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The promotion of neuronal maturation on soft substrates

Ana I. Teixeira [a,*,1], Shirin Ilkhanizadeh [a,1], Jens A. Wigenius [b], Joshua K. Duckworth [a], Olle Inganäs [b], Ola Hermanson [a]

[a] Department of Neuroscience, Karolinska Institute, Stockholm SE 171 77, Sweden
[b] Department of Physics, Chemistry and Biology, Biomolecular and Organic Electronics, Linköping University, Linköping SE 581 83, Sweden

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ABSTRACT

Microenvironmental mechanical properties of stem cell niches vary across tissues and developmental stages. Accumulating evidence suggests that matching substrate elasticity with *in vivo* tissue elasticity facilitates stem cell differentiation. However, it has not been established whether substrate elasticity can control the maturation stage of cells generated by stem cell differentiation. Here we show that soft substrates with elasticities commensurable to the elasticity of the brain promote the maturation of neural stem cell-derived neurons. In the absence of added growth factors, neurons differentiated on soft substrates displayed long neurites and presynaptic terminals, contrasting with the bipolar immature morphology of neurons differentiated on stiff substrates. Further, soft substrates supported an increase in astrocytic differentiation. However, stiffness cues could not override the dependency of astrocytic differentiation on Notch signaling. These results demonstrate that substrate elasticity *per se* can drive neuronal maturation thus defining a crucial parameter in neuronal differentiation of stem cells.

1. Introduction

Mechanical properties vary across tissues and developmental stages in living organisms and are increasingly being recognized as relevant cellular stimuli [1]. Defects in processing mechanical cues are thought to be involved in cancer progression as well in diseases such as muscular dystrophies and cardiomyopathies [2]. The impact of stiffness on cell behaviors is, predictably, cell type dependent. Epithelial cells [3] and fibroblasts [4] were found to migrate in the direction of increasing substrate stiffness whereas prostate carcinoma cells migrated in the direction of decreasing stiffness [5]. Processing of mechanical stimuli, mechanotransduction, is partly mediated by integrins that link the extracellular physical environment to the cytoskeleton [6,7]. Further, it has been shown that cells tune internal stiffness with the microenvironmental stiffness [8]. These physical responses are converted into biochemical alterations that modulate cellular behaviors such as spreading [9], migration [3], and differentiation [10].

Stem cells may be poised to respond to mechanical stimuli. Mesenchymal stem cells (MSCs) have shown remarkable responsiveness to substrate elasticity in cell culture. MSCs cultured on substrates with elasticities that matched native tissue elasticity showed elasticity dependent cell fate choices [10]. Soft substrates promoted neuronal differentiation, stiffer substrates were myogenic and substrates with bone-like stiffness promoted osteogenic differentiation [10]. Here we show that while substrate stiffness did not affect the number of neurons differentiated from neural stem cells, neuronal maturity was promoted by soft substrates. In addition, a decrease in substrate stiffness was associated with a robust increase in astrocytic differentiation. We conclude that modulation of substrate stiffness is a promising strategy for improved control of stem cell differentiation and maturation.

2. Materials and methods

2.1. Materials

The sources of the materials used in this work were as follows. Dow Corning PDMS Sylgard 184 Silicone from GA Lindberg. Serum-free DMEM:F12 and FBS were from Invitrogen. T3, PBS, poly-l-ornithine, fibronectin, paraformaldehyde (PFA), Triton-X, and bovine serum albumin (BSA) were purchased from Sigma–Aldrich. CNTF and FGF2 were from R&D Systems. Vectashield containing DAPI was obtained from Vector Laboratories and DAPT was from Calbiochem. The primary and secondary antibody sources and dilutions were as follows: mouse anti-Nestin from BD Biosciences Pharmingen (1:500), rat polyclonal anti-Myelin Basic Protein (MBP) from Chemicon (1:250), mouse monoclonal anti-Neuronal Class III B-Tubulin (TuJ1) from Nordic Biosite (1:500), mouse monoclonal anti-α-smooth muscle actin (SMA) from Sigma (1:1000); rabbit poly-clonal anti-glial fibrillary acidic protein (GFAP) from DAKO (1:500), rabbit anti-synaptotagmin (raised against the cytosolic part of rat synaptotagmin I and affinity purified) was a gift from Peter Löw, Karolinska Institute, Sweden (1:10000). Species-specific Alexa-488, Alexa-546, Alexa-549,

