUJ1+ (red) neurons with transgenic expression of Ascl1 or Brn2, Ascl1, Myt1l, and NeuroD1 (BAMN), with BAMN expression leading to greater numbers of and morphologically more developed neurons.

We used this system to test if substrate stiffness affected neuronal reprogramming. Cells were infected with reprogramming factor constructs, and plated on Matrigel/poly-D-lysine-coated substrates of 100, 700, 75000 Pa and polystyrene. One day later doxycycline was added to induce expression. 6 days after induction, cells were stained for TUJ1. Overall reprogramming efficiency was low, resulting in ~0.8% TUJ1+ BAMN-infected cells and ~0.4% TUJ1+ Ascl1-infected cells. Softer substrates exhibited significantly fewer TUJ1+ cells than stiffer substrates.

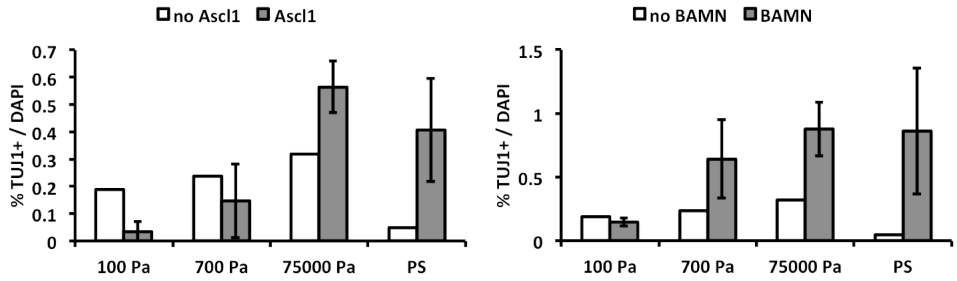

Figure 4. Stiffer substrates increase neuronal reprogramming from IMR-90 cells infected with Ascl1 (left) or BAMN (right).

Discussion

Both iPSC and neuronal reprogramming appear mechanosensitive. It will be important to first assess the exact cellular mechanisms (cell death, cell clustering, cell proliferation, reprogramming efficiency) leading to observed differences in either pluripotent colony formation or neuronal reprogramming. A direct method to study these potential mechanisms would be time-lapse microscopy with cells expressing fluorescent pluripotent or neuronal promoter-

reporters. Furthermore, isolating individual cells in micropatterned surfaces and hydrogels to restrict their migration will allow the longitudinal temporal tracking without requiring timelaspe imaging. This strategy will also prevent clustering and enable tracking of cell proliferation and correlation with reprogramming efficiency.

Once cellular mechanisms are understood, molecular mechanistic insights will be of considerable interest, especially if intersections between the responsible mechanosensitive pathways and known pathways important in reprogramming are found. These could include Rho GTPases, Rho-associated Kinase, myosin activity, E-cadherin, and Focal Adhesion Kinase. These mechanotransductive proteins have been implicated in stem cell differentiation (Chapter 2), the maintenance of human pluripotent stem cell, and cellular reprogramming [5, 11-17]. Furthermore, cellular forces could be correlated with reprogramming efficiency by using traction force microscopy [18] or internal tension sensors [19]. These studies will have implications for improving cell culture systems for generating cell populations for cell replacement therapies, as well as the development of materials to promote potential in vivo reprogramming efforts.

Acknowledgements

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Appendix A
Supplementary Information for Chapter 2

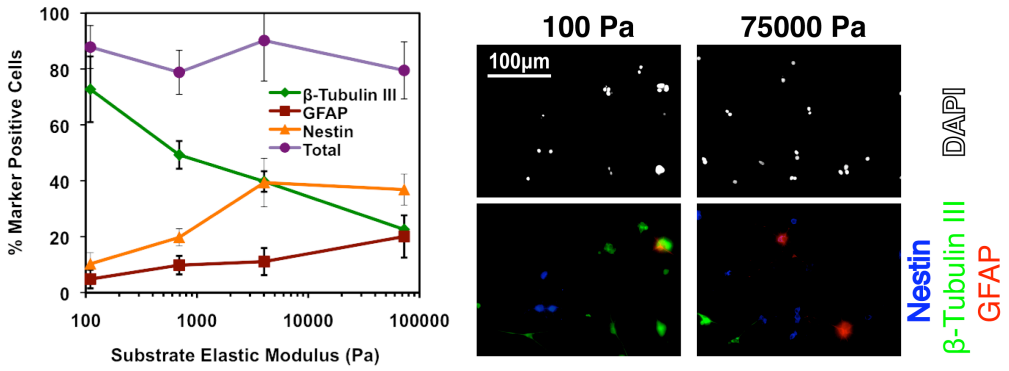

Figure 1. (Left) Lineage marker distributions as a function of ECM stiffness for NSCs differentiated in mixed media for 6 days shows that more undifferentiated, Nestin positive cells remain on stiff substrates, with an additional 10-20% marker negative cells on all stiffnesses. (Right) Immunofluorescence images of cells cultured on 100 Pa and 75000 Pa ECMs.

