Elastic modulus affects the growth and differentiation of neural stem cells

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Abstract

It remains poorly understood if carrier hardness, elastic modulus, and contact area affect neural stem cell growth and differentiation. Tensile tests show that the elastic moduli of Tiansu and SMI silicone membranes are lower than that of an ordinary dish, while the elastic modulus of SMI silicone membrane is lower than that of Tiansu silicone membrane. Neural stem cells from the cerebral cortex of embryonic day 16 Sprague-Dawley rats were seeded onto ordinary dishes as well as Tiansu silicone membrane and SMI silicone membrane. Light microscopy showed that neural stem cells on all three carriers show improved adherence. After 7 days of differentiation, neuron specific enolase, glial fibrillary acidic protein, and myelin basic protein expression was detected by immunofluorescence. Moreover, flow cytometry revealed a higher rate of neural stem cell differentiation into astrocytes on Tiansu and SMI silicone membranes than on the ordinary dish, which was also higher on the SMI than the Tiansu silicone membrane. These findings confirm that all three cell carrier types have good biocompatibility, while SMI and Tiansu silicone membranes exhibit good mechanical homogenization. Thus, elastic modulus affects neural stem cell differentiation into various nerve cells. Within a certain range, a smaller elastic modulus results in a more obvious trend of cell differentiation into astrocytes.

Key Words: *nerve regeneration; neural stem cells; carrier; mechanical properties; elastic modulus; cell differentiation; neurons; immunofluorescence; astrocytes; neural regeneration*

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Introduction

Neural stem cells (NSCs) can differentiate into neurons and glia cells, and NSC transplantation promotes recovery of neurological function following traumatic brain injury and spinal cord injury (Ma et al., 2004; Caprini et al., 2013). A number of extracellular factors involving the biological and physical environment impact upon proliferation, differentiation, maturity, and turnover of NSCs (McBeath et al., 2004; Wrage et al., 2008; Evans et al., 2009). Biochemical approaches combined with growth factors have limitations in inducing directional differentiation of stem cells *e.g.*, differentiated cells do not have all the characteristics of mature neurons, a low survival rate *in vivo*, and uncertainty in the type of differentiated cells (Cattaneo and McKay, 1990; Wrage et al., 2008; Qian et al., 2010). The physical microenvironment is strongly associated with proliferation and differentiation of NSCs (Engler et al., 2006; Guilak et al., 2009; Dado et al., 2012). Substrate height can be altered using

three-dimensional nanofiber meshes and induce neuronal differentiation of NSCs (Kshitiz et al., 2012). Elastic modulus of the extracellular matrix (ranging from 1–100 Pa) noticeably contributes to expression of tubulin III and 18S ribosomal RNA in NSCs. Moreover, the differentiation rate is decreased with an increase in elastic modulus. Previous studies have shown that migration, proliferation and differentiation of NSCs is suppressed with a substrate strength of 10 Pa. In addition, with substrate strengths of 100–500 Pa, NSCs differentiate into neurons, while at 1,000–10,000 Pa, they differentiate into glial cells (Engler et al., 2006; Guilak et al., 2009; Dado et al., 2012). The elastic modulus is an important parameter of engineering materials, and specifically, a number that measures an object or substance's resistance to being deformed elastically, which is slightly affected by the external environment. The elastic modulus can affect cell proliferation and the direction of differentiation (Banerjee et al., 2009), but current studies have only investigated the

effect of high elastic modulus on cells, with controversial results (Engler et al., 2006; Guilak et al., 2009; Dado et al., 2012). Indeed, there are no detailed studies regarding the effect of low elastic modulus on growth and differentiation of NSCs. Therefore, in this study, we sought to compare the effect of different materials with low elastic modulus on the growth and differentiation of NSCs, and to further improve directional differentiation of NSCs, investigated the effect and subsequent mechanisms of physical factors on proliferation and differentiation of NSCs.