* Corresponding author. Tel.: +46 8 5248 7081; fax: +46 8 341 960; mobile: +46 76 234 3676.
E-mail address: ana.teixeira@ki.se (A.I. Teixeira).
[1] Equal contribution.

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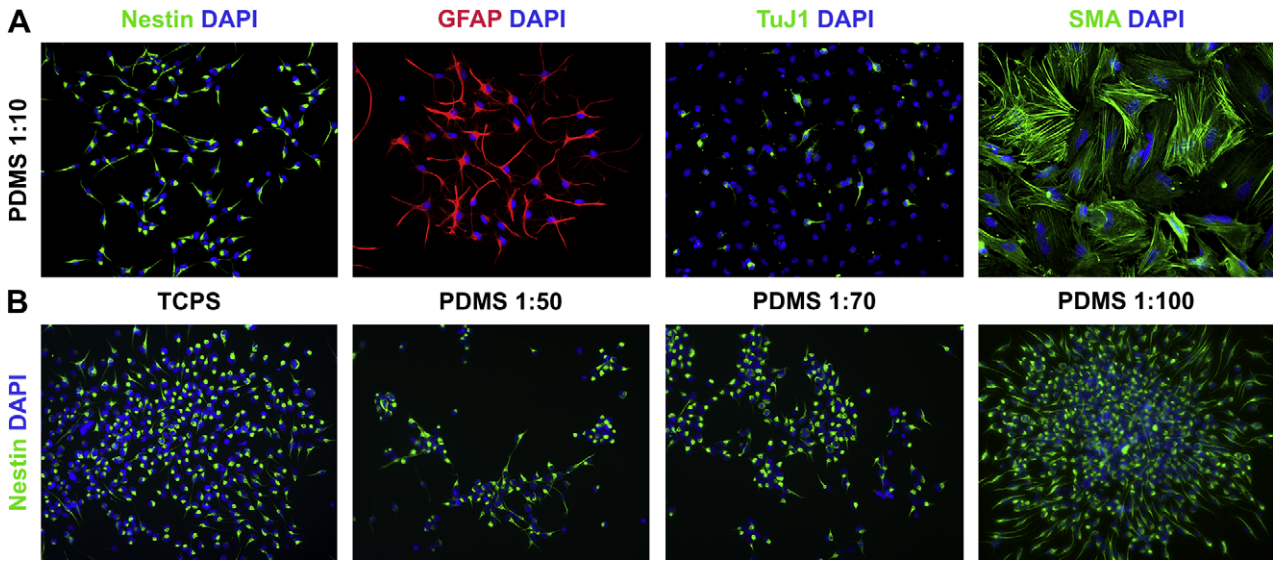

Fig. 1. NSCs cultured as monolayers on PDMS were able to maintain the stem cell state and differentiate in response to soluble factors, according to established protocols. (A) NSCs grown in N2 medium supplemented with 10 ng/ml FGF2 for 3 days were immunoreactive to nestin, a neural stem cell marker. Stimulation with 10 ng/ml ciliary neurotrophic factor (CNTF) for 3 days caused NSC differentiation into astrocytes, labeled for GFAP. Withdrawal of FGF2 for 5 days caused some cells to spontaneously differentiate into TuJ1 expressing neurons. NSCs treated with 10% fetal bovine serum (FBS) for 5 days became smooth muscle-like and expressed Smooth Muscle Actin (SMA). PDMS substrates were prepared with a dilution of crosslinker to base of 1:10, the standard dilution recommended by the manufacturer. Images were acquired with 20× magnification. (B) NSCs proliferated and expressed nestin when grown in medium supplemented with FGF2 on PDMS substrates prepared with ratios of crosslinking agent to base of 1:10, 1:50, 1:70 and 1:100. Images were acquired with 20× magnification.

