INVITED ARTICLE

TECHNOLOGY

Signal transduction of the physical environment in the neural differentiation of stem cells

Ryan Thompson[1] & Christina Chan[1,2,3]

Neural differentiation is largely dependent on extracellular signals within the cell microenvironment. These extracellular signals are mainly in the form of soluble factors that activate intracellular signaling cascades that drive changes in the cell nucleus. However, it is becoming increasingly apparent that the physical microenvironment provides signals that can also influence lineage commitment and very low modulus surfaces has been repeatedly demonstrated to promote neurogenesis. The molecular mechanisms governing mechano-induced neural differentiation are still largely uncharacterized; however, a growing body of evidence indicates that physical stimuli can regulate known signaling cascades and transcription factors involved in neural differentiation. Understanding how the physical environment affects neural differentiation at the molecular level will enable research and design of materials that will eventually enhance neural stem cell (NSC) differentiation, homogeneity and specificity.

***Keywords*: NRSF/REST; SMAD; Mechanotransduction; Neural; YAP/TAZ; Rho/ROCK; Modulus.**

INTRODUCTION

Stem cells hold much promise in regenerative medicine, in particular, for cell replacement therapy in the treatment of degenerative diseases including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis (ALS), type I diabetes and macular degeneration. Stem cell differentiation is a multistep, complex process involving the execution of numerous gene expression programs consisting of hundreds to thousands of genes specific to a particular cell type. Stimuli from morphogens, growth factors and hormones in the cell microenvironment dictate cell lineage determination. A number of studies have delineated the major signaling cascades that these extracellular factors induce to drive stem cell differentiation. Central to specifying cell fate is the composition of signaling molecules and transcription factors expressed and activated in the cell by these factors. For instance, exogenous expression of transcription factors has been shown to transdifferentiate mature cells into other cell types including embryonic stem cells (ESCs)[1–5].

Advances in biomaterial research have made great strides in the development of substrates and 3D scaffolds that mimic the physiological microenvironment to encourage stem cell differentiation. Indeed, incorporating growth and differentiation factors into scaffolds promoted the growth of osteocytes into bone-lesion models while 3D electrospun fibers provided maturing neural stem cells (NSCs) with tracks along which they can extend neurites[6–10]. Scaffolds can help control the availability of nutrients and oxygen while providing a physical matrix to assist in the development of cell morphology. Several physical factors in a cell's environment, such as the surface modulus, topography, porosity and anisotropy, have been shown to influence its behavior, e.g., growth rate, migration, and morphology[11–19]. It is increasingly evident that the physical environment contributes to cell lineage determination[20–22], as demonstrated by the neural markers expressed in stem cells cultured on soft (low-modulus) surfaces. Therefore, understanding the signaling mechanisms that contribute to these changes could benefit stem cell-based therapies. How the cell senses and responds to the physical environment to induce or alter stem cell differentiation has remained an enigma until recently. In particular, several areas of research have shown the physical environment affects the function of well-known signaling pathways important in differentiation. Instrumental to the mechano-transduction appears to be the cytoskeleton and the Rho GTPases[23–25]. Additionally, differential expression of well-known cell surface receptors, such as the transforming growth factor (TGF) family members[26,27], provide possible explanations for how the physical environment affects canonical cell signaling pathways in development. The effect of surface properties on the receptors of the TGF superfamily and their signaling components, in particular, the SMAD family have been demonstrated to play key roles in normal development and differentiation of cells and tissues[28].

TGFs are composed of a large family of proteins including TGF-βs, bone morphogenetic proteins (BMPs), activin, nodal and growth and differentiation factors (GDFs)[29,30]. These signal by translocating cytosolic SMAD proteins to the nucleus and activate genes important in the growth and development of tissues. Concomitantly, studies have demonstrated that physical properties also can elicit changes in SMAD signaling to drive differentiation of stem cells. This review highlights findings on cell signaling events induced by the physical environment (e.g., surface modulus) that influence stem cell differentiation to a neuronal lineage.

Ultimately, differentiation of stem cells into a mature phenotype relies on changes in the composition of nuclear transcription factors

[1]Cell and Molecular Biology Program, [2]Department of Chemical Engineering and Materials Science, [3]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824, USA. Correspondence should be addressed to: C.C. (krischan@egr.msu.edu)

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that control gene expression programs responsible for the final fate of the cell. Several transcription factors have been shown to be important for neural development and control the expression of neural-specific genes including, *Sox1*, *Pax6* and *NeuroD*[31–33]. Additionally, the neural phenotype is strongly repressed by the master transcriptional repressor, neuron restrictive silencer factor (NRSF). Downregulation of NRSF is a critical event during the maturation of neurons and activation of its target genes is enough to induce neuronal differentiation[34,35].

