Static pressure-induced neural differentiation of mesenchymal stem cells

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Growing experimental evidence suggests that physical cues play an important role in regulating the fate of stem cells and stimulating their differentiation behavior. We report here that static pressure enables the differentiation of rat bone marrow-derived mesenchymal stem cells (MSCs) into neural-like cells within several hours in the absence of disruptive bio-factors or chemicals. The realization of such differentiation is supported by the observation of characteristic morphology of neural-like cells with neurites, and an up-regulated expression level of neural-specific markers. Our finding also demonstrates the utility of the static pressure-based approach for in situ and specifically localized creation of neural cell systems, thereby providing profound implications for developing therapeutic application of stem cells.

Introduction

Adult or tissue-specific stem cells, responsible for normal regenerative processes, are believed to be able to differentiate into some or all of the major specialized cell types of tissues and organs. As a result, the therapeutic potential of adult stem cells in regenerative medicine and tissue engineering has been the focus of much scientific debate.† Bone marrow-derived mesenchymal stem cells (MSCs) can self-renew and develop into multiple cell types both in vitro and in vivo, including neurons,3–5 myoblasts,6 osteoblasts,2 chondrocytes,2 adipocytes2 and other lineage cells. Thus the investigation of MSCs in response to biological, chemical and physical stimuli is crucial in understanding the mechanisms underlying the lineage fate and governing the cell modes of development.

It has been well established that the differentiation of stem cells can be modulated by introducing biochemical supplements such as cytokines, growth factors, and small-molecule compounds.2,3 Apart from chemical stimuli, control over physical characteristics of the materials have also proved effective for regulating the process of cell differentiation in vitro.7–10 For example, nanotexture,11,12 nanotopography,13–15 stiffness16–19 and surface charge20,21 of the biomaterials have been found to play a significant role in dictating the fate of stem cells. Notably, considerable evidence shows that the differentiation of the stem cells can respond to external physical mechanical forces, such as local cyclic stress22 and fluid-flow-induced shear stress.23 To regulate and determine MSC fate, most researchers focus on differentiation into mesoderm lineages especially osteoblasts and muscle. Surprisingly, few studies have contemplated how neurogenic spasm may affect cell differentiation.16 As rehabilitating nerve regeneration is beneficial for patients with neurodegenerative disease,24,25 a controllable regulation method that can lead to efficient neuron-directed differentiation is thus desirable.

Herein, we demonstrate that the regulation of MSC differentiation can be achieved using a fused silica glass slide. We found that the glass slide, when placed onto the MSCs, can provide a static pressure under its own weight. More importantly, under such a static pressure the MSCs are able to differentiate into neural-like cells within only several hours, which is almost inaccessible by conventional biochemical3,26,27 and electrical simulation methods.28 Because neurons are the main components of the central nervous system,29,30 this finding will not only improve our understanding of the biological process of neurogenesis, but also provide the possibility to direct neural tissue repair and regeneration.

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Materials and methods

Cell culture
MSCs derived from bone marrow, isolated from the femurs and tibias of four week-old Wistar rats, were gifts from Shandong University. Cells were cultured in low glucose (1.0 g L⁻¹) Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin (Gibco) and 10 ng ml⁻¹ FGF2 (Peprotech).

Flow cytometry analysis of the isolated MSCs
About 5 × 10⁵ MSCs at the third passage were collected and incubated with 0.5 μg of fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD45, CD54, CD90 and mouse IgG1 (BD) for 1 h at 4 °C. After a wash, the samples were analyzed using a BD FACSCalibur flow cytometer.

Static pressure-induced MSC differentiation
MSCs were seeded on the FSG wafers (10 × 10 × 0.3–3 mm) at a density of 20 000 cm⁻² and cultured overnight for the adhesion process. The next day, FSG wafers were taken out, the wafers were turned over, and placed in a new tissue culture plate to realize applying static pressure to MSCs.