Alexa-633, and Alexa-699-conjugated secondary antibodies were used as appropriate and were obtained from Molecular Probes (1:500).

2.2. Substrate preparation

The substrates used were either regular tissue culture plates (TCPS) or PDMS with different stiffnesses. A Sylgard 184 cross-linking agent was added to the PDMS Sylgard 184 base at ratios of 1:10 (recommended from the manufacturer), 1:50, 1:70 and 1:100. The polymer and cross-linking agent were mixed and put in a vacuum chamber in order to remove bubbles [11]. Next, 250 μl of each PDMS mix was pipetted into 35 mm tissue culture plates and baked at 60 °C for 1 h. The PDMS substrates were then left in water over night. Before using for cell culture all PDMS substrates were put under UV for 10 min. All plates (TCPS and PDMS) were first coated with poly-l-ornithine (15 μg/ml) for 1 h, and then rinsed with PBS three times. Next, the plates were coated with fibronectin (1 μg/ml) for 1 h and then rinsed once in PBS.

2.3. Characterisation of PDMS substrates

The elasticity (Young modulus, E) was determined by placing a sphere with a known mass and radius on top of the PDMS substrate (1:10, 1:50 and 1:70) and measuring the resulting indentation. The indentation of a hard sphere on a flat surface with elastic properties is described by Hertz model:

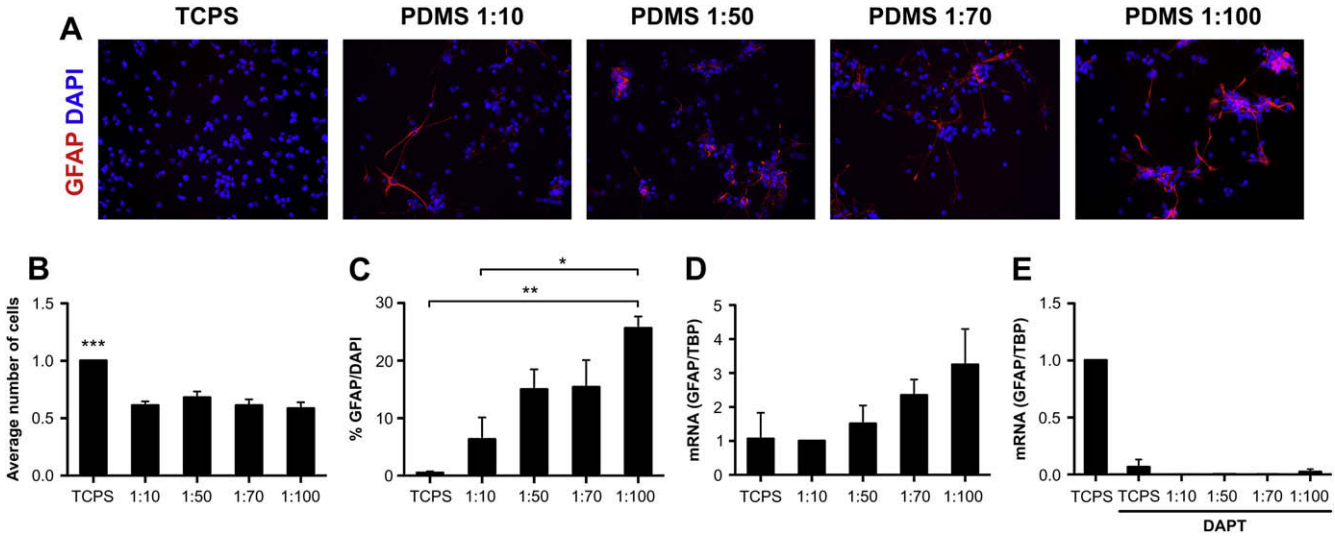

Fig. 2. Soft substrates promote astrocytic differentiation but cannot override Notch requirement for astrocytic differentiation. (A) NSCs were grown on TCPS or PDMS with different elasticities for 7 days in N2 conditions. On TCPS substrates, few cells displayed GFAP immunoreactivity, a marker for astrocytic fate. On the PDMS substrates there was an elasticity dependent increase in the numbers of GFAP-positive cells. (B) The total number of cells after culture for 7 days in N2 medium without added growth factors. All DAPI positive nuclei were counted in five random 20× images in three independent experiments. The higher average number of cells on TCPS compared to all PDMS substrates was statistically significant. No differences in average cell numbers were observed amongst the different PDMS substrates. (C) Quantification of the percentages of GFAP-positive cells in (A). Five 20× images were acquired in five random fields per plate in three independent experiments. Stars indicate statistical significance. (D) Real-time PCR showing mRNA levels of GFAP expression relative to tata binding protein (TBP) of NSCs grown on TCPS and PDMS substrates in N2 conditions. Three independent experiments were conducted. (E) Real-time PCR showing mRNA levels of GFAP expression relative to TBP of NSCs grown on TCPS treated with or without the γ-secretase inhibitor DAPT and PDMS substrates treated with DAPT in N2 conditions.

$$E = \frac{3(1-\nu^2)F}{4h^{3/2}\sqrt{r}},$$

where F is the force acting on the sphere (equal to the mass of the sphere multiplied by the gravitation acceleration, 9.82 m s^{-2}), r is the radius of the sphere and h is the indentation of the sphere into the substrate. ν is the Poisson's number which for PDMS is ~0.5 [12]. E was calculated as an average from multiple measurements on three substrates with two different spheres for each type of PDMS (>20 measurements on each mixing ratio). The accuracy of the indentation measurements was determined to ±5 μm, mass ±5 mg and the radius to ±1 μm. The PDMS substrate with dilution of 1:100 behaves as a non-Newtonian fluid with elasticity close to zero. The mass of PDMS displaced by the sphere was found to be equal to the mass of the sphere (assuming a density of the PDMS to 0.97 g/cm^{-3}), hence there was no or very small elastic contribution to the upward force keeping the sphere afloat.

2.4. Cell culture and immunocytochemistry

NSCs were dissociated and isolated from the cerebral cortices of E15.5 embryos of timed pregnant Sprague-Dawley rats [13]. Animals were treated in accordance with institutional and national guidelines (Ethical permit no. N310/05). NSC cultures were expanded until 80% confluent. Cells were used at passage one in all experiments. For immunocytochemistry, the plates were first rinsed once in PBS and then fixed in 10% formaldehyde for 20 min. The formaldehyde was aspirated and the plates were washed three times, 5 min each, in PBS/0.1% Triton-X 100. The slides were then incubated with respective primary antibodies in PBS/0.1% Triton-X 100/1% BSA overnight at 4 °C. The samples were then washed six times, 5 min each, in PBS/0.1% Triton-X 100. Secondary antibodies (1:500) in PBS/0.1% Triton-X 100/1% BSA were incubated with the samples at room temperature for 1 h. The samples were then washed three times in PBS and mounted with Vectashield containing DAPI. Fluorescent images were acquired using a Zeiss Axioskop2 coupled to an MRm (Zeiss) camera at 10×, 20×, and 40× magnifications with Axiovision software. Time-lapse imaging was performed on an Axiovert 200M microscope (Carl Zeiss Microimaging, Inc.) with OpenLab 3.1.7 software (Improvision Ltd.) as previously described [14].

2.5. Real-time PCR

Total RNA was extracted from cells using RNeasy (Qiagen) and contaminating DNA digested using RNase free DNase kit (Qiagen). cDNA was synthesized using 200 ng of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 1:25 dilution of the cDNA was used for real-time PCR. Platinum SYBR Green qPCR Supermix UDG (Invitrogen) was used for real-time PCR analysis with the 7500 PCR system (Applied Biosystems). Primers are available on request.

2.6. Statistical analysis

Statistical analysis and graphs were performed using the software Prism 4 (Graph Pad). 1-Way ANOVA analysis of variance with Bonferroni's multiple comparison test was used. The threshold value for statistical significance was set at 0.05. In all graphs, results are expressed as mean ± S.E.M.