Understanding the signaling mechanisms that contribute to these changes would benefit applications that rely on stem cell differentiation. This review addresses how physical stimuli signal through Rho GTPases and the BMP–SMAD signaling pathway, pathways well known to function in neural development and recently shown to respond to physical stimuli through changes in the cytoskeleton and surface receptors, as well as highlights the NRSF transcription factor, given its function as a master regulator of the neural phenotype, and how surfaces might transduce mechanical stimuli to regulate its expression.

Biomaterials for stem cell differentiation

Tissue engineering and differentiation

Tissue engineering, the integration of cells and synthetic or natural materials to support the growth and replacement of damaged tissues in pathological conditions, frequently involves precise and controlled delivery of biochemical and physicochemical factors to encourage regeneration of damaged tissue. Scaffolds can be loaded with growth factors and extracellular matrix (ECM) components to mimic the physiological niche of the tissue to be repaired; for example, in bone repair the scaffolds are pre-loaded with BMPs to promote the growth and differentiation of osteocytes into the lesioned region[6–8]. Scaffolds have also been used to promote central nervous system (CNS) repair by inducing regeneration of severed axons[15]. Stem cells are routinely manipulated *in vitro*; however, control of their fate *in vivo* suffers from a lack of efficiency. To this end, controlling the stem cell environment by tuning its mechanical properties has been successful in enhancing stem cell differentiation and growth in injury models. Electrospun fibers have been used to promote the differentiation of myoblasts into muscle tissue as well as guiding induced pluripotent stem cells (iPSCs) to neural differentiation[36,37]. Compressive forces have been shown to enhance chondrogenesis from MSCs[38]. Additionally, cyclic strain on MSCs as well as pre-stretched surfaces can induce mesenchymal stem cell (MSC) differentiation into smooth muscle cells (SMCs) without the addition of growth factors[17,18]. Stem cell differentiation, normally controlled by extracellular factors such as growth factors, hormones and cytokines, appears to be directed or enhanced by the physical properties of its environment[20]. Seminal work by Engler *et al.*[20] showed that the substrate modulus could directly influence MSC differentiation along neural, myogenic or osteo-lineages depending on the stiffness of the substrate, with very low surface modulus promoting neural and higher surface modulus promoting osteogenic differentiation. The modulus of the surface has also been shown to impact neuronal growth, enhance neural function such as cell–cell communication via synaptic currents and promote neurite extension[39,40]. Despite these advances, continued development in this field is needed to further improve the robustness and specificity of stem cell differentiation.

Neural differentiation induced by soft surfaces

Discher *et al.* reported that culturing MSCs on very soft surfaces promoted neural gene expression as well as neural morphology change[20]. Specifically, MSCs grown on polyacrylamide gels with a surface modulus of <1 kPa, reflecting that of brain tissue, increased expression of neural markers including microtubule-associated protein 2 and neural structural proteins such as β-III tubulin (Tuj1) and neurofilament. Since then, others have shown that soft surfaces have a neural-inducing effect on several types of stem cells including ESCs, iPSCs, and adult NSCs[37,41–43].

More importantly, soft surfaces have been shown to increase neuronal function. Keung *et al.* reported that soft ECM promotes dopaminergic differentiation of human PSCs (hPSCs) as assayed by the expression of the dopaminergic marker and dopamine biosynthetic enzyme, tyrosine hydroxylase[44]. Application of this finding could specifically lead to use of soft surfaces to enhance dopaminergic neuron studies. Similarly, soft surfaces were recently used to promote the generation of motor neurons from PSCs improving overall yield and specificity, and shortening the generation time[43]. Importantly, soft surfaces assisted in the development of neuronal morphology and improved the cell's ability to generate action potentials. Given that generation of subtypes of neurons is a tedious and inefficient process, soft surfaces could be applied to promote *in vitro* culture of all neural cells.

Interestingly, very soft surfaces specifically favor neuronal differentiation over glial differentiation[42,45]. Leipzig *et al.*[46] corroborated Saha's[42] results and demonstrated that NSCs could be specifically differentiated into oligodendrocytes (~7 kPa), astrocytes (1–3 kPa) or neurons (<1 kPa) with varying degrees of Young's modulus on photopolymerizable methacrylamide chitosan (MAC) surfaces[46]. To date, surface modulus has been shown to affect a variety of cell lineages and these are summarized in **Table 1**. These findings suggest that soft surfaces, i.e., with surface modulus of <7 kPa, could also be used to improve the specificity and homogeneity of differentiated neural cell cultures.