Live cell imaging
A commercial product was used to label MSCs with actin-RFP (CellLight Actin-RFP, Life Technology), according to the manufacturer’s instructions. A Leica Confocal Microscope SP8 was used to observe and record the actin-RFP behavior in live cells. Movies (Movies S10–121) were recorded under Laser Confocal Scanning Microscopy (Leica SP8) and the time interval during recording is 5 min between each picture.

qPCR
The total RNA was extracted from the cells using an RNeasy Plus Mini Kit (Qiagen), according to the manufacturer’s instructions. The RNA samples were reverse-transcribed into cDNA for qPCR, using a PrimeScript™ reagent Kit with a gDNA Eraser (Takara). qPCR was performed with SYBR Premix Ex Taq™ with ROX (Takara), according to the manufacturer’s instructions. The signals were detected using an ABI 7500 Fast Real Time PCR system (Applied Biosystems) for analyzing the expression of nestin, Tuj1, NSE and MAP2. The gene expression was normalized to beta-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal standard. The information of primers is provided in ESI Table S2.†

Immunofluorescence
After applying static pressure to MSCs for 24 hours, immunostaining was performed to detect the expression of neural-specific markers. The fixed and permeable cells were incubated with the primary antibody of Alexa Fluor 488 conjugated nestin (1 : 100, Millipore), Alexa Fluor 488 conjugated MAP2 (1 : 500, Millipore), NSE (1 : 200, Abcam) and Tuj1 (1 : 1000, Abcam). The second antibody was Alexa Fluor 488-conjugated goat anti-mouse or rabbit IgG (1 : 100; Jackson ImmunoResearch). Actin filaments were labeled with Alexa Fluor 594 conjugated phalloidin (Life Technology), and nuclei were stained by 4’,6-diamidino-2-phenylindole (DAPI, Life Technology) respectively.

Microarray analysis
The global gene expression was performed by CapitalBio Corporation (Beijing, China). Briefly, MSCs were cultured on fused silica glass, applying static pressure on the cells or not for 24 hours. Total RNA was extracted using TRizol (Life Technology). The RNA samples were labeled and hybridized to Affymetrix Rat Genome 230 2.0 Arrays (Santa Clara, USA). After scanning, data were exported using Affymetrix GeneChip Command Console software, and analyzed using Molecule Annotation System 3.0.

Results and discussion
A facile method was designed to realize applying static pressure on high purity MSCs, which is illustrated in Fig. 1. MSCs were isolated and derived from rat bone marrow with purity over 95%, which was assessed by flow cytometry analysis, as shown in Fig. 2a. For applying static pressure on the cells, MSCs cultured on the bottom of the tissue culture plate (TCP) were gently covered by a well-polished cyto-compatible fused silica glass (FSG) slide (surface topography and cyto-compatibility assessment of FSG are discussed in ESI S1†).

We chose FSG with different thicknesses (0.3, 0.5, 1, 2 and 3 mm) to assess the effect of quantity of static pressure on the fate of MSCs, and take a blank TCP as a control. The static pressure applied on the cells is difficult to be measured by using a high sensitive pressure sensor due to the liquid
environment and large size of the glass slide. Therefore, we calculated the static pressure applied on the cells from the total statistical area of the cells under the glass slide and the difference value between the weight of the FSG and its buoyancy in culture media. As is discussed in ESI S2,† the static pressure applied on the stem cells cultured under FGS with a thickness of 0.3, 0.5, 1, 2 and 3 mm is calculated to be about 13.0, 16.5, 37.7, 42.9 and 57.7 Pa, respectively. To make the experimental results more clear, we just show the time-dependent morphology evolution of the stem cells cultured under FSG with thicknesses of 0.3, 1, and 3 mm, which correspond to the static pressures of 13.0, 37.7 and 57.7 Pa. In Fig. 2b, we can find that without static pressure (rho 0.0 Pa), no morphological variation of MSCs can be observed even after being cultured for 1440 min. When the culture time is very short (20 min), all MSCs under the FSG with different static pressures still maintained the typical spindle-like morphology of MSCs. Obviously, numerous cells under high pressure (57.7 Pa) exhibit typical neural cell morphology, composed of somas, dendrites and neurites. After 180 min culture, although some MSCs under a pressure of 13.0 Pa still maintain a spindle-like morphology, almost all the cells under higher pressure exhibit a neural-like morphology. In particular, the neural-like cells connected with each other to form a neural network under high pressure (57.7 Pa). From these results, we can find that the speed of evolution of MSC morphology is highly dependent on static pressure applied on the stem cells, which illustrates that static pressure can determine neural differentiation of MSCs without any bio or chemical growth factors.