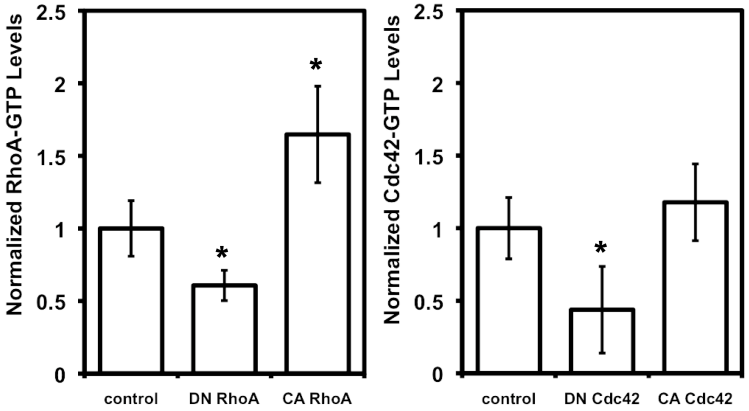

Figure 2. RhoA-GTP and Cdc42-GTP levels of NSCs expressing DN and CA RhoA and Cdc42 cultured in proliferating conditions on laminin-coated tissue-culture polystyrene. Levels are normalized to control, as determined by GLISA assays of samples loaded with equivalent total protein levels. Error bars are 95% confidence intervals. *$p < 0.05$ (Student's unpaired two-tailed t-test).

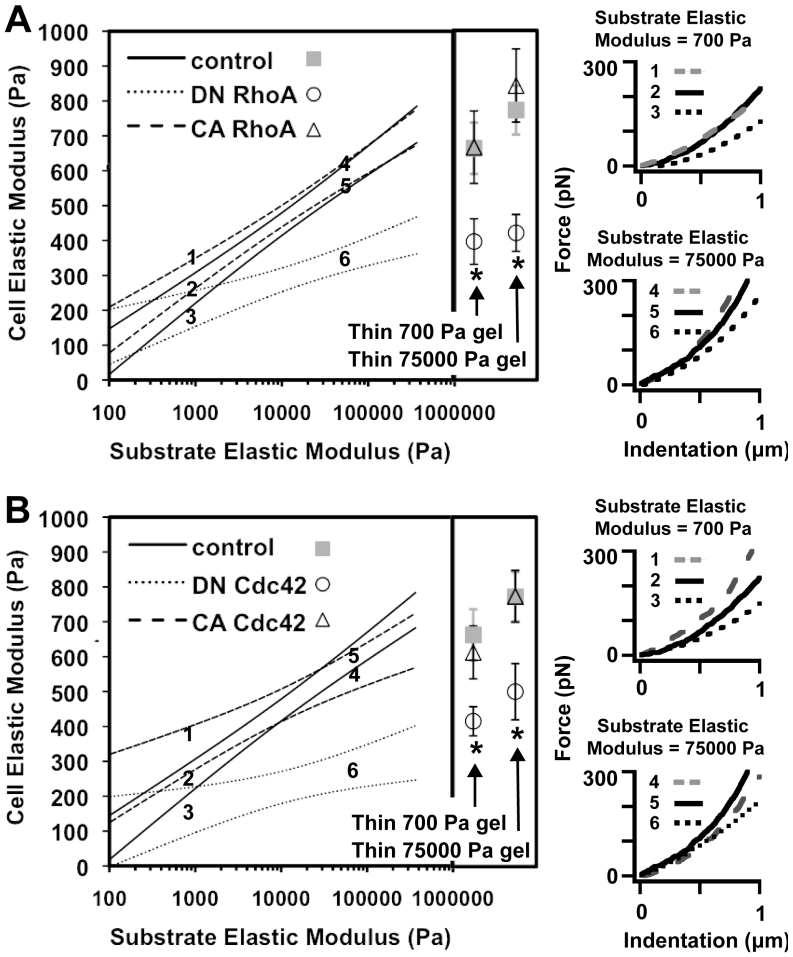

Figure 3. 95% confidence bounds, (A, B) solid lines – control, dotted lines – (A) DN RhoA and (B) DN Cdc42, dashed lines – (A) CA RhoA and (B) CA Cdc42, generated from one-way analysis of covariance on log-transformed substrate elastic modulus and cell elastic modulus data shown in Figure 3, reveal lower stiffnesses of NSCs expressing DN RhoA and DN Cdc42 compared to control on substrates above 1000 Pa. Error bars for NSCs on thin gels are 95% confidence intervals for n = 17-50 cells. *$p < 0.05$ (ANOVA-TK) for comparisons to control on each ECM elastic modulus (control data previously shown in Figure 2A).

