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A microfluidic array for quantitative analysis of human neural stem cell self-renewal and differentiation in three-dimensional hypoxic microenvironment

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ABSTRACT

We report a microfluidic array for investigating and quantitatively analyzing human neural stem cell (hNSC) self-renewal and differentiation in an *in vivo*-like microenvironment. NSC niche conditions, including three-dimensional (3D) extracellular matrices and low oxygen tension, were effectively reconstituted in the microfluidic array in a combinatorial manner. The array device was fabricated to be detachable, rendering it compatible with quantitative real-time polymerase chain reaction for quantifying the effects of the biomimetic conditions on hNSC self-renewal and differentiation. We show that throughput of 3D cell culture and quantitative analysis can be increased. We also show that 3D hypoxic microenvironments maintain hNSC self-renewal capacity and direct neuronal commitment during hNSC differentiation.

1. Introduction

Neural stem cells (NSCs) may provide effective therapeutics for treating neurodegenerative diseases, ischemic stroke, spinal cord injury, and traumatic brain injury [1–4]. NSCs have the capacity to self-renew and differentiate into neurons, astrocytes, and oligodendrocytes [5]. Transplantation of NSCs has shown therapeutic potential by regenerating neuronal tissue and recovering functional behavior in diseased animals and humans [6–9]. Recent studies highlight the importance of the NSC niche [10–12], which, in the brain, is made of three-dimensional (3D) extracellular matrix (ECM), vasculature, and neighboring cells such as neurons and glial cells [13–15]. These niches can improve the efficacy of NSC therapeutics because they maintain NSC self-renewal and, under certain circumstances, guide tissue specific differentiation of NSCs [13,16].

A critical component of the *in vivo* NSC niche, which is often been overlooked, is low oxygen tension. The midbrain's physiological oxygen concentration ranges from 1% to 8%, indicating that NSCs in the brain reside in a relatively hypoxic microenvironment [17,18]. Previous studies used hypoxic conditions for NSC culture to mimic the *in vivo* environment in the vapor phase [19,20]. They report that a 3–5% oxygen concentration greatly enhances differentiation and proliferation of NSCs [19,20]. Another critical component of the *in vivo* NSC niche is the 3D ECM. Cells cultured on 3D ECM exhibit altered morphology, structure, and gene expression compared to those in two-dimensional (2D) culture [21]. It is also believed that the physicochemical distribution of soluble factors or gases in a 3D environment is different from those in a 2D environment [22,23]. Although stem cell researchers recognize the roles of solid matrices and gas in NSC proliferation and differentiation, the effects of these niche conditions on NSC behavior have only been investigated individually and, most commonly, in 2D culture conditions. To date, no studies report the simultaneous effects of solid and gas components on regulating NSC behavior in a 3D microenvironment.

In this study, we examined the simultaneous effects of 3D NSC niche conditions created by solid 3D ECM and gas content (hypoxia) on NSC self-renewal and differentiation. To this end, we developed a microfluidic array (Fig. 1) that creates *in vivo*-like 3D microenvironments, with reduced oxygen content, from specific ECM proteins. The device also increases the throughput of 3D cell cultures for quantitative analysis. The ECM proteins tested in this study

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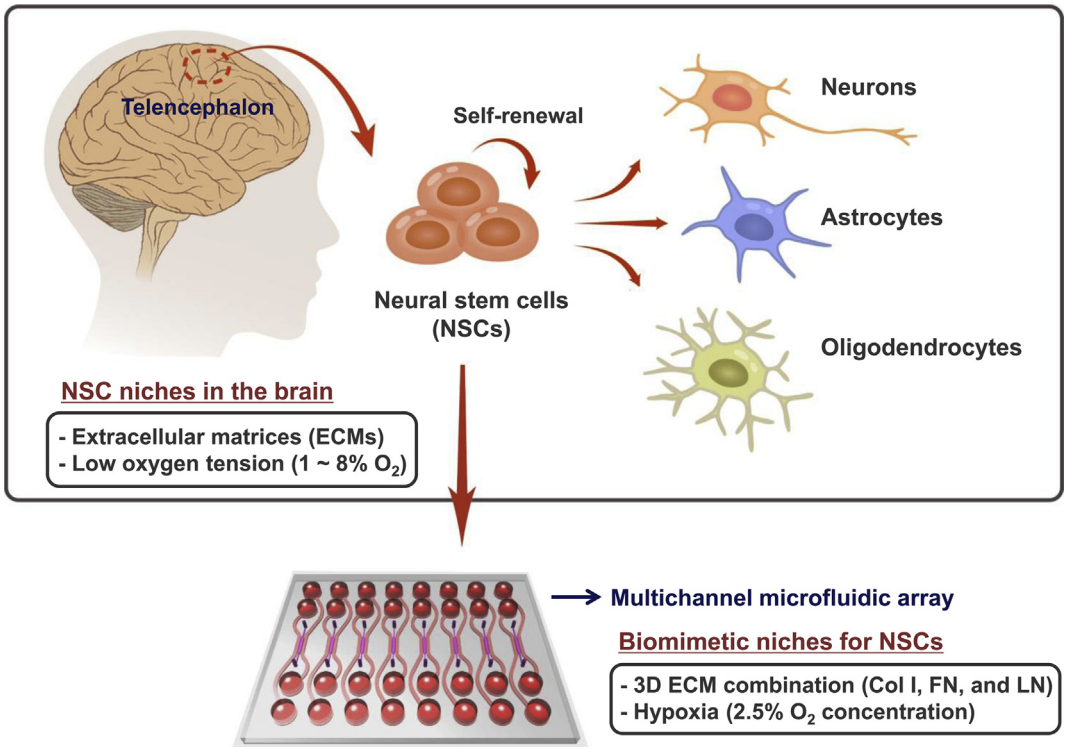