Materials and Methods
Determination of mechanical properties
To measure the mechanical properties of Tiansu silicone membrane (Tianjin Plastics Research Institute, Tianjin, China) and SMI silicone membrane (SMI, Denver, CO, USA), tensile tests were performed using a mechanical tester (INSTRON 5865; Instron, Boston, MA, USA) (**Figure 1**). Silicone membrane thickness was measured. The tensile parameters were: length of stretching, 50 mm; stretching rate, 10 mm/s; preload, 0.1 N; and maximum compressive strain, 2,000%; for a total of 3 cycles. Stress-strain curves were generated, and elastic moduli of both silicone membranes were obtained. Before experiments, both silicone membranes received Cobalt-60 irradiation for sterilization, and were then cut into circles of 5 cm diameters and fixed in autoclaved culture chambers with black aprons to prevent air leakage.

NSC culture
Embryonic day 16 (E16) Sprague-Dawley rats were provided by the Experimental Animal Center of Academy of Military Medical Sciences in China (license No. SCXK (Army) 2012-0004). Experiments were approved by the Ethics Committee of Affiliated Hospital of Logistics University of People's Armed Police Force in China. Rats were immersed and sacrificed in 75% ethanol. Fetal rat cerebral cortex was cut into approximately 1 mm^3 blocks, triturated, filtered with a 200-mesh sieve, and centrifuged at $100 \times g$ for 5 minutes. After removal of the supernatant, samples were precipitated and digested with 0.25% trypsin. Digestion was terminated by addition of 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). Following centrifugation and supernatant removal, cells were resuspended in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco), single cell suspensions were obtained and seeded in 25 cm^2 culture dishes, and then incubated at 37°C. Once every 3 days, half the medium was replaced. Primary cultures were maintained for approximately 6 days. After supernatant removal, cultured NSCs were collected, centrifuged in sterile centrifuge tubes, digested with 0.25% trypsin, and terminated with 10% FBS. Following centrifugation and supernatant removal, fresh medium was added for agitation, resuspension, and quantitation. All samples were then incubated in 25 cm^2 culture flasks, with half the medium replaced once every 3 days. Cells were subcultured approximately once every 6 days. Third passage NSCs (1 ×

10^5 cells/mL) were incubated on different carriers: ordinary dish (Corning, Shanghai, China) (control group), Tiansu silicone membrane (Tiansu silicone membrane group), and SMI silicone membrane (SMI silicone membrane group). Twenty-four hours later, DMEM/F12 medium containing 10% FBS was replaced by serum-free DMEM/F12 medium with B27, recombinant human epidermal growth factor, and recombinant human basic fibroblast growth factor (Gibco).

Biocompatibility of silicone membranes
NSCs received mechanical separation and enzymatic digestion, and single cell suspensions were prepared. Three days later, cell morphology was observed. Cells were subcultured approximately every 6 days. Third passage NSCs were incubated in DMEM/F12 medium supplemented with 10% FBS, which promotes adherence and differentiation of NSCs. At 24 hours and 7 days after culture, alterations in cell morphology were observed using an inverted phase contrast microscope (Olympus, Tokyo, Japan).

Immunofluorescence staining
Immunofluorescence staining for neuron specific enolase (NSE), glial fibrillary acidic protein (GFAP), and myelin basic protein (MBP) was performed after 7 days of adherent growth. Cells from each group were fixed, permeabilized, blocked with bovine serum albumin, and incubated with rabbit anti-rat NSE monoclonal antibody (1:150; Pharmingen, San Diego, CA, USA), rabbit anti-rat GFAP monoclonal antibody (1:1,000; Pharmingen), and rabbit anti-rat MBP monoclonal antibody (1:100; Abcam, Cambridgeshire, UK) at 4°C overnight, and then incubated with green fluorescent protein-labeled goat anti-rabbit secondary antibody (1:200; Sigma, San Francisco, CA, USA) at 37°C for one hour. 4′,6-Diamidino-2-phenylindole (DAPI) dye was used for nuclear staining. Coverslips were mounted using antifade fluorescence mounting medium. Specimens were observed using an inverted fluorescence microscope (Leica, Solms, Germany). Images were processed using Image-Pro Plus 7.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Flow cytometry
Cells from each group were induced for 48 hours. Cells at a density of 3×10^6 cells/cm^2 were seeded onto 6-well plates, digested, centrifuged, and fixed. After membrane disruption, cells were placed in tubes, and incubated with goat anti-rat GFAP antibody (1:500; Sigma) at 4°C overnight. The rate of NSC differentiation into astrocytes was determined by flow cytometery (BD, Frankfort, NJ, USA).