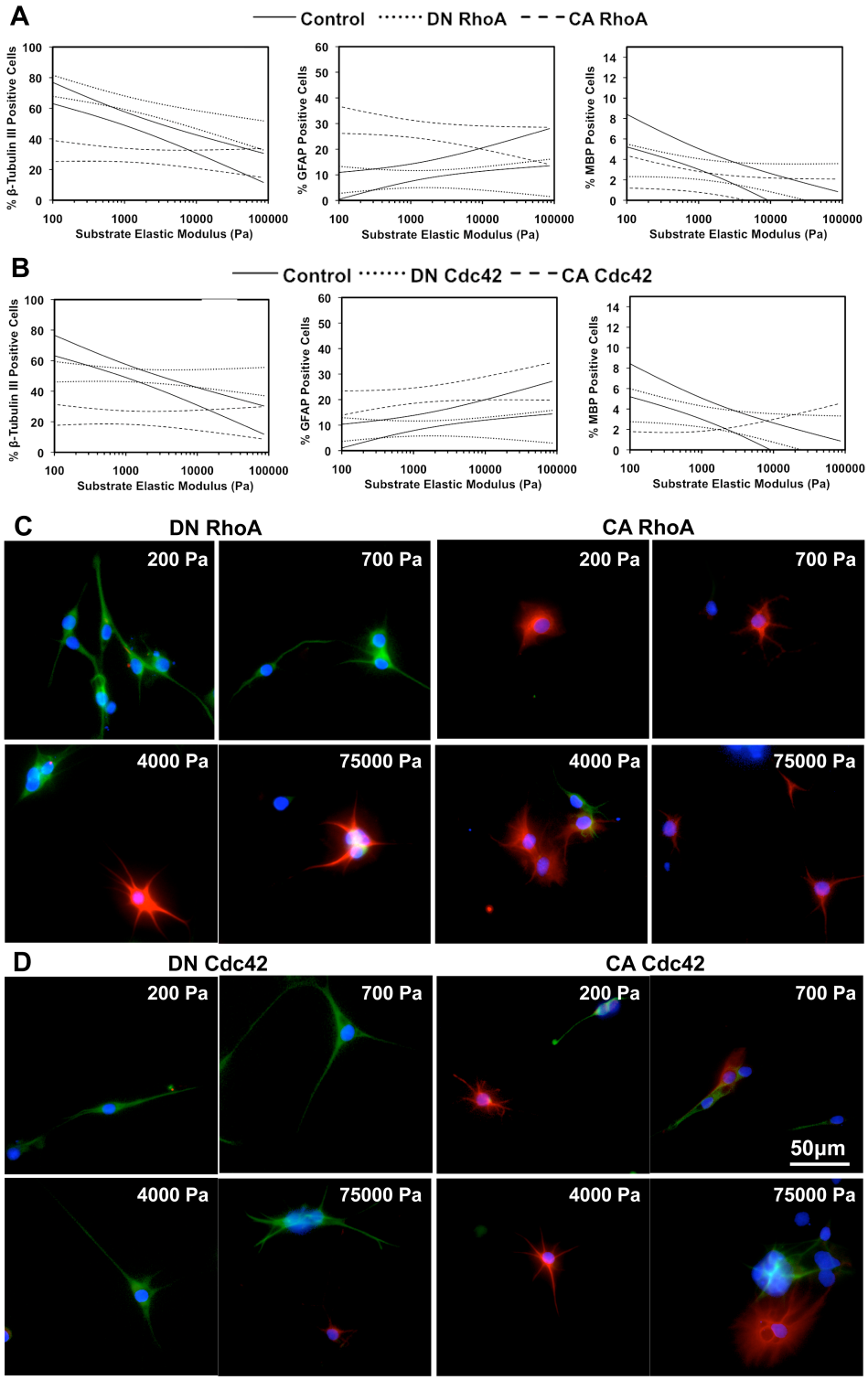

Figure 4. 95% confidence bounds, (A, B) solid lines – control, dotted lines – (A) DN RhoA and (B) DN Cdc42, dashed lines – (A) CA RhoA and (B) CA Cdc42, generated from one-way analysis of covariance on log-transformed substrate elastic modulus and cell immunostaining data shown in Figure 3, show that (A) DN RhoA and (B) DN Cdc42 increase the proportion of neurons and decrease the proportion of astrocytes on stiffer ECMs compared to control after 6 days. In contrast, (A) CA RhoA and (B) CA Cdc42 decrease the proportion of neurons and increase the proportion of astrocytes on softer ECMs compared to control. (C) Higher power images of insets from Figure 3C-D.

