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Short communication

Soft surfaces induce neural differentiation via the neuron restrictive silencer factor

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ABSTRACT

The physical and mechanical environment influence the differentiation and culture of stem cells. Early research in this field showed that mesenchymal stem cells (MSCs) adopt preferential lineages when cultured on surfaces of varying stiffness. Culture on very soft surfaces promoted neural differentiation. This effect has been observed in mesenchymal and embryonic stem cells and was shown to increase development of neural stem cells. However, providing further information on the molecular characterization of this phenomenon could help to guide the development of materials that enhance neural differentiation. Previously, our laboratory showed that chemical cues, forskolin and IBMX, induced neural differentiation of MSCs by downregulating the Neuron Restrictive Silencer Factor, leading to de-repression of neural gene expression. We sought to determine if the mechanism whereby soft surfaces induce neural differentiation could also involve NRSF function. We show that MSCs cultured on soft polydimethylsiloxane (PDMS) surfaces have reduced expression of NRSF as well as altered localization of NRSF. This suggests the modulation of transcription factors by surface substrate could be capitalized upon in designing materials for neural cell culture.

1. Introduction

1.1. Soft surfaces induce differentiation through transcription factors

Stem cell differentiation is a complex molecular biology process controlled by responses to growth factors and morphogens. These activate signaling cascades leading to the nucleus where gene expression programs are initiated and maintained by expression of terminal transcription factors that often control expression of genes specific to a mature cellular phenotype. It is well established that stem cells could be coaxed into different cell fates by adjusting the physical microenvironment [1], such as the stiffness [2] or anisotropy [3] of the surface on which they are cultured, that is, physical forces are relevant features in the design of biomaterials for the enhancement of cell culture. However, the molecular events that underlie this physical-based induction are still actively being characterized. Master transcriptional regulators are crucial for specifying cell fate [4–8]. Exogenous expression of these molecules have the power to directly reprogram cells from one type to another. It was previously shown that the master transcriptional regulator MyoD, a marker for myogenic differentiation, can be modulated by mechanical cues, such as surface stiffness [2] and anisotropy [3]. Concomitantly, very low surface modulus was able to induce expression of Tuj1, a structural molecule specific to neural lineages [2]. Further work by others has shown that neural and embryonic stem cells show enhanced neural differentiation when cultured on very soft surfaces [9–14]. However further characterization of the changes in neural specific transcription factors can further aid our understanding of the acquisition of certain phenotypic characteristics.

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1.2. NRSF downregulation can induce neural differentiation

Previously, we showed that a combination of cAMP-inducing molecules, forskolin and IBMX, was able to induce neural marker expression and sensitivity to dopamine of mesenchymal stem cells (MSCs) [15], which was reliant on the downregulation of the master transcriptional repressor, Neuron Restrictive Silencer Factor (NRSF) [16]. This work was interesting as it demonstrated that MSCs could be induced to transdifferentiate from their mesodermal origin to acquire neural characteristics of ectodermal origin. Since induction of neural characteristics in MSCs by substrate surface modulus noted by Engler et. al. [2] here, we aimed to determine whether soft surfaces could modulate NRSF. Using PDMS that can be tuned to stiffnesses ranging from low kilo- to mega-pascals we observed that MSCs have altered NRSF expression and activity.

2. Materials and methods

2.1. Materials

A list of the materials are provided in Table 1.

2.2. Mesenchymal stem cell culture and isolation

MSCs were isolated from animals using procedures approved by the Institutional Animal Care and Use Committee at Michigan State University. MSCs were derived from bone marrow isolated from 4 to 6 week-old Sprague-Dawley female rat as previously described [17]. Briefly, femurs and tibias were removed from 4 to 6-week-old rats. The two ends of the bone were cut open and the marrow was flushed with 10 mL of DMEM using a 25 g needle and syringe. The cell suspension was passed through a 70-um nylon mesh to remove bone debris and blood aggregates. Cells were cultured in low glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and free of antibiotics. Cells were incubated in a humidified atmosphere containing 5% CO2 at 37 C. Non-adherent cells from the flushed marrow were removed after 48 h after isolation. Media was replaced every 3 days until the cells reached 80–90% confluence. Confluent cells were washed with PBS (no CaCl2) detached by 0.25% trypsin–EDTA (Invitrogen) and plated for further experiments.

2.3. PDMS preparation

PDMS was prepared from the Sylgard 184 manufacturer's kit. We prepared regular PDMS at the specified crosslinker to base ratio of 1:10 and also very soft PDMS at a crosslinker to base ratio of up to 1:80. This is the lowest ratio we could use and still have the PDMS cure properly.

Table 1
List of items and reagents.