Statistical analysis
Data were processed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Measurement data are expressed as the mean ± SD. Intergroup comparison was performed by one-way analysis of variance and independent-samples *t*-test. *P* values < 0.05 were considered statistically significant.

Results

Mechanical properties of Tiansu and SMI silicone membranes

The ordinary dish was made of polystyrene, with an elastic modulus ranging from 3,000–3,600 MPa (Wang et al., 2007). The elastic moduli of Tiansu silicone membrane and SMI silicone membrane were 4.255 ± 0.344 and 2.256 ± 0.096 MPa, respectively. Stress-strain curves showed that Tiansu and SMI silicone membranes behaved according to Hooke's law (*i.e.*, a linear relationship between stress and strain). Moreover, elastic modulus variability was smaller in the SMI silicone membrane group than in the Tiansu silicone membrane group. SMI silicone membrane exhibited good stability (**Figure 2**).

Biocompatibility of Tiansu and SMI silicone membranes

After 3 days of culture, inverted phase contrast microscopy showed that in the control group, NSCs proliferated and formed neurospheres containing tens to hundreds of cells. Twenty-four hours after adherent differentiation, NSCs proliferated and formed neurospheres. Gradually, cells extended from the edge of neurospheres and formed dendritic processes. Neurospheres connected with each other through processes. Compared with the control group, many regular processes were visible between neurospheres in both the Tiansu and SMI silicone membrane groups. After 7 days of adherent growth, NSCs differentiated into nerve cells with different shapes (**Figure 3**).

Effect of Tiansu and SMI silicone membranes on NSC differentiation

NSE, GFAP, and MBP are specific immune markers for neurons, astrocytes, and oligodendrocytes, respectively (Sommer and Schachner, 1981; Noetzel and Agrawal, 1985; Sterk et al., 1999). After 7 days of culture, immunofluorescence expression of NSE, GFAP, and MBP was detected in cells from each group, thereby demonstrating NSC differentiaton (**Figure 4**).

Flow cytometry showed a significantly higher rate of NSC differentiation into astrocytes after 7 days of adherent culture in the Tiansu and SMI silicone membrane groups compared with the control group ($P < 0.05$, $P < 0.01$). Moreover, the rate of cell differentiation was greater in the SMI silicone membrane group than in the Tiansu silicone membrane group ($P < 0.01$; **Figure 5**).

Discussion

In neural tissue engineering, adhesion, migration, proliferation, and differentiation of stem cells are affected by physical and chemical factors and carrier surface properties (Chen et al., 2012; Lu et al., 2012). As cell carriers, tissue-engineered scaffolds provide a three-dimensional environment for nerve cells and affect cell turnover (Chen et al., 2013). The effects of elastic modulus, as inherent physical scaffold properties, have been gradually attracting the attention of scientists.

In this study, NSCs from the cerebral cortex of fetal Sprague-Dawley rats were incubated on carriers with different elastic moduli, and found to adhere to different carriers.

Twenty-four hours after adherence, NSCs proliferated and formed neurospheres. Cells then gradually extended from the edge of neurospheres and formed dendritic processes. After 7 days of adherent growth, immunofluorescence detected specific immune markers for neurons, astrocytes, and oligodendrocytes. Morevoer, flow cytometry found that the majority of NSCs differentiated into astrocytes. These findings show that NSCs can grow and differentiate on three different carriers with different elastic moduli. Thus, the cultured NSCs are pluripotent, with both types of silicone membrane exhibiting good biocompatibility.