Very soft surfaces (<1 kPa) appear to have a neural-inducing effect on stem cells, but to date, it remains unclear the extent to which the cells mature. Current work shows that hESCs and hPSCs respond to soft surfaces by differentiating into functional neurons[43,47]; however, while very soft surfaces have a neural-inducing effect on NSCs and MSCs, it is unclear if soft surfaces alone can drive neural differentiation in these cell types to fully functional neurons. Indeed, Keung *et al.* found that very soft surfaces promoted expression of pan-neuronal markers including Tuj1, but did not promote differentiation of neuronal subtypes[48] suggesting that only early neuronal induction was occurring. A hallmark of differentiated cells is their inability to revert back to a more undifferentiated state with the exception of during disease states like cancer. Lee *et al.* demonstrated that neural differentiation of MSCs promoted by soft surfaces could be reversed simply by transferring the cells from a soft to stiff surface[49]. Additionally, MSCs grown on soft surfaces do not appear to alter their global DNA methylation patterns suggesting that the changes in gene expression while on soft surfaces is a transient effect[50]. Thus, not surprisingly, the cell background is largely important in how the cell responds to very soft surfaces. These effects are summarized in **Table 2**. These results suggest that while soft surfaces can promote neural differentiation, mechanical or physical cues impart function in combination with the rest of the cellular niche to form differentiated mature neurons. Indeed, research shows that soluble differentiation factors synergize with physical characteristics of the microenvironment to enhance stem cell differentiation[43,44]. To take full advantage of this synergy, future work is needed to better understand the molecular mechanisms by which physical and soluble cues interact to transduce signals that induce stem cell differentiation.

Table 1 Surface modulus of the substrates that stem cells are cultured on can direct them to specific lineages. Very soft surfaces (<1 kPa) favor neural differentiation in a wide range of stem cells including embryonic, pluripotent, adult neural and mesenchymal.

Stiffness	Modulus (kPa)	Lineage	Reference
Very Soft	<1	Neural	20,44,46
Soft	1–7	Glial	42,46
Intermediate	8–17	Myogenic	20
Stiff	25–40	Osteogenic	20

Table 2 Neural-inducing effects on hESCs, hiPSCs, NSCs and MSCs. Phenotypic effects range from increased neural gene expression, morphology changes and electrophysiological function.

Cell type	Effect	Stiffness	Material	Reference
MSC	Neural gene expression	0.1–1 kPa	Polyacrylamide	20
MSC	Neural gene expression	0.5 kPa	Polyacrylamide	49
NSC	Increased neurite length	<1 kPa	PDMS	41
NSC	Neuronal > Glial differentiation	<1 kPa	Polyacrylamide	42
NSC	Neuronal > Glial differentiation	<1 kPa	MAC	46
hESC	Enhanced neural gene expression; Increased electrophysiologic function	<5 kPa	PDMS	43
hESC, hIPSC	Increased neural differentiation; expression of TH	0.1 kPa–0.7 kPa	Polyacrylamide	44
hESC	Enhanced neural gene expression; Increased electrophysiologic function	0.7 kPa	Polyacrylamide	47

Molecular signaling mechanisms

For the physical environment to have an effect on a cell, the cell must transduce the physical cue into a molecular signal. It is not obvious how a cell performs this as the known molecular mechanisms involved in stem cell differentiation are predominantly derived from studies involving soluble factors. An important question is, does the physical environment influence known signaling pathways involved in stem cell differentiation? Recent research suggests that the physical environment affects well characterized signaling pathways relevant for stem cell differentiation, including the BMP–SMAD signaling as well as the cytoskeleton regulators YAP/TAZ. This section reviews studies that focus on how cytoskeleton regulators such as the Rho GTPases in combination with BMP–SMAD signaling and the YAP/TAZ regulators contribute to neural differentiation.

Basic BMP-SMAD signaling

BMP–SMAD is a critical signaling pathway involved in the development and differentiation of numerous tissues. Mutations that affect this signaling pathway are known to cause a variety of neurological defects including Alzheimer's, Parkinson's and motor neuron diseases[51]. BMPs belong to the TGF superfamily of proteins that also include TGFs, GDFs), activin and nodal. BMP–SMAD signaling has been comprehensively reviewed and readers are referred to Massague[28] for details. In brief, BMPs initiate their signaling cascade upon binding to and dimerizing a type I and type II TGF receptor. Dimerization of the receptors leads to activation of the type I receptor through autophosphorylation which enables it to phosphorylate SMAD proteins to then transmit their signal to the nucleus. Regulatory SMADs (R-SMADs) SMADs 1, 2, 3, 5 and 8 are the primary substrates of TGF receptors, with SMADs 1, 5 and 8 activated by BMP signaling and SMADs 2 and 3 activated by TGF. Phosphorylation of the R-SMADs activates them to become substrates for the common SMAD (co-SMAD), SMAD4. Binding of R-SMAD to SMAD4 forms a heterodimer that is then translocated into the nucleus where the SMADs exert their function as transcription factors. SMADs 6 and 7 are inhibitory SMADs and act to downregulate the activity of R-SMADs. SMAD signaling is regulated at multiple steps and can also be inhibited by phosphorylation at alternative sites or degraded by the ubiquitin-proteasome system[52]. BMP ligand activity can also be regulated through gene expression changes and sequestration by inhibitory proteins such as chordin, noggin and follistatin. SMAD signaling can also be regulated at the cell surface level through internalization of the TGF receptors by endocytosis[53,54].