3. Results

3.1. Maintenance of neural stem cells on PDMS substrates with different stiffnesses

Embryonic neural stem cells (NSCs) are commonly grown on hydrophilic polystyrene substrates (tissue culture polystyrene, TCPS) coated with poly-L-ornithine and fibronectin. NSCs grown on TCPS in culture medium supplemented with fibroblast growth factor 2 (FGF2) form uniform cultures of nestin positive cells (more than 95% nestin positive cells), indicating neural stem cell identity (Fig. 1) [15,16]. Polydimethylsiloxane (PDMS) is an elastomeric polymer widely used in micropatterning and microfluidics. It is particularly well suited for biological applications due to its biocompatibility and transparency. NSCs were able to grow on PDMS substrates and maintained the stem cell state in FGF2 supplemented medium, as assessed by nestin staining (Fig. 1A). NSCs grown on PDMS also kept the ability to differentiate into neural subtypes (neurons, astrocytes and oligodendrocytes) and smooth muscle cells in response to soluble factors, according to established protocols (Fig. 1A and Movies S1–S3) [15,17,18].

In order to investigate the effects of substrate stiffness on NSC state and fate, we prepared PDMS substrates with different stiffnesses. The stiffest PDMS substrate used in this study was prepared with a ratio of crosslinker to base of 1:10, the standard dilution recommended by the manufacturer. Increasing the ratio of crosslinking agent caused the stiffness of PDMS to decrease. We prepared PDMS substrates with ratios of crosslinking agent to base of 1:10, 1:50, 1:70 and 1:100. Young's Modulus of PDMS with ratios 1:10, 1:50 and 1:70 was 750 ± 170 kPa, 38 ± 15 kPa and 12 ± 4 kPa, respectively. The softest substrates (with ratio of 1:100) had a Young's Modulus close to zero. The stiffness of the adult brain parenchyma has been reported to be in the order of hundreds of Pa [19]. Therefore, the reported brain elasticities lie within the range defined by the samples with ratios of 1:70 and 1:100. NSCs grown on all PDMS substrates, in FGF2 supplemented medium, proliferated