Carrier micromorphology has a guiding role on cell growth and impacts upon biological properties of the cells (Noetzel and Agrawal, 1985). The present results showed that the elastic modulus of SMI silicone membrane was smaller and more stable than that of Tiansu silicone membrane, which indicates that SMI silicone membrane has good mechanical deformation characteristics with a uniform nature. After 7 days of adherent differentiation, the flow cytometry results suggest that elastic modulus affects NSC differentiation into astrocytes. Within a certain range, a smaller elastic modulus results in a more obvious trend of cell differentiation into astrocytes.

The extracellular matrix-integrin-cytoskeleton-nuclear matrix system is an important mechanical signaling pathway influencing cell turnover, while cell flexibility determines the sensitivity of cells to force (Wang et al., 2010). Different cells have different flexibilities. Within a certain range, during cell adherence, cells on carriers with small elastic modulus are easily deformed and a great tension force produced. Astrocyte flexibility may make them relatively sensitive to tension force (within a certain range), which indirectly indicates that cells on silicone membrane with a small elastic modulus are deformed, and a large local tension force generated. Thus, the direction of NSC differentiation into astrocytes can be affected, but these findings still be confirmed by a larger number of studies.

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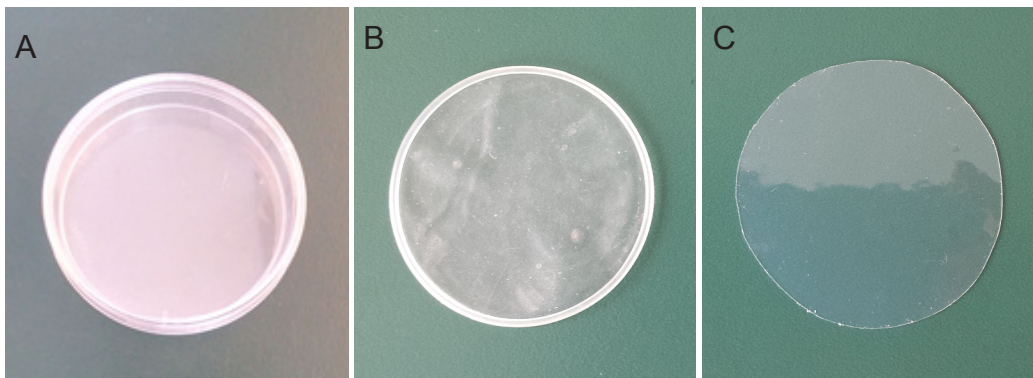
Figure 1 Carriers with different elastic moduli. (A) Ordinary dish. (B) Tiansu silicone membrane. (C) SMI silicone membrane.
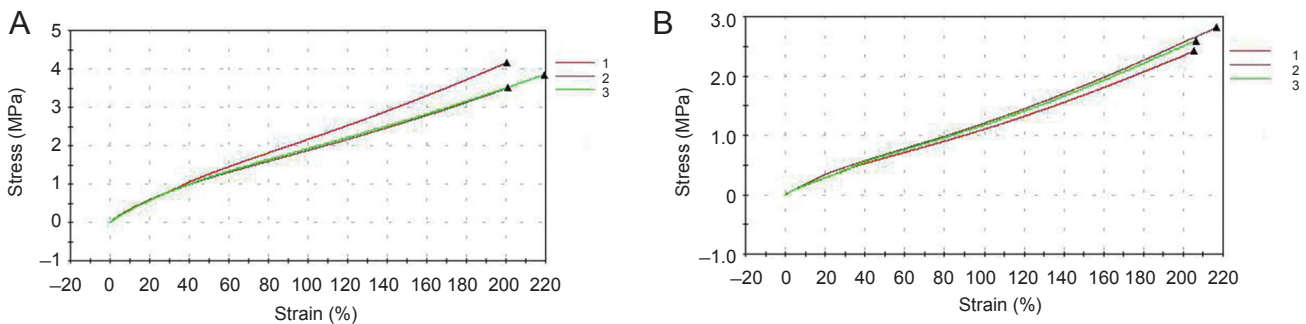