Although higher static pressure is beneficial to accelerate the variation of neural-like cells, the pressure over about 40 Pa can cause cell death in the central part of the FSG (Fig. 2b, column 1440 min) after 24 hours culture. To balance the differentiation efficiency and the cytoactivity of the stem cells, we selected a 1 mm thick FSG (37.7 Pa) slide to apply static pressures on the stem cells cultured.
pressure on MSCs for 24 h to check the pressure controlling process of MSCs in the following study.

To observe the morphological evolution process of MSCs from spindle shape to neural-like cell morphology more clearly, the MSCs transfected with stably expressed actin-red fluorescent protein (RFP), and the morphological variation of individual stem cells was in situ recorded by using a confocal laser scanning microscope at a time-lapse mode (see ESI S3† and Fig. 3a). From Fig. 3a, we can see that under static pressure, the shape of spindle-like MSCs changed gradually, neurites grow and extend, and finally exhibit a classic neural cell morphology with a round body and neurites. The area of MSCs was about 4200 µm², under static pressure; MSCs differentiated into neural-like cells, with the area of about 1000 µm² (Fig. 3b). A time-lapse video recorded the evolution process of MSCs cultured under pressure for above 16 hours in a bright field (see ESI S10, Movie S1†). The movie clearly showed the process of cell spreading, shape changing, appearance of somas and dendrites, and the process of cells getting connected together to be in a network. The in situ observation and the time-lapse video gave visual and powerful evidence, suggesting that the static pressure can control MSCs to differentiate to neural-like cells.

To confirm the differentiation induced by a physical static pressure without the effect of the chemical composition of the covered wafer, a single crystalline LiNbO₃ wafer (a good cytocompatibility transparent material³¹,³²) with a size of 0.8 × 10 × 10 mm (according to the formula of ESI S2† pressure is about 50 Pa) was used to repeat the above experiments, and can also induce the same neural differentiation (ESI S11, Movie S2†). At least 4 batches of MSCs extracted from different rats were used to repeat the above experiments, and all the parallel experiments proved that static pressure could control MSC differentiation toward neural-like cells. Different from the chemical induction process, preinduction³ or starvation²⁷ is not necessary for static pressure control neural differentiation in this work, and high speed and high efficiency of neural differentiation of MSCs is more challenging.

Following the observed significant morphological effect of static pressure on MSCs, we detected the neural associated gene expression levels to further characterize the differentiated cells, including nestin, tubulin-beta 3 (Tuj1), neuron-specific enolase (NSE), microtubule-associated protein-2 (MAP2) and glial fibrillary acidic protein (GFAP) by the quantitative polymerase chain reaction (qPCR) (primer sequence details in ESI S9, Table S2†). The qPCR results showed that the expression of nestin, Tuj1, NSE and MAP2 in the cells cultured under a static pressure of 37.7 Pa for 24 h is up-regulated compared to MSCs without pressure, and the fold of the gene expression level was 1.2, 1.2, 2.8 and 3.1, for nestin (Fig. 4a), Tuj1 (Fig. 4b), NSE (Fig. 4c) and MAP2 (Fig. 4d), respectively. Glia specific gene-GFAP could not be detected by qPCR (no data was observed). Nestin is a neural stem cell marker, as proved by the previous literature.²⁷,³³,³⁴ During the early process of MSCs’ differentiation into neural-like cells, the expression of nestin is up-regulated. In this study, the result of nestin expression is consistent with the previous studies with chemical compound induction. The expression of neuronal specific markers Tuj1, MAP2 and NSE increases significantly by the induction of static pressure, and the results verify that the static pressure can induce neural differentiation of MSCs.

Immunofluorescence was used to detect the expression of neural associated proteins – nestin, Tuj1, NSE and MAP2 (Fig. 4e). The cultured MSCs without static pressure exhibit the classical morphology of MSCs – spindle and fibroblastic appearance, and negligible expression of nestin, Tuj1, NSE and MAP2. In contrast, under static pressure, MSCs exhibited a neural morphology and a strong expression of neural specific markers (nestin, Tuj1, NSE and MAP2).