SMAD signaling plays a central role in the induction of neural differentiation

SMAD signaling has been recognized to be an important component to neural development for decades. In the "default model" of neural differentiation in the developing embryo, removal of TGFs and BMPs is sufficient to initiate neuralization[55]. Indeed, suppression of SMAD1 and SMAD2 is a key step during neural induction[56]. Studies of SMADs in embryonic development have transferred to cell culture models and SMADs are now known to contribute to neural differentiation of ESCs and iPSCs[56] and are generally pro-growth transcription factors that help maintain stem cells in the undifferentiated state. Inhibition of the SMAD signaling pathway initiates neural development of stem cells in early embryonic development. While it is not entirely clear how every *SMAD* gene contributes to neural differentiation, it is evident that BMP signaling through SMAD1/5/8 play a more important role in the development of the nervous system as compared to SMAD2/3. Soluble neural inducers generally function by downregulating SMAD activity or expression. This has led to the inclusion of SMAD inhibitors in neuronal differentiation protocols including noggin, a BMP inhibitor and SB431542, a TGF-β signaling inhibitor. In fact, dual inhibition of SMAD signaling was found to promote up to 80% conversion of hESCs to Pax6+ neural cells[56]. Importantly, SMAD inhibition also promotes neural differentiation of MSCs[57] demonstrating that SMAD signaling is a common mechanism for neural differentiation in several different types of stem cells. Soluble factors, including insulin-like growth factor (IGF) and fibroblast growth factors (FGFs), induce neural differentiation of MSCs by activating the MAPK signaling cascade[58], which phosphorylate the linker region of SMAD1 to inhibit SMAD1's activity and its translocation to the nucleus, providing another pathway to the traditional neural induction machinery.

BMP–SMAD and surface signal transduction

Given that disruption of SMAD signaling is important for neural differentiation, it is not surprising that SMAD activity is downregulated in cells grown on soft surfaces. Soft surfaces may regulate SMAD signaling at the cell surface level by having an effect on the availability of BMP receptors[26]. Du *et al.* showed that MSCs cultured on soft surfaces reduce the amount of surface BMPR1A, which co-localize with B1 integrin leading to receptor internalization and reduced phosphorylated SMAD and nuclear translocation[26]. Additionally, Zouani *et al.* provided evidence that MSCs grown on soft surfaces show disrupted BMP–SMAD signaling[27]. These MSCs did not respond to BMP2 treatment and showed reduced translocation of p-SMAD to the nucleus. Taken together, these studies suggest that soft surfaces might be rendering MSCs incompetent to receive soluble signals.

Others have also noted that SMAD localization can shift from nuclear to cytosolic when cultured on stiff *vs.* soft surfaces, respectively. In the differentiation of hPSCs into motor neurons, Sun *et al.* showed that SMAD is predominantly nuclear in cells cultured on stiff surfaces, shifting to cytoplasmic when cultured on softer surfaces[43]. SMAD localization was also shown to be dependent on how the surface modulus affected the Hippo/YAP signaling axis[43]. YAP was shown to shuttle phosphorylated SMAD into the nucleus when cultured on rigid surfaces; however, when cultured on soft surfaces, YAP becomes phosphorylated by lats1/2 causing its sequestration by 14-3-3 in the cytosol and disrupting its ability to import SMAD into the nucleus. These results support that soft surfaces

can inhibit SMAD's activity and prevent its translocation to the nucleus. Furthermore, by inhibiting the traditional SMAD signaling pathway this could explain the preference for stem cells to undergo neural differentiation on very soft substrates.

Roles of Rho GTPases and mechanotransduction in neural differentiation

Rho GTPases are widely known effectors for inducing changes in the cell cytoskeleton and have been extensively reviewed in the context of cell migration and morphology[59]. The three main members of the Rho superfamily, RhoA, Rac and Cdc42 have been characterized for their ability to elicit cytoskeletal changes in cellular development, immune response and cancer metastasis. Not surprisingly, given the morphological complexity of neuronal cells, Rho GTPases are instrumental in the development and function of mature neurons. (See Govek[59] for a comprehensive review on this topic.) Rho GTPases also play an important role in mechano-transduction, transducing physical signals to the nucleus to affect cellular differentiation, including neural differentiation on soft surface.