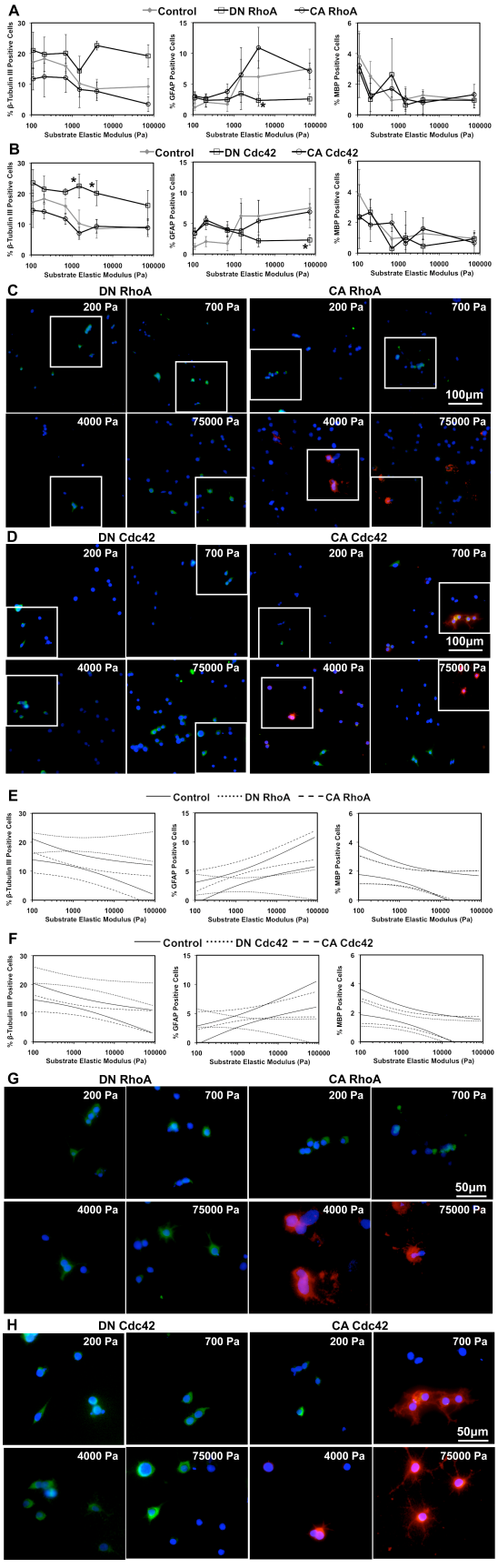
A
Control
DN RhoA
CA RhoA
% β-Tubulin III Positive Cells
% GFAP Positive Cells
% MBP Positive Cells
Substrate Elastic Modulus (Pa)
B
Control
DN Cdc42
CA Cdc42
C
DN RhoA
CA RhoA
200 Pa
700 Pa
4000 Pa
75000 Pa
100μm
D
DN Cdc42
CA Cdc42
E
F
G
50μm
H

Figure 5. Rho GTPases modulate the effect of ECM elastic modulus on the proportions of neurons and astrocytes in survival conditions. Error bars are 95% confidence intervals, n = 5-6. *p < 0.05 for comparisons to control for each substrate elastic modulus (control data previously shown in Figure 1B) (ANOVA-TK). β-tubulin III *(green)*, GFAP *(red)*, DAPI *(blue)*, MBP *(white)*. 95% confidence bounds, (E, F) solid lines – control, dotted lines – (E) DN RhoA and (F) DN Cdc42, dashed lines – (E) CA RhoA and (F) CA Cdc42, generated from one-way analysis of covariance on log-transformed substrate elastic modulus and cell immunostaining data shown in (A) and (B). (G, H) Higher power images of insets from (A, B).

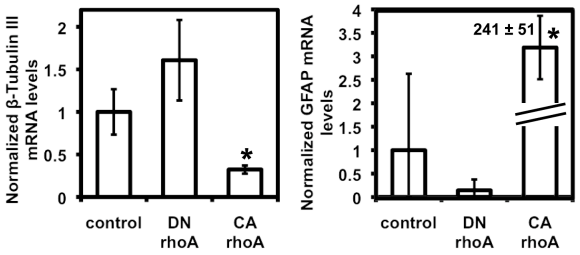

Figure 6. Quantitative RT-PCR measurements of the increases in mRNA levels for neuronal and astrocytic markers in NSCs expressing CA or DN RhoA cultured on laminin-coated tissue culture polystyrene for 6 days in mixed conditions over the same NSCs cultured in self-renewal conditions (20 ng/ml FGF-2). Results show similar trends to immunostaining (Figure 4), with CA RhoA exhibiting a smaller increase in neuronal mRNA levels and a greater increase in astrocytic mRNA levels and DN RhoA exhibiting greater neuronal and smaller astrocytic increases in mRNA levels compared to control. Error bars are 95% confidence intervals, n = 3. *p < 0.05 for comparisons to control NSCs (ANOVA-TK).

