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Maintenance of the self-renewal properties of neural progenitor cells cultured in three-dimensional collagen scaffolds by the REDD1-mTOR signal pathway

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ABSTRACT

Three-dimensional (3-D) culture, compared with traditional two-dimensional (2-D) cell culture, can provide physical signals and 3-D matrix close to the *in vivo* microenvironments. Here, sponge-like collagen scaffolds were used to assess how 3-D culture would affect the differentiation and self-renewal of neural progenitor cells (NPCs). Cultured in differentiation medium without growth factors, cells in 3-D collagen scaffolds yielded much higher clone formation efficiency and expressed less neuron marker, TUJ1, compared with cells cultured on 2-D plates. mTOR inactivation was identified and showed to supported the self-renewal of NPCs in 3-D culture. At the same time, REDD1 was highly expressed in cells cultured in 3-D conditions, which blocks the activity of mTOR. Moreover, knocking-down REDD1 induced the differentiation of NPCs in 3-D collagen scaffolds. These results indicated that mTOR inactivation by REDD1 mediated the self-renewal regulation of NPCs in 3-D cultures. Thus, 3-D collagen scaffolds maintained self-renewal properties of NPCs, and the inhibitory regulator of mTOR (such as REDD1) played an important role in the regulation of self-renewal and differentiation of NPCs.

1. Introduction

Stem cells (SCs) give rise to tissues and organs. The balance of differentiation and self-renewal of SCs are sophisticatedly harmonized by physical, soluble, and solid signals in stem cell niches [1]. However, the *in vivo* microenvironments are too complicated to be simulated by the current approaches. The physical signals, cell–cell, and cell–matrix interaction, which are important regulating signals to stem cells, cannot be well characterized by the two-dimensional (2-D) *in vitro* culture [2]. The three-dimensional (3-D) culture system bridges the gap between *in vivo* system and the *in vitro* 2-D system.

In the central nervous system (CNS), neural progenitor cells (NPCs) give rise to all kinds of types of neural cells. The fate of NPCs is decided by the cooperation between extracellular cues and intercellular factors [3]. A group of transcription factors with basic helix-loop-helix (bHLH) motifs are found to be critical to the behaviors of NPCs. These neuro-active bHLH factors include the members of NeuroD, Neurogenin, Mash, Olig, Id and Hes families. Among them, two classes of inhibitory bHLH factors, Id and Hes, play an important role in maintenance of NPC self-renewal and inhibition of neurogenesis. Both of them expressed at a high level in the ventricular zone of the telecephalon [4,5]. In *Id1*: *Id3* double mutant mouse model, NPCs exited the cell cycle and accelerated the neurogenesis prematurely during development [6]. In the subventrical zone of adult brain, high level of Id1 expression was necessary for the self-renewal of Type B1 neural progenitor cells [7]. Hes proteins, which were highly expressed in NPCs, maintained the self-renewal property of NPCs by inhibiting neuronal differentiation [8,9].

In additional to intercellular factors, the differentiation and self-renewal of NPCs are also under control of extracellular signals. Notch [9,10], BMPs [11,12], sonic hedgehog [13,14], Wnt [15–17], insulin and insulin-like growth factor-I (IGF-I) signals are identified to be vital to determine the fate of NPCs [18–21]. Mammalian target of rapamycin (mTOR), one downstream factor of insulin/IGF-I signal, is reported to be involved in the regulation of differentiation and development of CNS in several species. In *Drosophila*, TOR supported the temporal system development [22]. In mouse, mTOR inhibition resulted in embryonic development arrest and telecephalon depletion [23], and TOR signal was necessary for chick neuronal differentiation initiation [24]. We have previously found mTOR mediated the neurogenesis enhancement induced by insulin

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[25], which extended the understanding of mTOR functions in regulating NPCs differentiation. Our subsequent experiments further discovered that in the 2-D *in vitro* culture the inhibition of mTOR by rapamycin could improve the self-renewal of NPCs (Fig. S1).

The 3-D culture of NPCs is getting more attention. However, the mechanisms that control the differentiation and self-renewal of 3-D cultured NPCs remained largely uncharacterized. In this study, through comparing NPCs cultured on 2-D plates and in 3-D collagen scaffolds, the differentiation and self-renewal characters of NPCs were analyzed. To profoundly understand the behavior of NPCs 3-D culture, the signal mechanisms directing NPC differentiation and self-renewal in 3-D culture was further investigated.