Item	Vendor	Catalog
DMEM	Gibco	11965
Nylon Mesh	Corning	352350
FBS, Qualified	Gibco	26140
0.25% Trypsin-EDTA	Gibco	25200
Sylgard 184	Ellsworth	184SIL ELAST
NE-PER Kit	Thermo Scientific	78833
Anti-Tuj1	Cell Signaling Tech	4466
Anti-GAPDH	Cell Signaling Tech	2118
Anti-NRSF	EMP Millipore	07–579
DAPI	Invitrogen	D3571
Alexa 488	Invitrogen	A11008
Alexa 546	Invitrogen	A11035
ProLong™ Gold Antifade Mountant	Invitrogen	P36930
Goat Anti-Mouse HRP	Invitrogen	A16078
Goat Anti-Rabbit HRP	Invitrogen	A16110
Super Signal West Femto	Thermo Scientific	34096

Crosslinker and base was mixed for 5 min and degassed in a vacuum chamber for ~10 min before pouring. For cell lysates, PDMS was poured into 10 cm pyrex dishes. For immunostaining and calcium imaging, PDMS was poured onto 1 mm thin glass coverslips. 1:10 PDMS was cured in a dry oven overnight at 70 C. 1:80 PDMS was cured on top of a hot plate at 150 C for about 4–6 h. After curing, PDMS was O_2 plasma treated for 60 s, 30 W. Immediately following plasma treatment, PDMS was then incubated with poly-L-lysine for 1 h at room temperature. PLL was then removed then washed several times with PBS. After drying, PDMS was sterilized under UV light for 30 min

2.4. Nuclear isolation

Nuclear fractionation for protein lysates was performed according to NE-PER Nuclear and Cytoplasmic Extraction Reagents. Briefly, cells were harvested by washing once with PBS then incubating with.05% trypsin-EDTA until cells lifted off substrate. Detached cells were collected, and residual trypsin was neutralized with cell medium containing 10% FBS. Cells were pelleted in a centrifuge at 200 *g* for 5 min and the excess trypsin was aspirated. Cell pellet was resuspended and washed with PBS and pelleted again to remove traces of FBS. After the wash was aspirated, the cell pellet was incubated in ice cold CER I solution and vortexed to lyse cell plasma membrane. Samples were then centrifuged at 16,000*g* to separate the cytosolic fraction from the insoluble nuclear pellet. After removal of the cytosolic fraction, nuclei were suspended in buffer NER and incubated on ice for up to 1 h to extract the nuclear fraction.

2.5. Immunostaining

Cells were washed with PBS and then fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. After washing 3 times with PBS, fixed cells were permeabilized with.1% triton-X100 in PBS for 15 min at room temperature. Cells were further washed 3 times with PBS to remove the triton X-100. Cells were then blocked in PBS with 10% normal goat serum for 1 h at room temperature. After blocking, cells were incubated for primary antibody overnight at 4 C. Following overnight incubation, cells were washed 3 times with PBS and incubated for 1 h at room temperature with appropriate secondary antibody. Cells were washed once more then fixed to a microscope slide with Prolong Gold with DAPI. This was allowed to bond overnight before imaging.

2.6. Western blotting

Whole-cell extracts were prepared by lysing cells with RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% IGEPAL (NP-40), 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate) on ice for 30 min. Lysates were mixed with 5X SDS protein loading buffer (50 mM Tris pH 7.0, 25% glycerol, 2% SDS, 0.025% bromophenol blue) and denatured at 95 C for 5 min 20 ug of each sample lysate was separated by electrophoresis on an 8% Tris–HCl gel and transferred to a nitrocellulose membrane. Membranes were then blocked in 5% milk and 0.05% Tween 20–TBS (Tris buffered saline) for 1 h and incubated with primary antibodies against Tuj1 or GAPDH (Cell Signaling) or NRSF/REST (Millipore) overnight at 4°C. Anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Thermo Scientific) was added the second day after primary antibody incubation. The blots were incubated for 90 min and then washed three times with 0.05% Tween 20–TBS. The blots were then visualized by chemiluminescence with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

2.7. Preparation of very low modulus PDMS

PDMS stiffness can be tuned by adjusting the ratio of crosslinker to base. Mixing is recommended at a ratio of 1:10 which can be cured in a

70 C for ~2 h. However, to obtain a very low surface modulus, the ratio of crosslinker to base must be dramatically lowered and can present problems for curing. When mixing at a ratio of 1:70 and 1:80 sample preparations had to be cured on a hotplate @ 150 C. 1:70 PDMS curing took at least 3 h while 1:80 PDMS curing took at least 6 h.