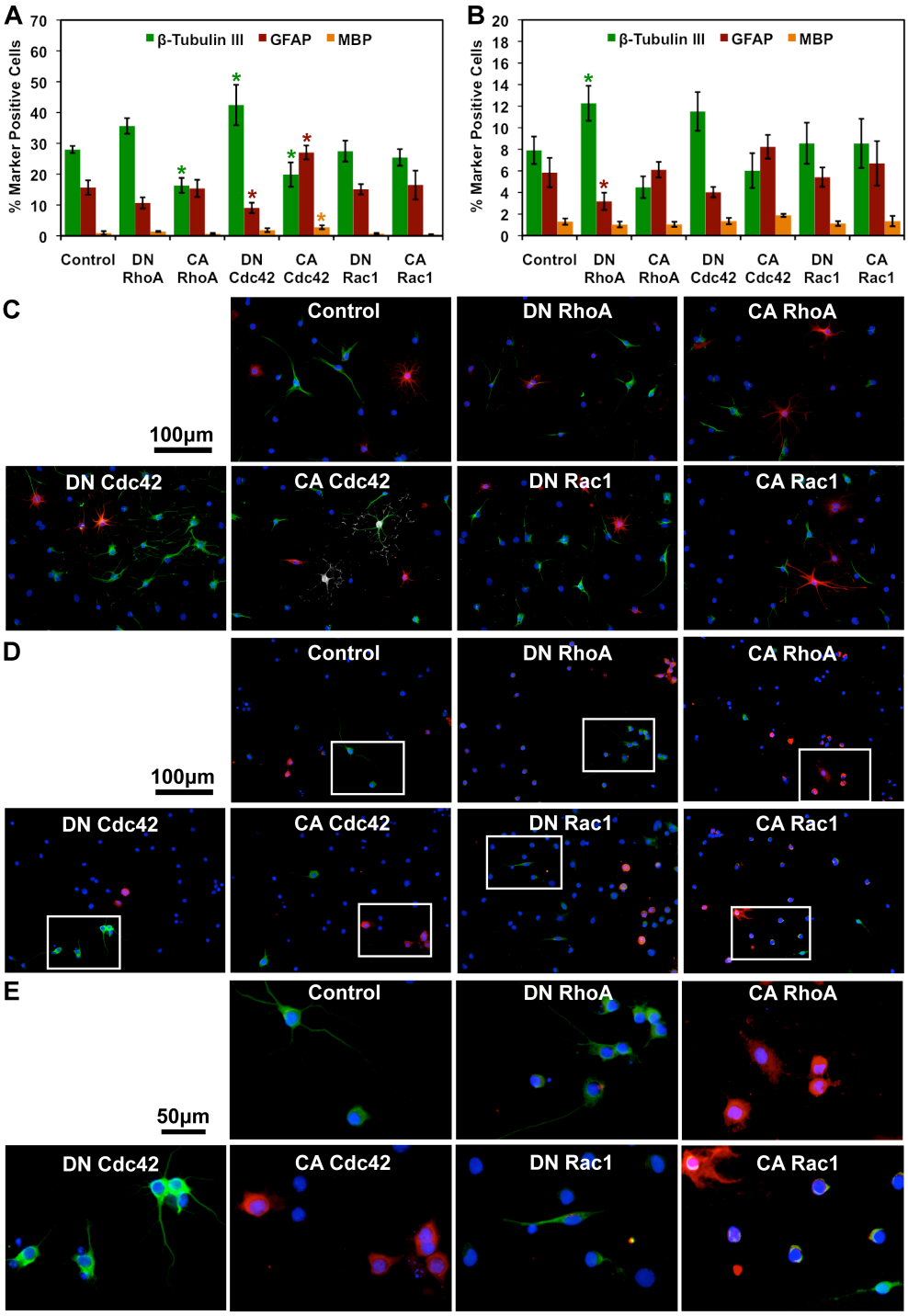

Figure 7. Rho GTPases modulate NSC lineage compositions on laminin-coated glass. Immunostaining against β-tubulin III (*green bars*), GFAP (*red bars*), and MBP (*orange bars*) of cells cultured on glass show that DN RhoA and Cdc42 increase the percentage of neurons and decrease the percentage of astrocytes while CA Cdc42 slightly increases the percentage of oligodendrocytes over control cells after 6 days of differentiation in (A) mixed conditions and (B) survival conditions. DN and CA Rac1 do not affect NSC lineage compositions in either media condition. Error bars are 95% confidence intervals, n = 5-6. *p < 0.05 for comparisons to control (ANOVA-TK). Representative immunofluorescence images of NSC lines after 6 days of differentiation in (C) mixed conditions and (D) survival conditions. β-tubulin III *(green)*, GFAP *(red)*, DAPI *(blue)*, MBP *(white)*. (E) Higher power images of insets from (D).