Rho GTPases mediate changes in cell morphology by regulating components of the cytoskeleton, while cell morphology in turn, can impact cell lineage. Early work by McBeath *et al*. showed that the morphology of MSCs could predetermine cell fate to the adipocytic or osteocyte lineages[60]. Using micropatterned substrates to control cell size and shape, they showed that cell shape could dictate cell lineage mediated through RhoA–ROCK signaling. Importantly, lineage commitment was dependent on actin–myosin tension generated within the cell. However, the changes induced at the transcriptional level were unclear. Recently, it was demonstrated that cell geometry had a profound impact on the localization and activity of YAP/TAZ[61,62]. On unpatterned surfaces, the cells spread over the substrate and localized YAP/TAZ to the nucleus to drive proliferation. Cells grown on a smaller restricted substrate area showed minimal YAP/TAZ nuclear localization. This effect was mediated by actin–myosin tension generated within the cell and severing of actin filaments interfered with YAP/TAZ nuclear localization.

Research has shown that activation of Rho–ROCK signaling inhibits neural differentiation and neurite formation[63–66]. On very soft surfaces, Rho GTPases have also been demonstrated to play a role in mechano-induced neural differentiation of adult NSCs. A direct relationship was shown between substrate stiffness and RhoA and Cdc42 activation leading to inhibition of neural differentiation[25]. Disruption of RhoA or its downstream effectors including ROCK restored the neuronal differentiation potential of NSCs on soft surfaces.

How Rho GTPases transduce signals from soft surfaces to induce neural differentiation remains ambiguous. However, better characterization of YAP/TAZ has revealed a pathway through which Rho GTPases signal to the nucleus. RhoA activation of ROCK induces stabilization of actin filaments, an important event for localizing genes to their respective cellular compartments. Indeed, nuclear localization of YAP/TAZ was found to be dependent on the polymerization of actin[67,68] which in turn was dependent on cytoskeletal tension[67]. ROCK inhibitors destabilize actin filaments, inhibit YAP/TAZ nuclear localization and promote neural differentiation. Taken together, RhoA activation on stiffer surfaces activates ROCK to stabilize filamentous actin resulting in nuclear localization of YAP/TAZ (**Fig. 1**). While YAP/TAZ is under the control of RhoA and actin, it remains to be determined whether this also affects SMAD localization.

Role of YAP/TAZ on neural differentiation

YAP/TAZ proteins were originally identified as modulators of organ size and have since been shown to play a role in stem cell and cancer biology[69]. They have also been implicated as mechanosensors for MSCs and emerging research has demonstrated that YAP/TAZ can convert cell cytoskeleton tension into nuclear signals[43,47,67], and act as mechanosensors to alter gene expression.

Whether YAP/TAZ induces neural gene expression directly or engages other signaling pathways and transcription factors is actively being investigated. Han *et al*. showed that YAP/TAZ signaling is important for NSC maintenance through activation of the TEAD transcription factor[70].