2.8. Measurement of mechanical properties

The mechanical properties of the cross-linked PDMS substrate was determine by rheological measurements on a rotational rheometer (Anton Paar MCR 302) at T = 20ºC. The testing geometry is parallel plates with diameter of D = 8 mm in the measurements. Strain sweeps from 0.01% to 30% at 1 rad/s were performed to identify both the shear modulus and the linear response region. Then, frequency sweeps from 100 rad/s to 0.1 rad/s at a constant strain of 0.3% (within the linear response region) was performed to check the shear modulus at different frequencies. Both strain sweeps and frequency sweeps yield to identical shear modulus, G. The Young's modulus is thus identified through E = 3 G.

2.9. Statistical analysis

Statistical analyses for differences in mean were performed using two factor ANOVA followed by Tukey Test to show significance between means for n = 3–4. For colocalization data, student t test was performed for the two data sets (n = 19 and n = 14) with P < .01.

3. Results

3.1. PDMS modulus determination

A rheostat was used to assess the stiffness of the samples. 1:10 PDMS had a Young's Modulus of ~1 MPa while 1:70 and 1:80 PDMS had a Young's Modulus of 6.1kPa and 1.8kPa, respectively (Fig. 1). This is in line with published values [11,18,19]. Although our preparation of 1:80 PDMS had a Young's Modulus near that of brain tissue [20], we noted cell attachment was poor, complicating some of the experiments. Therefore, we used 1:70 PDMS as our softest substrate.

3.2. Soft surface induced changes in gene expression

Culture of MSCs on soft PDMS resulted in reduced expression of NRSF protein levels by western blotting (Figs. 2a and S1). Previously, we demonstrated that induction of neural differentiation of MSCs included expression of pan-neural markers. MSCs can be induced to express Tuj1 in the presence of cAMP-elevating compounds IBMX and forskolin [15]. Here, we show that culture of MSCs on soft surfaces causes them to

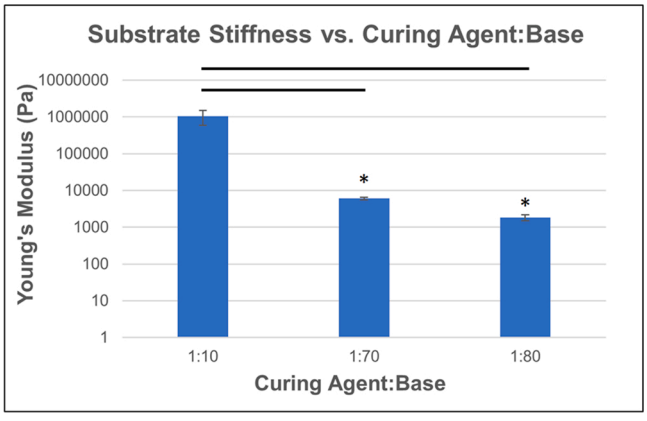

Fig. 1. Young's Modulus of PDMS prepared with varying ratios of cross-linker to base (N = 4), *= p < .01.

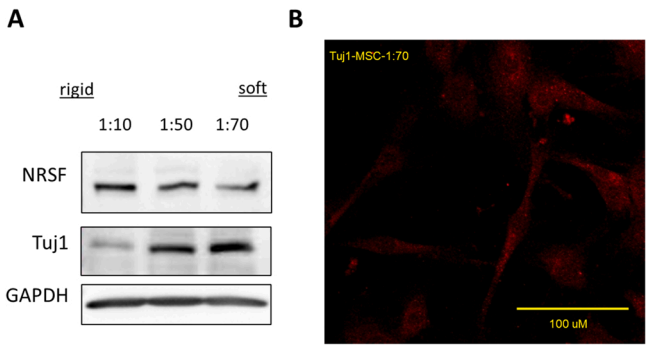

Fig. 2. Expression of neural marker in MSCs cultured on soft surfaces. A) MSCs grown on softer surfaces show a decrease in total NRSF expression and an increase in expression of neural marker Tuj1. B) Immunofluorescence of neural markers in MSCs grown on soft PDMS.

spontaneously express Tuj1 (Fig. 2a,b). A major molecule involved in the induction of neural differentiation are members of the SMAD-1/5/8 family. SMAD signaling inhibits differentiation [21] and has been shown to directly regulate gene expression of NRSF [22]. In practice, SMAD inhibition by small molecules is used in in vitro neural differentiation protocols. However, when grown on soft PDMS, we observed no change in the relative expression of SMAD (Fig. 2a).

Since the primary function of NRSF is transcriptional regulation we next sought to determine its subcellular localization. Staining for NRSF and imaging with confocal microscopy shows a difference in nuclear localization (Fig. 3a). NRSF is mainly nuclear in cells cultured on stiff surfaces while it is predominantly cytosolic in cells cultured on soft surfaces (Fig. 3a). Colocalization analysis run on FV1000 software showed higher Pearson's correlation of DAPI vs. alexa-546 signal in cells grown on 1:10 PDMS (n = 19) than compared to 1:70 (n = 14), p < .01 (Fig. S2). This is further confirmed by analyzing the protein expression of NRSF by western blotting (Fig. 3b). The cells were fractionated into cytosolic and nuclear fractions and their relative amounts of NRSF were compared. NRSF protein levels are higher in the nucleus in the cells grown on stiff surfaces (1:10) and show higher expression in the cytosol when cells are cultured on soft surfaces (1:70).