Figure 2 Stress-strain curves of Tiansu silicone membrane (A) and SMI silicone membrane (B).
1, 2, 3: Results of cycles 1, 2, and 3, respectively, at a velocity of 10 mm/min. A triangle represents the end of tension, and mainly contains data before rupture.
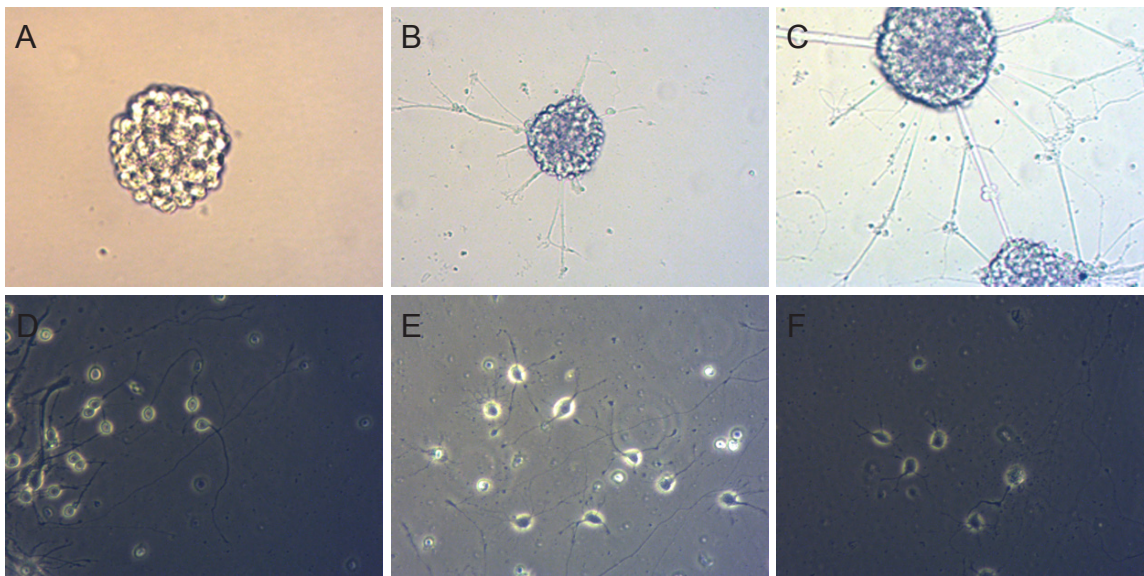

Figure 3 Morphology and differentiation of NSCs.
(A) NSCs at 3 days after culture (× 100). (B) NSCs at 24 hours after adherent differentiation (× 100). (C) Connection of processes at 4 days after culture (× 100). (D–F) After 7 days of adherent growth, NSCs differentiated into neuron-, astrocyte-, and oligodendrocyte-like cells (× 250). NSCs: Neural stem cells.