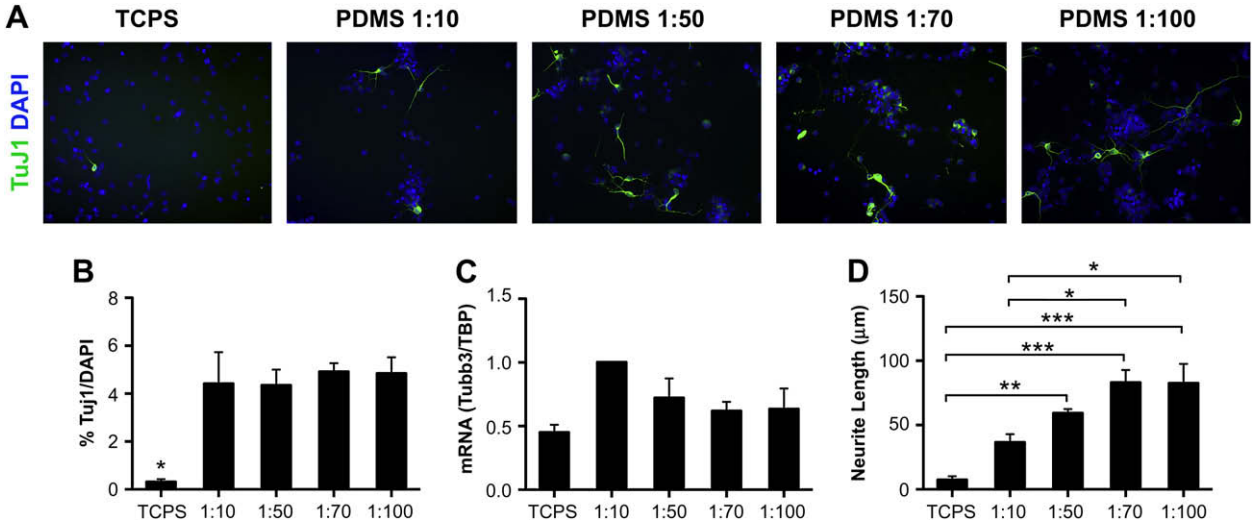

Fig. 3. NSC derived neurons show a more mature morphology on soft substrates. (A) NSCs grown on TCPS or PDMS with different elasticities were stained for the neuronal marker TuJ1 after 7 days. NSCs grown on TCPS substrates in N2 conditions showed few TuJ1-positive cells. All PDMS substrates showed higher numbers of TuJ1-positive cells than the TCPS controls but the numbers of TuJ1-positive cells between the different PDMS substrates were similar. (B) Quantification of the percentages of TuJ1-positive cells in (A). Five 20× images were acquired in five random fields per plate in three independent experiments. The percentage of TuJ1-positive cells was statistically significantly higher on all PDMS substrates than on TCPS. (C) Real-time PCR showing mRNA levels of Tubb3 expression relative to TBP of NSCs grown on TCPS and PDMS substrates in N2 conditions. Three independent experiments were conducted. (D) The longest neurite of all neurite-bearing cells in five random 20× images in four independent experiments was measured using ImageJ. Stars indicate statistical significance.