Fig. 1. NSC niche conditions in the brain include 3D ECM proteins and low oxygen tension. *In vivo*-like microenvironments are effectively reconstituted to mimic NSC niches in the microfluidic array.

included collagen type I (Col I), fibronectin (FN), and laminin (LN), which are key regulators of stem cell proliferation, differentiation, and migration [24]. ECM proteins are easily incorporated into the microfluidic device and formed into an *in vivo*-like 3D microenvironment to provide spatiotemporal control of microscale fluid behavior (e.g., laminar flow and molecular transport by diffusion), which is distinct from macroscale fluid behavior [21]. This system also allows for accumulation of soluble factors secreted from the embedded cells [25,26]. We thought that the 3D ECM proteins used here created gas diffusion profiles physiologically similar to those of *in vivo* profiles, compared with 2D culture and macro 3D ECM created in tissue culture plates [23].

2. Materials and methods

2.1. Fabrication of the microfluidic array

Microfluidic array devices were fabricated with poly(dimethylsiloxane) (PDMS) by a conventional soft lithography process, as previously described [27]. The microfluidic array was designed to contain eight units in a single device. Each unit contains one central channel for NSC culture in ECMs and two side channels, at both sides of the central channel, for supplementation with growth medium. The process for bonding the PDMS microchannel component to the flat PDMS membrane (~80 μm thick; Amed Co., Seoul, Korea) was modified to allow for easy disassembly of the device [27]. Both the channel component and membrane were treated with oxygen plasma (CUTE; Femto Science, Seoul, Korea), exposed to air for several minutes, and assembled. This modified process leads to formation of reversible bonding between the PDMS microchannel component and the PDMS flat membrane. The bonded devices were placed in a drying oven for 12 h at 80 °C to allow them to recover their hydrophobicity. In devices constructed using this process, the PDMS membrane can be readily detached from the channel component because of its elastic properties and the relatively weak bonding strength between the plasma-treated surfaces produced by additional exposure to air [27].

2.2. Culture of human neural stem cells (hNSCs)

Human fetal NSCs were isolated from the telencephalon (HFT13) as previously described [28]. The cells were plated in dishes at a seeding density of 6.0×10^5/mL. hNSCs were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) medium (Gibco, Gaithersburg, MD, USA) and supplemented with basic fibroblast growth factor (bFGF, 20 ng/mL; Sigma, St. Louis, MO, USA), leukemia inhibitory factor (LIF, 10 ng/mL; Sigma), and N-2 supplement (Gibco) in humidified air with 5% CO_2 at 37 °C, which allows hNSCs to grow as neurospheres. The culture medium was changed every 3 days.

2.3. hNSC culture in the microfluidic extracellular matrix (ECM) hydrogels

Four types of ECM solutions were prepared for hNSC culture in the 3D ECM hydrogels [24]: Col I, Col I + FN, Col I + LN, and Col I + FN + LN. Col I (3.44 mg/mL, rat tail collagen type I; BD Bioscience, CA, USA) was mixed with 10× phosphate-buffered saline (PBS; Thermo Scientific, MA, USA), 0.5 N NaOH, and distilled deionized water to adjust the pH to 7.4 and the concentration (2.0 mg/mL) for gelation (Col I group). For the combined ECM groups (Col I + FN, Col I + LN, and Col I + FN + LN), Col I solution (2.0 mg/mL, pH 7.4) was mixed with FN (from human plasma, 1.0 mg/mL, Sigma) and/or LN solution (from engelbreth-holm-swarm murine sarcoma basement membrane, 1.0 mg/mL, Sigma) and the final concentration of each ECM was adjusted to 25 μg/mL. After preparation of the hydrogel solutions, hNSCs were suspended in each ECM hydrogel (5.0×10^6 cells/mL). ECM hydrogel solutions containing hNSCs were allowed to gel in the channels of the microfluidic array devices for 3D microscale cultures. hNSCs in the 3D ECM hydrogels were cultured in DMEM/F12 medium without mitogenic factors (bFGF and LIF) under normoxic (20% O_2) or hypoxic condition (2.5% O_2). For hypoxic culture of hNSCs in the 3D microfluidic ECM hydrogels, the array devices were placed in a multi-gas incubator (MCO-5M, Sanyo, Osaka, Japan) with air condition of 2.5% O_2 and 5% CO_2 at 37 °C. Low oxygen tension was maintained through the controlled supply of N_2 gas to the incubator. The medium was changed daily.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

hNSCs were collected from the disassembled microfluidic array devices as previously described [27]. qRT-PCR was performed as previously reported [28]. Total RNA was isolated from each sample ($n = 3$ per group) using the RNeasy Mini kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. A reverse transcription reaction was performed with 5 ng pure total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). qRT-PCR measurements of gene expression were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and Fast Universal PCR Master Mix (Applied Biosystems). Gene expression in hNSCs was quantified using TaqMan Gene Expression Assays (Applied Biosystems) for each target (nestin;