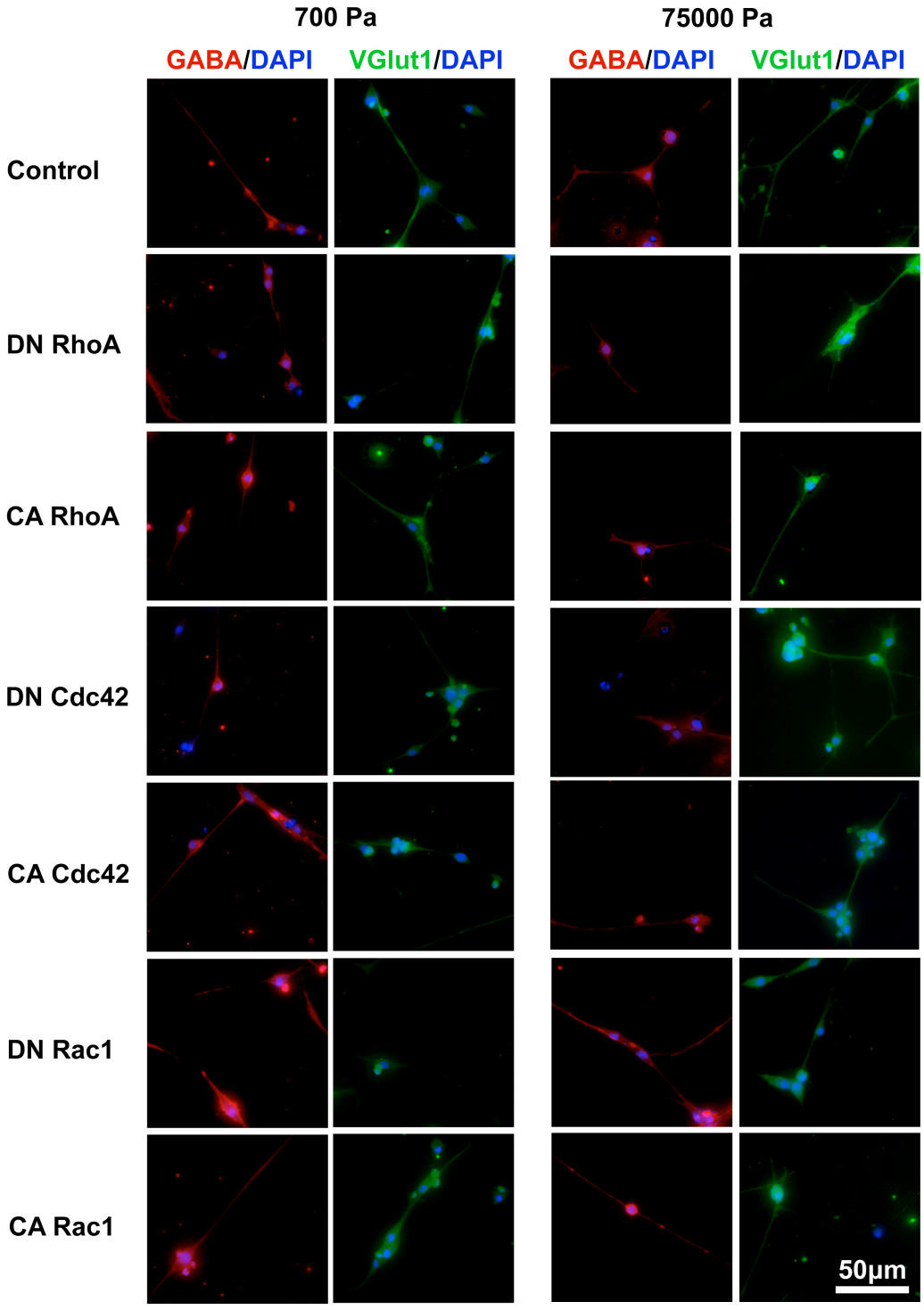

Figure 8. Neither expression of DN and CA Rho GTPases nor variation of ECM stiffness compromises later stages of neuronal maturation and subtype marker expression, with GABAergic (GABA, *red*) and glutamatergic (VGlut1, *green*) neurons detectable on substrates of 700 and 75,000 Pa and all RhoA/Cdc42 genotypes (DAPI, *blue*). Cells were cultured in 1 μM forskolin and 5 μM all-trans retinoic acid for 6 days then switched to 20 ng/ml brain-derived neurotrophic factor with 1 μM forskolin for another 6 days.

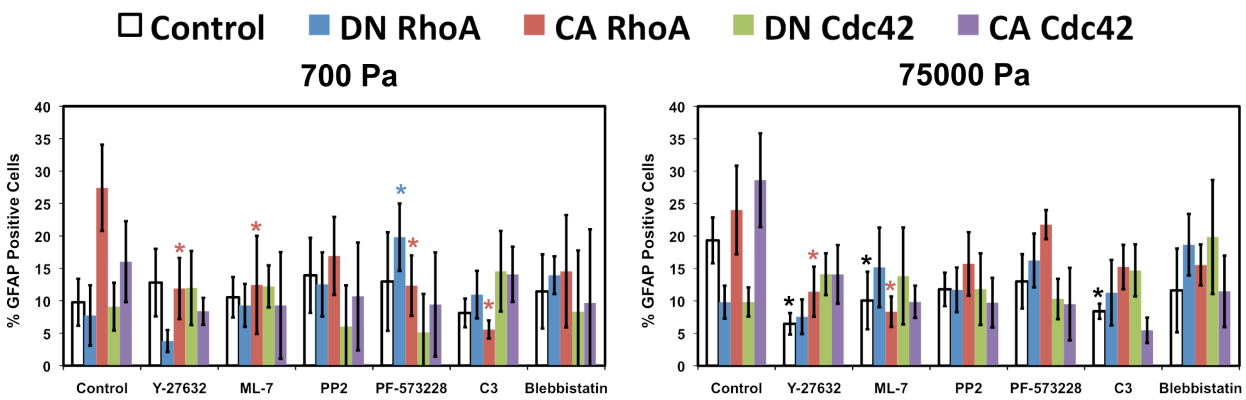

Figure 9. Inhibition of proteins that regulate cellular contractility reduces astrocytic differentiation in mixed conditions on soft and stiff ECMs. Error bars are 95% confidence intervals, n = 5-6. *p < 0.05 for comparisons to the same NSC population in control media conditions (control data previously shown in Figure 1A) (ANOVA-TK).