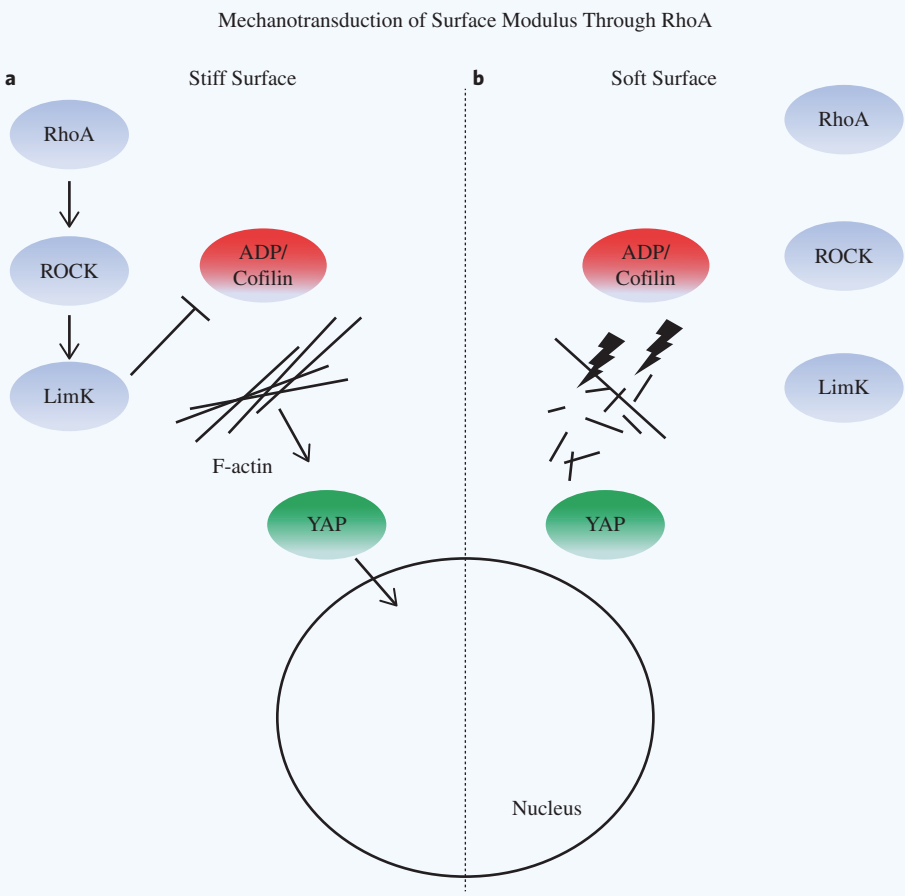

Figure 1 RhoA acts as a mechanotransducer of surface modulus by promoting actin polymerization. (**a**) On stiff surfaces, RhoA activation indirectly stabilizes F-actin by inhibiting actin severing proteins ADP/Cofilin through ROCK and LimK kinases. F-actin is required for YAP nuclear translocation. (**b**) On soft surfaces, reduced activation of RhoA de-represses actin severing proteins resulting in actin depolymerization and reduced translocation of YAP.

This SMAD-independent induction of neural gene expression through YAP/TAZ with respect to physical cues remains to be determined.

Transcriptional regulation

The neuron restrictive silencer factor

SMADs are well known inhibitors of neural differentiation, and understanding the regulation of their target genes during neural differentiation is essential to understanding how extracellular signals induce neural lineage. Given that increased nuclear levels of SMAD is inversely associated with neural differentiation, it is important to identify what factors SMADs induce that *inhibit* neural differentiation. The neuronal phenotype is known to be strongly repressed by the NRSF. First identified nearly two decades ago, NRSF is now characterized as a master transcriptional regulator of the neural phenotype[71]. During normal development and maturation of NSCs to fully functioning neurons, NRSF is downregulated and de-represses hundreds of neuronal genes required for differentiation[35]. Among the genes repressed, include neuron-specific structural proteins, neuron-specific biosynthesis enzymes, ion channels and neurotrophic factors[72–77]. Silencing of NRSF in MSCs can induce several neuronal characteristics[77] including generation of spontaneous Na^+ currents while forced expression of a constitutively active NRSF mutant (VP16) initiates neural differentiation in NSCs[79]. NRSF is of special interest because it could serve as a primary transcriptional target for mechano-transduction in neural differentiation. Notably, the promoter region of NRSF contains two SMAD-binding elements (SBEs) and SMAD1 localization to the nucleus during BMP2 induction induces expression of NRSF in astrocytes[80]. Given that soft surfaces can induce neural characteristics in stem cells in a SMAD-dependent manner, we hypothesize that soft surfaces interfere with SMAD-induced expression of NRSF to induce neural characteristics by de-repressing NRSF target genes. In our lab, we found that NRSF expression is downregulated in MSCs cultured on soft PDMS surfaces. Particularly, we observe a strong decrease in NRSF in the nuclear fraction (unpublished data). In further support of this, downregulation of NRSF in stem cells phenocopies several effects induced by soft surfaces. Downregulation of NRSF supports generation of action potentials in stem cells[78] while, conversely, upregulation decreases action potential generation by repressing critical Na^+ channel genes[81]. Downregulation of NRSF is also important for generation and maintenance of neuronal morphology and axon pathfinding[82]. Taken together, they suggest that soft surfaces could encourage neuronal morphology and neurite branching[13] through downregulation of NRSF. An interesting observation regarding neural differentiation on soft surfaces and SMAD signaling is that the cells can acquire tyrosine hydroxylase expression indicative of the dopaminergic neuronal subtype[44]. Tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, is also a gene that is directly repressed by NRSF[83] supporting the notion that soft surfaces are inducing neural differentiation by downregulating NRSF.