2. Materials and methods

2.1. Scaffolds preparation

The 3-D collagen scaffolds were made from bovine collagen of spongy bone as described previously [26]. Briefly, the spongy bones were separated from the head of a long bone, cut into appropriate size, and soaked in acetone for 48 h. After removing the fatty composition by acetone, the samples were washed by ddH_2O completely. Then, the samples were demineralized by 0.6 M HCl following enzyme treatment and ddH_2O washing. Finally the 3-D collagen scaffolds were obtained from freeze-drying approach. In this study, the scaffolds were further cut into 1 mm × 5 mm × 5 mm cubes for NPCs growth.

2.2. Neural progenitor cells culture

NPCs isolation was referred to the previous procedure with slightly modification [25]. Briefly, the telecephalons were dissected from neonatal Sprague–Dawley rats. Then tissues were cut into 1 mm^3 pieces and removed to 0.25% trypsin, incubated at 37° C for 40 min. Trypsin inhibitor was added to stop the digestion, and the sample was centrifuged for 5 min at 250 g. The remained pellet were resuspended in neurosphere medium which contains 20 ng/mL bFGF (Peprotech Asia, Rehovot, Israel), 20 ng/mL EGF (Peprotech Asia), 2% B27 (Invitrogen, GIBCO, NY, USA), 30% glucose (Sigma, MO, USA), and 1.83 μg/mL heprin (H3149, Sigma) dissolved in DMEM-F12 1:1 medium (Invitrogen). Cells were seeded to T25 flask (Corning, NY, USA) at the density of 200 cells/μL and incubated at 37 °C with 5% CO_2. At the 4th day, neurospheres were collected by centrifugation at 250 g, then the pellets were suspended in fresh neurosphere medium. At the 7th day, the neurospheres were collected and digested in 0.25% trypsin for 15–20 min at room temperature. NPCs were resuspended in adhesion medium which contains 10% FBS (Invitrogen) in DMEM medium (Invitrogen). NPCs were then seeded onto 60 mm dishes and 6-well plates precoated with poly-D-lysine (PDL) or type I collagen for 2-D culture and seeded into 3-D collagen scaffolds for 3-D cultured. In detail, NPCs were plated at the density of 8–15 × 10^4 cells/cm^2 for 2-D culture. For 3-D culture, the scaffolds were sterilized by gamma ray radiation, and soaked in DMEM medium overnight. Then the filter paper was used to absorb the excessive medium of scaffolds. 2 × 10^6 cells in 50–80 μL adhesion medium were absorbed by the scaffolds. Subsequently, the scaffolds were incubated at 37 °C for 4 h to allow cells to attach onto the inner surface of the scaffolds, and new adhesion medium was added for further incubation.

After 24 h adhesion, the adhesion medium was change by differentiation medium containing 2% B27 and 30% glucose in DMEM-F12 1:1 medium. Cells were collected at the indicated time for different assays. For detecting the phosphorylation of p70S6K, rapamycin (Sigma) was pretreated for 2 h.

2.3. Western blotting

NPCs were digested by trypsin from 2-D and 3-D culture systems, and the trypsin inhibitor was used to terminate the digestion. The collected cells were washed by phosphate buffered saline (PBS, Thermo, Hyclone, UT, USA) for 2 times, and lysed by RIPA buffer (Sigma) supplemented with proteinase inhibitor cocktail (04693116001, Roche Applied Science, Mannheim, Germany) for 30 min on ice. The whole-cell lysates were harvested from the supernatant of centrifugation 14,000 g for 30 min. BCA assay was used to measure the protein concentration, and the equivalent quantity protein lysates were electrophoresed in SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane (GE, Amershame, Buckinghamshire, UK). The primary antibodies used in this study included: anti-Tuj1 (05-559, Millipore, NY, USA), anti-GFAP (MAB360, Millipore), anti-α-tubulin (T5168, Sigma), anti-phosphorylated-p70S6K (9204, Cell Signaling, MA, USA), anti-total-p70S6K (9202, Cell Signaling), anti-GADPH (AB-P-R 001, Goodhere, Hangzhou, China), anti-REDD1 (10638-1-AP, ProteinTech, IL, USA), and anti-Id1 (sc-488, Santa Cruz, CA, USA). And the HRP-linked-secondary antibodies including anti-mouse (SouthernBiotech, Alabama, USA) and anti-rabbit (Thermo, PIERCE) IgG antibodies were used according to the corresponding primary antibody.