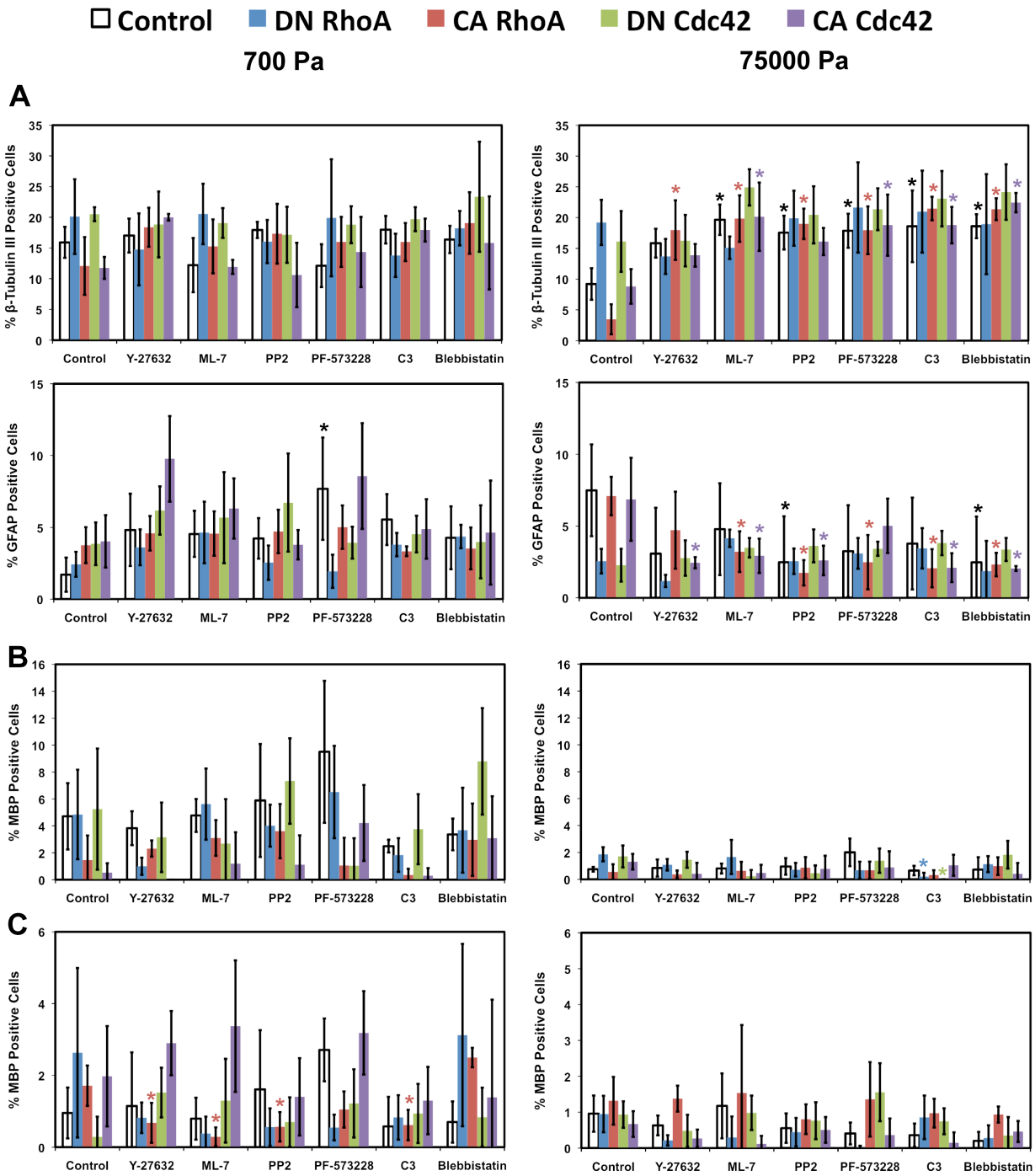

Figure 10. (A) Inhibition of proteins that regulate cellular contractility rescues neuronal differentiation in survival conditions on soft and stiff ECMs. Error bars are 95% confidence intervals, n = 6. *p < 0.05 for comparisons to the same NSC population in control media conditions (ANOVA-TK). (B and C) Inhibition of proteins that regulate cellular contractility and adhesion do not appear to modulate the proportion of oligodendrocytes generated from NSCs. (B) mixed and (C) survival conditions. Error bars are 95% confidence intervals, n = 6. *p < 0.05 for comparisons to the same NSC population in control media conditions (ANOVA-TK).

Supplemental Experimental Procedures

Neural Stem Cell Culture

Standard cultures were grown on tissue culture polystyrene coated with 10 μg/ml poly-ornithine (Sigma-Aldrich, St. Louis, MO) and 5 μg/ml mouse laminin (Invitrogen, Carlsbad, CA), in Dulbecco's modified Eagle medium (DMEM)/F-12 (1:1, Invitrogen) supplemented with N2 supplement (Invitrogen) and 20 ng/ml recombinant human basic fibroblast growth factor (Peprotech, Rocky Hill, NJ). Glass substrates were coated with 20 μg/ml poly-ornithine and 10 μg/ml mouse laminin.

Viral Production, In Vitro Transduction, and In Vivo Delivery

cDNAs were subcloned into the murine retroviral vector plasmid CLGPIT (Peltier et al., 2007) for in vitro transduction and into pCAG-IRES-GFP (modified from Addgene Plasmid 16664 (Zhao et al., 2006)) for in vivo delivery. The resulting vectors were packaged, concentrated, and purified as described (Peltier et al., 2007). In vitro, NSCs were infected at a multiplicity of infection of 1 IU/cell and were selected with 0.6 μg/ml puromycin (Sigma) for 4 days. For in vivo studies, eight-week-old adult female Fisher 344 rats were anesthetized prior to 3 μL bilateral intrahippocampal stereotaxic injections of retrovirus. Injection coordinates were -3.5 mm anteriorposterior and ± 1.8 mm mediolateral relative to bregma and -3.3 mm dorsoventral relative to dura. BrdU (Sigma) was administered intraperitoneally at 50 mg/kg dissolved in saline. All animal protocols were approved by the Animal Care and Use Committee of the University of California Berkeley.