The assessment of immunofluorescence gives very strong evidence for supporting the neural-differentiation of MSCs.
under static pressure. The assessment results of both qPCR and immunofluorescence strongly support that static pressure can control MSCs toward neural differentiation.

Furthermore, the gene microarray was performed on the cells after being cultured for 24 h under a static pressure of 37.7 Pa to test the global gene expression. The up-regulation/down-regulation of genes was determined in relation to the gene expression profile of undifferentiated MSCs at 24 h without pressure. The gene profile of cells cultured under static pressure dramatically changes compared to the normal cultured MSCs, and the expressions of 716 genes were up-regulation-marked by red dots, and 248 genes were down-regulation-marked by green dots (Fig. 5a). Molecule Annotation System (MAS) analysis exhibited biological processes that static pressure induced. At \( p < 0.05 \) cut off, the neuron associated biological process enrichment is found, such as neuron differentiation, development, maturation, neural tube development and neurological system processes (Fig. 5b).

We also tried some other stem cells to test the effect of general applicability of static pressure on the fate of stem cells (details in ESI S4†). The results showed that human dental pulp stem cells (hDPSCs), human periodontal ligament stem cells (hPDLSCs), human mesenchymal stem cell derived umbilical cord (hMSC-UB) and human induced pluripotent stem cells (hiPSCs) didn’t exhibit obvious neural differentiation upon applying a static pressure for 24 hours. Mouse mesenchymal stem cells derived from bone marrow (mMSCs) showed a typical neural-like morphology under pressure for several hours. It appears that neural differentiation under static pressure is mostly special for bone marrow-derived MSCs.

Another experiment to prove the static pressure can induce neural-like differentiation of MSCs is using a grooved glass slide to apply static pressure on cultured MSCs with an alternate pattern. It is found that in one culture system, the part of the solid part can induce neural differentiation, and the cells at the blank part still retain typical morphology of stem cells.
These results further confirm the static pressure induced neural differentiation of MSCs, and the patterned slides can realize localized and committed differentiation.

However, discovering the mechanism of MSC differentiation into neural cells under static pressure is a great challenge because of its highly complex and multicomponent signaling milieu present under these conditions (see ESI S7†). Normally, the reason for MSC differentiation into neural cells can be divided into two groups: chemical compound induction, or growth factors and cytokines involved in neural development. For the chemical induction, a preinduction process is necessary and will take several hours to fulfill the differentiation. For cytokine induction, it will take several weeks to differentiate stem cells into neural cells. In our study, a starvation environment was formed and accompanied by static pressure, and we also detected the gene profiles of MSCs particularly derived under serum depletion conditions, which is often used as preinduction with chemical induction methods. The results showed a significantly different global gene expression profile compared to the control MSCs (ESI S5†), and some neural markers were up-regulated under serum depletion (see ESI S6†). It is possible that the microenvironment induced by static pressure could promote the MSC differentiation into neural cells. In our study, the differentiation process just takes several hours for applying static pressure on MSCs without preinduction, depletion serum, or assistant additional chemical molecules.

Static pressure induced neuron differentiation has some disadvantages. It is not easy to achieve live neuron-like cells by the trypsin-digestion method because the glass slide is tightly in contact with the TCP bottom. When the glass slide is peeled off from the bottom of TCP, most cells are damaged. So, it is very difficult to conduct an experiment for checking the electrical function of the differentiated cells. Recently, we tried to use the micro-fluid method to apply pressure on MSCs, and probably overcome the problem, and obtain living cells after the release of the pressure. It looked more like a stress reaction induced neural differentiation in response to static pressure conditions. Therefore, in the future, how to combine the static pressure and biological methods to induce MSCs into real functional neurons will be important for neural development.

**Conclusion**

Our study delineates the importance of static pressure in MSC differentiation into neural-like cells, which opens a new route to regulate neural differentiation of MSCs. In the future, by specially designed bio-devices, static pressure can not only be used to accelerate neural-like differentiation, development and maturation processes, but can also be used to control the location and time of the differentiation, and may realize pinpoint controllability for building the neural cell system at regular time and quantity. It is a novel strategy for neural differentiation under physiological conditions and will be widely applicable in tissue engineering and regenerative medicine.

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Notes and references