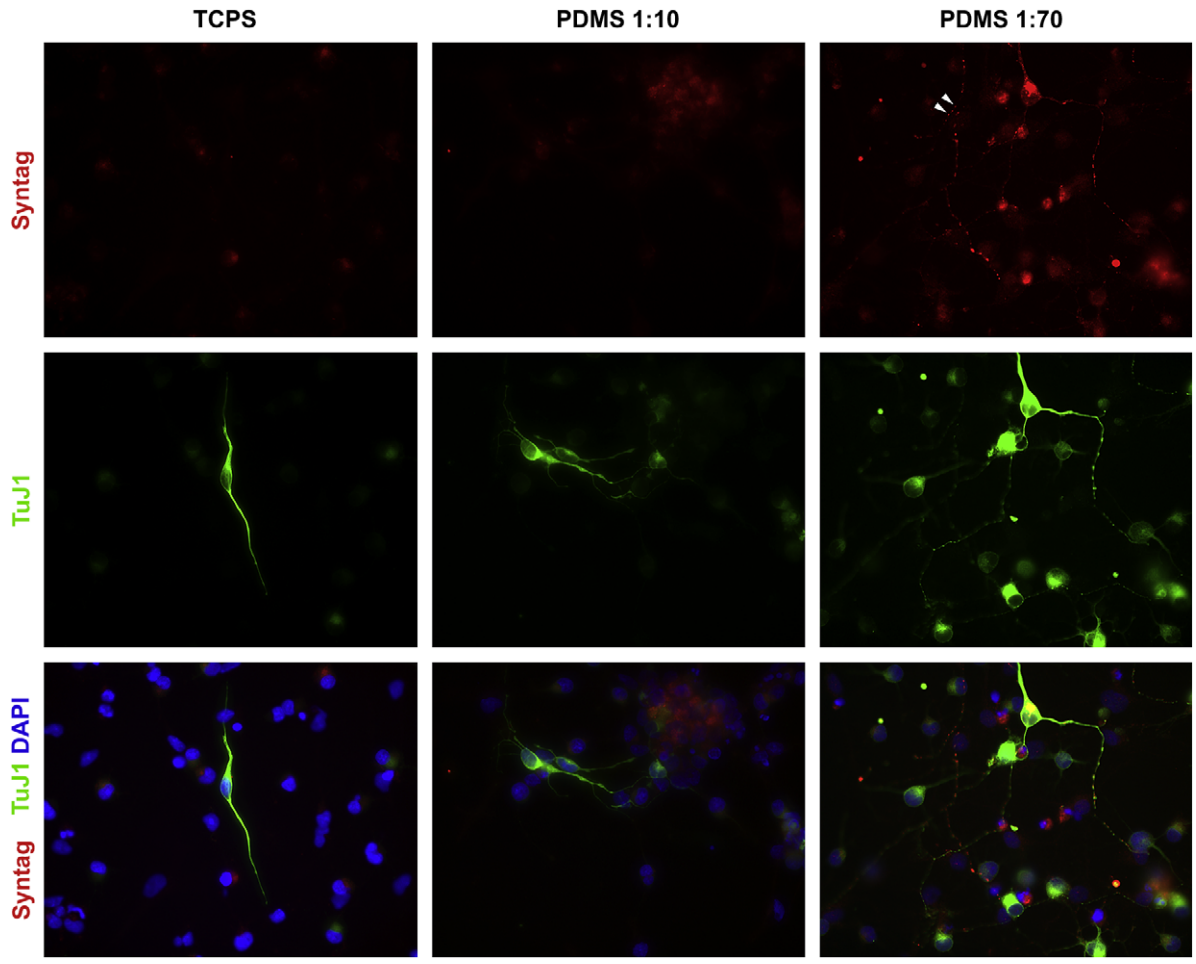

Fig. 4. NSC derived neurons showed assembly of presynaptic terminals only on soft substrates. Representative 40× images of TuJ1 immunoreactive cells cultured on TCPS substrates and PDMS substrates with ratios of crosslinking agent to base of 1:10 and 1:70. TuJ1-positive cells showed mostly a bipolar immature morphology on TCPS and the stiffest PDMS substrates (PDMS 1:10). On soft PDMS substrates (PDMS 1:70), neurons that showed mature morphology with long neurites were often also immunoreactive for the presynaptic marker synaptotagmin. Arrowheads point to examples of synaptotagmin positive presynaptic terminals.

and expressed nestin at levels comparable to cells grown on TCPS (Fig. 1B). Together, these data validate PDMS as a substrate for NSC maintenance and directed differentiation.

3.2. Dependence of glial differentiation on PDMS substrate stiffness

After FGF2 withdrawal, NSCs differentiate into a mixed population of neurons, astrocytes and oligodendrocytes [15]. Total cell numbers after 7 days of culture on PDMS without added growth factors were independent of substrate stiffness. Cell numbers on all PDMS substrates were however lower than on control TCPS surfaces (Fig. 2B).

A remarkable increase in astrocytic differentiation was observed with decreasing PDMS substrate stiffness (Fig. 2A and C). After 7 days without added growth factors, the softest PDMS substrates supported approximately three times more astrocytes, immunoreactive to glial fibrillary acidic protein (GFAP), than the stiffest PDMS substrates. Consistently, mRNA expression of GFAP increased monotonically with decreasing substrate stiffness (Fig. 2D). Furthermore, the extent of astrocytic differentiation, obtained by counting the percentages of GFAP-positive cells, was higher on all PDMS substrates than on TCPS.

Functional Notch/CSL signaling has been shown to be required for astrocytic differentiation of NSCs [18,20]. Accordingly, inhibition of Notch signaling by the γ-secretase inhibitor DAPT abolished astrocytic differentiation of NSCs grown on control TCPS surfaces (Fig. 2E). DAPT also prevented astrocytic differentiation on PDMS substrates, irrespective of substrate stiffness (Fig. 2E). Therefore, the astrogliogenic effect of decreasing substrate stiffness was not able to override the requirement for Notch signaling in astrocytic differentiation. These results show that substrate stiffness by itself can direct NSC fate decisions but the dependency of astrocytic differentiation on Notch signaling is maintained.

NSC cultures treated with thyroid hormone (triiodothyronine, T3) become enriched in oligodendrocytes. Substrate stiffness did not affect oligodendrocyte cell numbers after 7 days in culture without added growth factors or with T3 treatment (Fig. S1). However, oligodendrocytic differentiation on all PDMS substrates was lower than on TCPS substrates, measured by counting percentage of myelin basic protein (MBP) positive cells as well as MBP mRNA content. Therefore, substrate chemistry (TCPS vs PDMS) showed different effects on oligodendrocytic and astrocytic differentiation.