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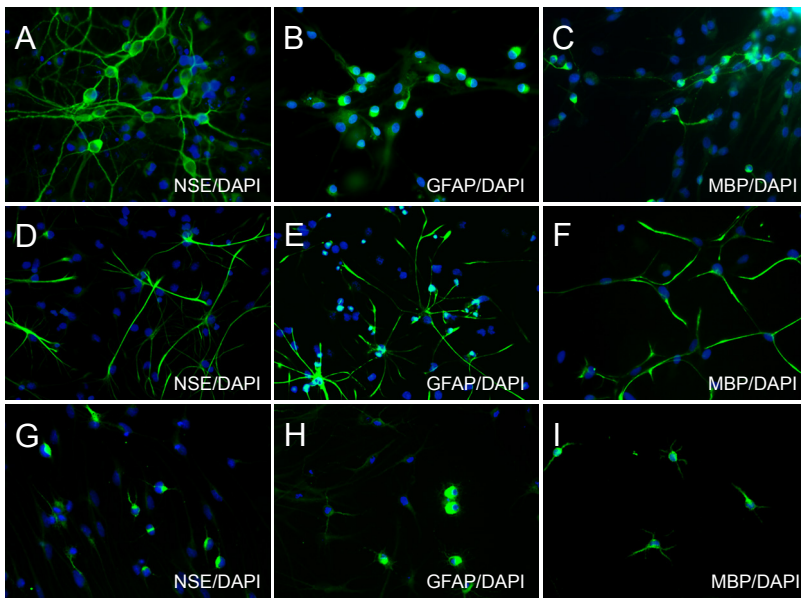
Figure 4 Effect of Tiansu and SMI silicone membranes on NSC differentiation (immunofluorescences staining, × 400).
(A, D, G) Control group. (B, E, H) Tiansu silicone membrane. (C, F, I) SMI silicone membrane. NSE+ cells had small bodies and long processes, GFAP+ cells had many long processes, while MBP+ cells were smaller in number, with small bodies, and short processes. In the control group, mature neurons and a large number of oligodendrocytes were visible. In the SMI silicone membrane group, there was a low rate of neuronal differentiation, with immature neurons, mature glial cells, and a small number of oligodendrocytes detected. The morphology in the Tiansu silicone membrane group was a combination of the control and SMI silicone membrane groups. NSC: Neural stem cell; NSE: neuron specific enolase; GFAP: glial fibrillary acidic protein; MBP: myelin basic protein; DAPI: 4′,6-diamidino-2-phenylindole.
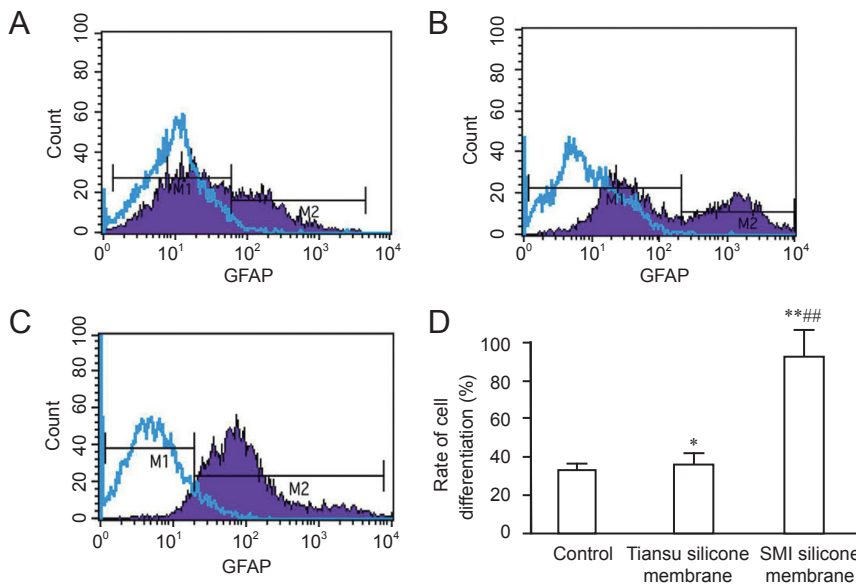

Figure 5 Effect of Tiansu and SMI silicone membranes on NSC differentiation into astrocytes (flow cytometry).
(A–C) GFAP expression in the control, Tiansu silicone membrane, and SMI silicone membrane groups, as detected by flow cytometry. (D) The rate of NSC differentiation into astrocytes. Data are expressed as the mean ± SD. *$P < 0.05$, **$P < 0.01$, *vs.* control group; ##$P < 0.01$, *vs.* Tiansu silicone membrane group (one-way analysis of variance and independent-samples *t*-test.). GFAP: Glial fibrillary acidic protein; NSC: Neural stem cell.

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