2.4. Scanning electron microscopy

3-D scaffolds with NPCs growing in it within 24 h and the 3-D collagen scaffolds without NPCs were washed by PBS for 3 times, and fixed in 2% glutaraldehyde at 4 °C for 4 h. After washing in PBS for 3 × 10 min, the fixed scaffolds were dehydrated through a graded ethanol series: 30%, 50%, 70%, 75%, 80%, 85%, 90%, and 95% for 10 min respectively, and 100% for 2 × 20 min. Ethanol was extracted in 3:1, 1:1, and 1:3 mixtures of ethanol and amyl acetate for 20 min each followed by 100% amyl acetate storage. Then the samples were dried by super critical CO_2 extraction, and coated with gold. The images were captured by HITACHI S-3000N scanning electron microscopy (HITACHI, Tokyo, Japan).

2.5. RT-qPCR and RNA interfering

Total RNA was extracted by Trizol reagent (Invitrogen) from cells culture in 3-D and 2-D conditions for the time indicated in the experiment. 1 μg total RNA reverse-transcribed with SuperscriptIII (Invitrogen) after digested by DNaseI (Invitrogen) according to the manufacturer's instruction. Quantitative real-time PCR was performed by ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Life Technologies, CA, USA) and SYBR Green Master Mix (Applied Biosystems) as instructions described by manufactures. Briefly, 0.5 μL cDNA mixed with 0.5 μL primer mixture, 5 μL Master Mix and 4 μL distilled water were denatured at 95 °C for 10 min followed by 40 cycles of PCR (95 °C for 15 s, 60 °C for 1 min). The sequences of the primers were as follow: Hes1: forward 5′ GCCGTGGCGGAACTGA 3′, backward 5′ GAGGTGGGCTAGGGAGTTTATGA 3′; Hes5: forward 5′ CCGCATCAACAGCAGCATT 3′, backward 5′ CGGTCCCGACGCATCTT 3′; Id1: forward 5′ GACGAACAGCAGGTGAACGTT 3′, backward 5′ TCCTTGAGGCGTGAGTAGCA 3′; Id3: forward 5′ GAGCTCACTCCG-GAACTTGTG 3′, backward 5′ CCGGGTCAGTGGCAAAAAC 3′; REDD1: forward 5′ CGCTCTTGTCCGCAATCTTC 3′, backward 5′ GGACGCTGGTTGATGAGGTT 3′. Relative quantitation of the expression level was analyzed by using the $2^{-\Delta\Delta Ct}$ method. The ΔCt value was resulted from the normalization by β-actin Ct value, and the ΔΔCt value was resulted from the normalization by the ΔCt value of 2-D PDL group.

REDD1 RNA interfering was conducted by lentivirus, and the target sequence was 5′ AGGACTCCTCATACCTGGATG 3′ [27]. The construction and envelope were undertaken by Shanghai GenePharma Company (Shanghai, China). After adhered for 24 h, NPCs were infected by RNAi lentivirus (control or REDD1 RNAi) using MOI of 15 without polybrene. Incubating for 24 h at 37 °C, lentivirus was absorbed and replaced by fresh adhesion medium. After adhesion medium was incubated for 24 h, medium was changed by differentiation medium. At 6 h after differentiation medium changed, cells were collected for detection of REDD1 expression, p70S6K phosphorylation and Id1 expression. Cells for colony formation assay were collected at the 7th day after differentiation medium changed.

2.6. Colony formation assay

Colony formation assay of NPCs was mainly conducted as described previously with slight modification [28]. Briefly, Cells cultured in 3-D and 2-D conditions for 7 days were digested to single cells and plated into 96-well plates at various cell densities (0.1–10 cell/μL) in neurosphere medium. B27, EGF, and bFGF were replenished every other day by adding appropriated quantity to the well. Plates were scored for neurosphere blinded to each treatment using phase-contrast microscopy at the 12th day. Linear regression analysis was used to determine the frequency of colony formation.

2.7. Statistic analysis

Data represented the mean ± s.d. Statistical differences between groups were performed by ANOVA. Student's *t* test was performed to value the significance of differences between means. * represented p value < 0.05, ** p value < 0.01, and *** p value < 0.001.

3. Results

3.1. Differentiation of NPCs in 3-D collagen scaffolds

The 3-D collagen scaffolds have a sponge-like shape (Fig. 1A). The surfaces of 3-D collagen scaffolds were visualized by scanning electron microscope (SEM). As shown in Fig. 1B, the pore size of 3-D collagen scaffolds was 50–200 μm in diameter. It would provide sufficient space for cells growing in the scaffolds. NPCs isolated from the telencephalons of neonatal rats proliferated and formed floating spheres in neurosphere medium containing growth factors (bFGF and EGF). The neurospheres were digested by trypsin to