Downregulation of NRSF and de-repression of neural genes is critical for neural differentiation; however, additional transcription factors mediate the progression of the neural gene program. Among these is the NeuroD family of genes. While NRSF is a master transcriptional *repressor*, NeuroD is a master transcriptional *activator* of the neuronal phenotype and overexpression of NeuroD can directly convert ectoderm tissue directly into neurons[84]. Importantly, NeuroD1, NeuroD2 and NeuroD4 are under repression by NRSF in the undifferentiated stem cell state[85,86], suggesting that NRSF could be restrain a transcriptional cascade important for driving differentiation. In support of this, it was observed that NeuroD gene expression is also responsive to varying degrees of surface stiffness[69,85] with the highest expression on the softest surfaces. Musah *et al.*[47] found a strong connection between soft surfaces, neuronal differentiation of iPSCs, localization of YAP and transcription factors important for neural differentiation including NeuroD1 and NeuroG2 (Neurogenin-2). The authors hypothesized that NeuroD1 and NeuroG2 were responsible for driving differentiation; however, whether their expression was mechanically induced independently of NRSF remains to be determined. Concomitantly, Gao *et al.* showed that NeuroD1 may be an important neuronal activator that is repressed in NSCs by NRSF[87]. Whether this differential expression of neural activators on soft surfaces is dependent on NRSF remains to be determined. Regardless, downregulation of NRSF combined with increased expression of NeuroD genes could explain why stem cells exhibit neural differentiation on soft surfaces.

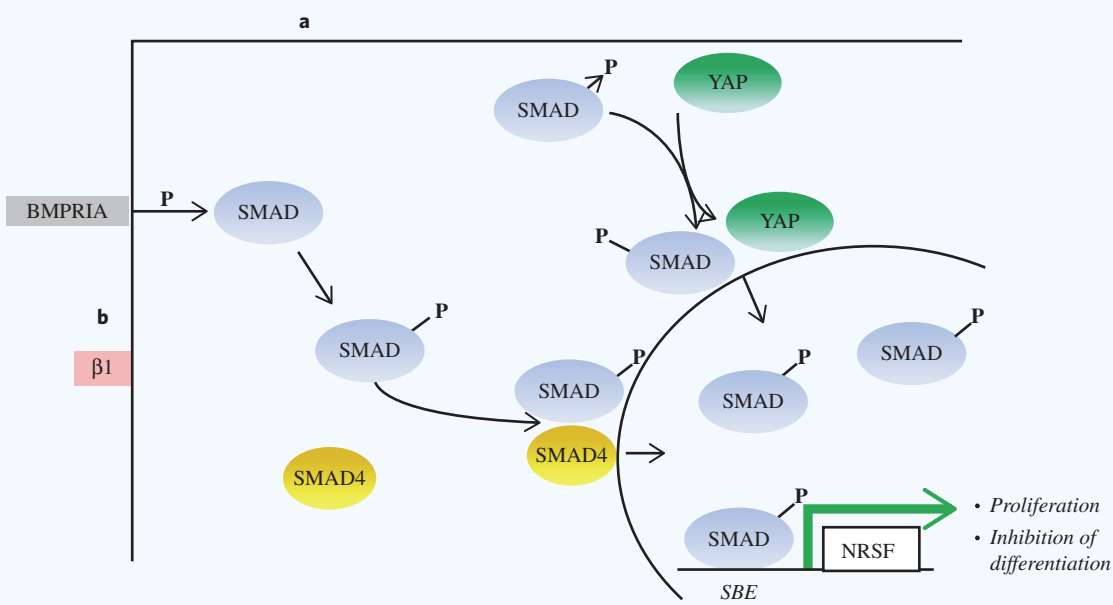

Figure 2 Molecular mechanisms explaining the role of SMAD during neural induction of stem cells on stiff surfaces. (**a**) YAP mediates SMAD import into the nucleus to promote proliferation and inhibit differentiation. (**b**) Phosphorylation of SMAD by BMPR1A permits SMAD's import into the nucleus by SMAD4 (the co-SMAD). SMAD accumulation in the nucleus activates expression of NRSF thereby inhibiting neural differentiation.