Immunofluorescence and Immunohistochemical Staining

Cells and tissue sections were fixed with 4% paraformaldehyde. BrdU-treated samples were incubated in 2 N HCl, neutralized with 0.1 M borate buffer prior to blocking and permeabilizing in 2% (5% for sections) goat serum (Sigma) and 0.3% Triton X-100 (Calbiochem, San Diego, CA) in pH 7.4 phosphate buffered solution at room temperature. Samples were incubated for 36 hours at 4°C with the following primary antibodies: rabbit anti-β-tubulin III (1:1000 dilution; Covance, Emeryville, CA), mouse anti-glial fibrillary acidic protein (GFAP, 1:1000 dilution; Advanced ImmunoChemical Inc., Long Beach, California), rat anti-myelin basic protein (MBP, 1:100 dilution; Abcam Inc., Cambridge, MA), rabbit anti-cleaved caspase 3 (1:400 dilution; Cell Signaling, Danvers, MA), rat anti-5-Bromo-2'-deoxyuridine (BrdU, 1:250 dilution; Abcam), guinea pig anti-γ-aminobutyric acid (GABA, 1:1000 dilution; Abcam), rabbit anti-vesicular glutamate transporter 1 (VGlut1, 1:2500 dilution; Synaptic Systems, Germany), rabbit anti-green fluorescent protein (GFP, 1:2000, Invitrogen, A11122), mouse anti-neuronal nuclei (NeuN, 1:100, Millipore, Billerica, MA), and guinea pig anti-doublecortin (DCX, 1:1000, Millipore). The primary antibody solution was removed, and cells were rinsed and incubated for 2 hours with the secondary antibodies FITC-conjugated goat anti-rabbit IgG, Cy3-conjugated goat anti-rat IgG or Cy3-conjugated goat anti-guinea pig, and Cy5-conjugated goat anti-mouse IgG at a dilution of 1:250 (all from Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Nuclei were stained with DAPI (Invitrogen) at 10 μg/ml. Cells were manually scored as positive or negative for lineage markers in regularly spaced and rastered fields of view. Images were collected at 20x magnification on a Nikon Eclipse TE2000-E microscope with a Photometrics Coolsnap HQ2 camera, and exposure settings were chosen to minimize background fluorescence as determined using control samples without primary antibodies. 300-1500 cells were counted per culture until at least 300 cells were obtained. 3-6

experiments were performed in parallel cultures for each study. 40 μm hippocampal sections were stained, and 15-20 confocal images obtained on a LSM710 (Carl Zeiss Inc, Oberkochen, Germany) were z-stacked and flattened in ImageJ. 200-600 GFP+ cells per rat were counted, which corresponded to 16 total hippocampii sections per animal. 4 rats were sacrificed for each condition.

Quantitative real time Polymerase Chain Reaction

Quantitative real time PCR (QRT-PCR) was used as a complementary technique to immunofluorescence staining to accurately quantify specific mRNA concentrations in cells. Cells were lysed and frozen in TRIZOL (Invitrogen), and mRNA was extracted and reverse transcribed to cDNA using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis (Invitrogen). Equivalent amounts of total RNA were transcribed into cDNA, which was subsequently used as template for each QRT-PCR reaction. The QRT-PCR assay used GFAP as a marker for astrocytics and β-tubulin III as a marker for neurons (utilizing a Bio-Rad Laboratories iCycler 5, Hercules, CA). To normalize any remaining variations in starting cDNA amounts, each reaction was carried out in duplex format with ribosomal 18S detected using Cal-dye TaqMan probes and the lineage marker was detected using FAM-dye TaqMan probes (Biosearch Technologies, Novato, CA). QRT-PCR reactions were run for each biological sample with n=5-6 for each condition.

The primers and TaqMan probes used are listed as follows: (GFAP, 5'-GACCTGCGACCTTGAGTCCT-3', 5'-TCTCCTCCTTGAGGCTTTGG-3', 5'-FAM490-TCCTTGGAGAGGCAAATGCGC-BHQ-3'), (β-tubulin III, 5'-GCATGGATGAGATGGAGTTCACC-3', 5'-CGACTCCTCGTCGTCATCTTCATAC-3', 5'-FAM490-TGAACGACCTGGTGTCTGAG-BHQ-3'), and (18S, 5'-GTAACCCGTTGAACCCCATTC-3', 5'-CCATCCAATCGGTAGTAGCGA-3', 5'-CAL610-AAGTGCGGGTCATAAGCTTGCG-BHQ-3'). Standards for performing QRT-PCR were pPCR4-TOPO plasmids (Invitrogen) containing the amplicon of interest as an insert. The plasmids were linearized by restriction digestion and quantified by absorbance, and tenfold serial dilutions from 1 ng/mL to 10^{-9} ng/mL were prepared to generate a standard curve.