3.3. Effects of substrate stiffness on neurite length of neural stem cell-derived neurons

The percentage of neurons (identified by labeling for neuron-specific class III β-tubulin, TuJ1) was higher on all PDMS substrates

than on TCPS, suggesting that the PDMS material *per se* promotes neuronal differentiation (Fig. 3A and B). The percentage of neurons on PDMS substrates was independent of substrate stiffness. Accordingly, Tubb3 mRNA levels were constant on the different PDMS substrates (Fig. 3C).

In order to investigate neuronal maturity, we measured the length of the longest neurite present in TuJ1-positive cells. A strong correlation was observed between decreasing substrate stiffness and increasing neurite length (Fig. 3D). Additionally, neurite length was significantly higher on all PDMS substrates than on the TCPS.

Together these results indicate that soft substrates, with brain-like stiffness, promote neuronal maturation.

3.4. Influence of PDMS substrate stiffness on the assembly of synaptotagmin positive presynapses

Synaptotagmin is a presynaptic protein widely used as a synaptic marker in primary neuronal culture. Synaptotagmin assembly into presynapses is characterized by presence of punctate synaptotagmin labeling along neurites as well as immunoreactivity in the soma. Presynaptic synaptotagmin was absent from neurons differentiated on TCPS as well as on stiff PDMS substrates that showed an immature morphology with short neurites. Synaptotagmin assembly into presynapses was observed primarily on neurons bearing long neurites present on soft PDMS substrates (Fig. 4).

These results show that under these experimental conditions, NSCs differentiate into mature neuronal populations only when cultured on soft substrates.

4. Discussion

Evidence is accumulating for the benefits of matching the substrate mechanical properties used for stem cell differentiation *in vitro* to the mechanical properties of the target tissue *in vivo*. Adult neural [21], cardiac muscle [22], and mesenchymal [10] stem cell differentiation was improved by using substrate elasticities similar to those of the native tissues. Here we show that differentiation of NSC on substrates with stiffness similar to the stiffness of the brain yields a clear enhancement in neuronal maturity compared to standard culture on rigid substrates. The length of the longest neurite, presumably the axon, was three times larger for cells differentiated on soft substrates than on stiff surfaces. The improved ability for neurons to extend processes on soft substrates was not due to a general mechanism whereby soft substrates facilitate spreading. Indeed, oligodendrocyte spreading followed the opposite trend with stiffer substrates supporting oligodendrocytic areas 2–3 times larger than soft substrates (Fig. S1). This observation parallels the opposite responses to surface stiffness reported for hippocampal neurons and fibroblasts [9].

NSCs grown on soft substrates differentiated into astrocytes to a larger extent than on stiff substrates, whereas oligodendrocytic and neuronal differentiation was quantitatively independent of substrates stiffness. Adult NSCs were previously shown to form more neurons on soft substrates than on stiff substrates but less astrocytes [21], suggesting that developmental stage affects stem cell responsiveness to substrate stiffness.

As mentioned above, the improved neuronal maturity on soft substrates was associated with an increase in astrocytic differentiation. Indeed, astrocytes have been proposed to regulate formation and maturation of synapses [23]. It is possible that the larger number of astrocytes on soft substrates creates an environment conducive to neuronal maturation. Together, these results show that stem cells integrate mechanical cues with intrinsic programs during differentiation.

5. Conclusions

Here we have shown that soft substrates promote the maturation of neural stem cell-derived neurons. In the absence of added growth factors, neurons differentiated on soft substrates displayed long neurites and synaptotagmin positive presynaptic terminals. In contrast, on stiff substrates neurons showed a bipolar immature morphology. Further, soft substrates supported an increase in astrocytic differentiation compared to stiff substrates. However, stiffness cues could not override the dependency of astrocytic differentiation on Notch signaling. These results demonstrated that substrate elasticity is crucial parameter in neuronal differentiation of stem cells.

Acknowledgments

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biomaterials.2009.05.013.

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