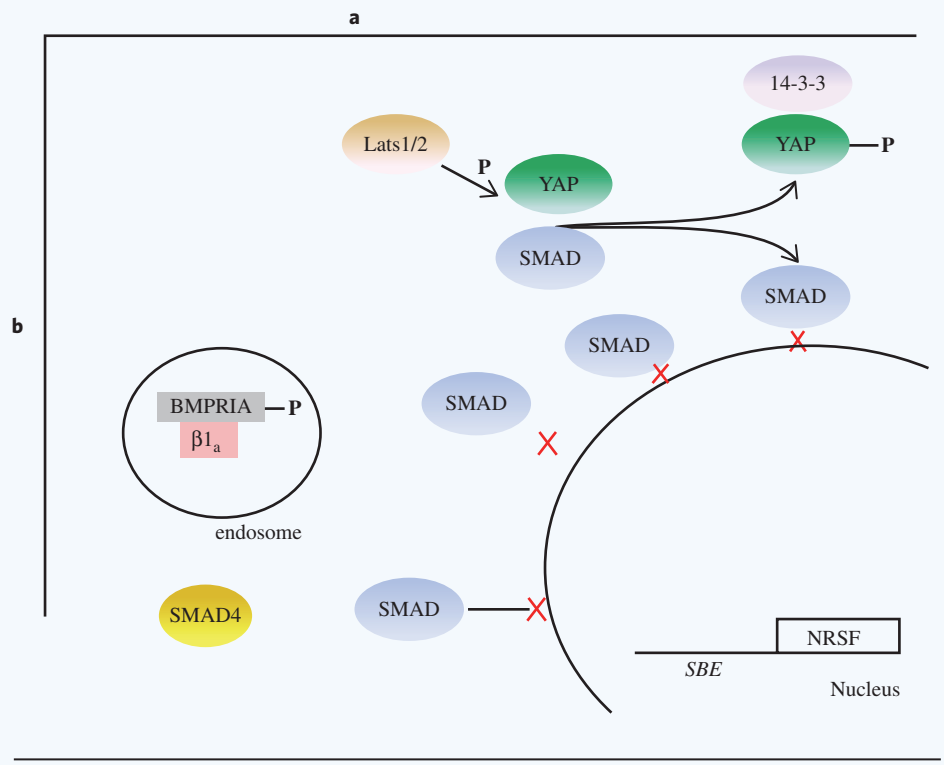

Figure 3 Molecular mechanisms explaining the role of SMAD during induction of neural differentiation of stem cells on soft surfaces. (**a**) Phosphorylation of YAP by Lats 1/2 causes YAP to be bound and sequester in the cytoplasm by 14-3-3, reducing the ability of YAP to shuttle SMAD to the nucleus. (**b**) Soft surfaces increase BMPR1A internalization by endocytosis and reduce its ability to phosphorylate SMAD, thereby reducing the localization of SMAD on soft surfaces and its upregulation of NRSF expression.

soft surfaces appear to promote early neural differentiation, causing stem cells to exhibit many pan-neuronal markers. However, it remains an open question whether soft surfaces can promote later differentiation into specific neuronal subtypes.

Although this review focused on neural differentiation, varying surface modulus can be used to promote differentiation into other lineages. Surfaces with intermediate stiffness promote myogenic or adipogenic differentiation while very stiff surfaces promote osteogenic differentiation. Characterizing the molecular signaling pathways and transcriptional regulators induced by different surface moduli could help in engineering surfaces for lineage-specific differentiation. Much like neural differentiation and NRSF, other lineages have their own master transcriptional regulators. For example, MyoD was the first such master regulator characterized to directly transform fibroblasts into skeletal muscle[5]. Since then, PPARγ and Runx2[88,89] have been shown to be master regulators of adipogenesis and osteogenesis, respectively. Thus, future research needs to be directed on the molecular mechanism that are activated or repressed by these transcription factors to induce their lineage-specific differentiation in response to physical cues.

CONCLUSIONS

Understanding the mechanisms involved in stem cell differentiation have largely been identified for soluble factors; however, the physical cues are increasingly recognized as an important component. Particularly, very soft surfaces favor neural differentiation in various types of stem cells, and increasingly evident that they cooperate with soluble factors to induce neural differentiation. Much of the focus to date has been on BMP–SMAD signaling, Rho GTPases and YAP/TAZ. Given that these pathways are known to function in physiological neural differentiation, it is not surprising that they are also affected by the physical environment. Rho GTPases can regulate YAP/TAZ through the cytoskeleton providing an important link between the signal imparted by the substrate to the cell and nuclear regulators. The association between YAP/TAZ and SMADs recently identified to be modulated by surfaces is significant since SMAD1/5/8 are known transcriptional regulators involved in neural development. Given that SMAD has been shown to regulate NRSF in astrocytes, provides a potential mechanism by which soft surfaces induce neural characteristics in a variety of stem cell types. Based on the current literature we propose a model that could explain how soft surfaces favor neural differentiation. Central to this model is the localization of SMAD to the nucleus. On rigid surfaces SMAD is imported into the nucleus (**Fig. 2**) while on soft surfaces it remains cytosolic (**Fig. 3**).

Knowing how the molecular signaling pathways are affected by soft surfaces could aid in the design of materials that can enhance neural differentiation, surfaces could then be better engineered to promote neural function. Nevertheless, an important question remains with regard to what extent neural differentiation is induced by soft surfaces. Currently,

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