4. Discussion

The results indicate that growing MSCs on soft surfaces has a negative effect on the expression of NRSF and impact the localization of NRSF. These observations combined impact the ability of NRSF to repress gene expression, as evidenced by the de-repression of its target gene, Tuj1 [23].

Given its role as a master regulator, it is not surprising that the regulation of NRSF expression and localization is itself complex. Among those known to affect neural differentiation are the bone morphogenetic proteins (BMPs). BMPs induce activity of SMADs to express NRSF and *prevent* neural differentiation. Removal of BMPs can cause spontaneous neural differentiation of embryonic stem cells. SMAD inhibition by small molecules is commonly used to induce neuronal differentiation from neural stem cells. Previous work by Du et. al. had shown that culture of bone marrow MSCs on soft surfaces resulted in downregulation of BMP receptor expression as well as a decrease in SMAD phosphorylation [24]. In a separate study, SMAD was shown to directly regulate the NRSF gene promoter region to maintain astrocytic identity and prevent neural differentiation [22]. The possibility for SMAD to regulate expression of NRSF may also implicate the known mechanotransducer, YAP/TAZ. This transcription factor is also known to inhibit neural differentiation in cells cultured on rigid surfaces. Interestingly, YAP/TAZ contributes to nuclear localization of SMAD in cells cultured on rigid surfaces [14] suggesting a relationship to NRSF expression on rigid vs. soft surfaces. Further research into the effect of soft surfaces on SMAD signaling could

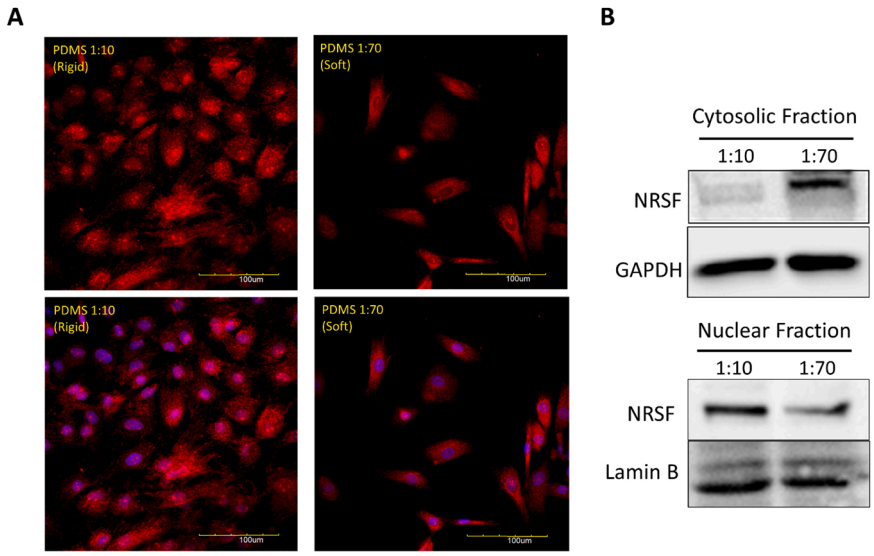

Fig. 3. Differential expression and localization of NRSF on tissue culture polystyrene vs. soft PDMS. A) NRSF (red) expression appears nuclear in MSCs grown on 1:10 (rigid) PDMS. MSCs grown on 1:70 (soft) PDMS show reduced expression in the nucleus visualized by DAPI stain (blue). B) NRSF shows differential levels of expression in MSCs grown on rigid vs. soft between cytosolic and nuclear fraction.

further synergize with the results of this study on NRSF in the design of materials that would promote and help maintain neuronal phenotypes in cell culture.

The results of this study show that the physical environment can have an effect on a transcription factor during induction of cell differentiation. This approach could be extended to other lineages to identify transcription factors, particularly master transcriptional regulators, that could be activated or repressed based on substrate stiffness. In support, medium stiffness surfaces [2] and anisotropic surfaces [3], which promote MYOD expression, could be beneficial for culturing and maintaining myogenic cells without the need for chemical factors, while stiff surfaces that induce RUNX2 could be useful for culturing osteogenic cells. Studying how the physical environment affects the activity of these key molecules that determine cell lineage may assist in the development of materials that could improve the robustness and viability of cell culture.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Christina Chan reports a relationship with Michigan State University that includes: employment.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2022.108724.

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