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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

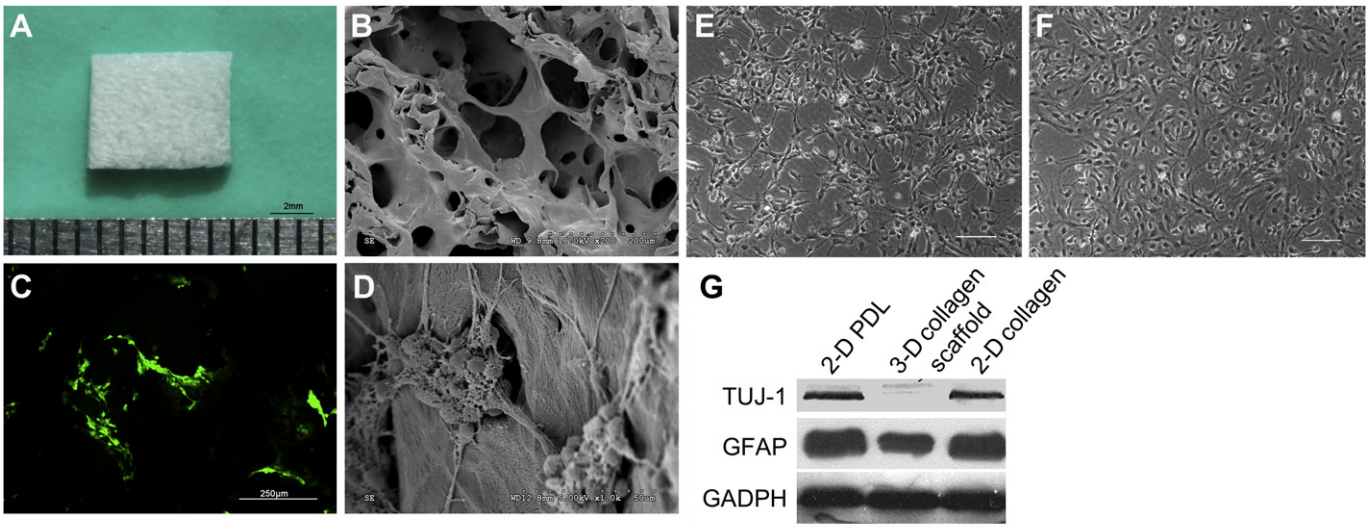

Fig. 1. The differentiation of NPCs in 3-D collagen scaffolds and on 2-D plates. (A) macroscopic view of collagen scaffolds, scale bar = 2 mm (B) collagen scaffold observed by SEM, scale bar = 500 μm. (C) Fluorescein diacetate (FDA) stained NPCs cultured in collagen scaffolds observed by LSCM, scale bar = 250 μm (D) collagen scaffolds embedded with NPCs observed by SEM, scale bar = 50 μm (E–F) phase-contrast image of NPCs seeded on PDL (E)- and collagen (F)- coating culture plate, scale bar = 100 μm. (G) NPCs differentiated in 3-D scaffolds and on 2-D plates for 7 days. Neuron marker TUJ1 was much lower expressed by NPCs in 3-D culture than those in 2-D culture. While the glia marker GFAP was expressed at a common level among the three groups.

single cells. The digested NPCs were seeded on 2-D culture plates pre-coating with PDL or type I collagen and in 3-D collagen scaffolds. NPCs tended to aggregate with each other in 3-D collagen scaffolds (Fig. 1C,D), but they spread out on 2-D plates within 24 h upon attachment. (Fig. 1E,F). Once NPCs attached to 2-D plates and 3-D scaffolds, the medium was changed by differentiation medium, which withdrew growth factors from neurosphere medium. The differentiation potential of NPCs in 2-D and 3-D conditions was inspected. After differentiating for 7 days, the expression of neuron marker TUJ1 in 3-D collagen scaffolds was much lower than those in 2-D culture. The TUJ1 expression was nearly the same on PDL-coating and collagen-coating 2-D plates. At the same time, the expression of glia marker GFAP in 3-D collagen scaffolds was similar to those in 2-D culture (Fig. 1G). It indicated that the neuronal differentiation of NPCs in 3-D collagen scaffolds decreased.

3.2. Expression of inhibitory bHLH factor

NPCs self-renewal and differentiation are controlled, to a large part, by transcription factors with basic helix-loop-helix (bHLH) motifs [29]. The subset of neuro-active bHLH factors (neuroD, neurogenin (ngn), mash, Id and Hes) have been proven to play an important part in cell fate decision of NPCs. qPCR demonstrated that *Id1, Id3, Hes1* and *Hes5* were expressed at a higher level in 3-D collagen scaffolds than those in 2-D conditions (Fig. 2). *Id* and *Hes* belong to inhibitory bHLH factors, which undertake the self-renewal maintenance of NPCs [6,9,30,31]. Thus, the high level expressions of inhibitory bHLH factors may explain the phenomenon that the differentiation of NPCs in 3-D culture was decreased.

3.3. Secondary colony formation ability

Id and Hes play an important role in maintaining NPCs self-renewal and inhibiting neuronal differentiation [6,9,30,31]. We identified that the differentiation of NPCs was down-regulated (Fig. 1G), and the expression of *Id* and *Hes* maintained at a high level in NPCs in 3-D collagen scaffolds (Fig. 2). Next, we analyzed the self-renewal abilities of NPCs cultured on 2-D plates and in 3-D scaffolds. NPCs were digested to single cells after differentiating for 7 days in 2-D and 3-D culture, then plated at cell densities of 0.1-5cells/μL in neurosphere medium with growth factors. NPCs cultured in 3-D collagen scaffolds generated more colonies at all densities than those in 2-D groups. There were no significant

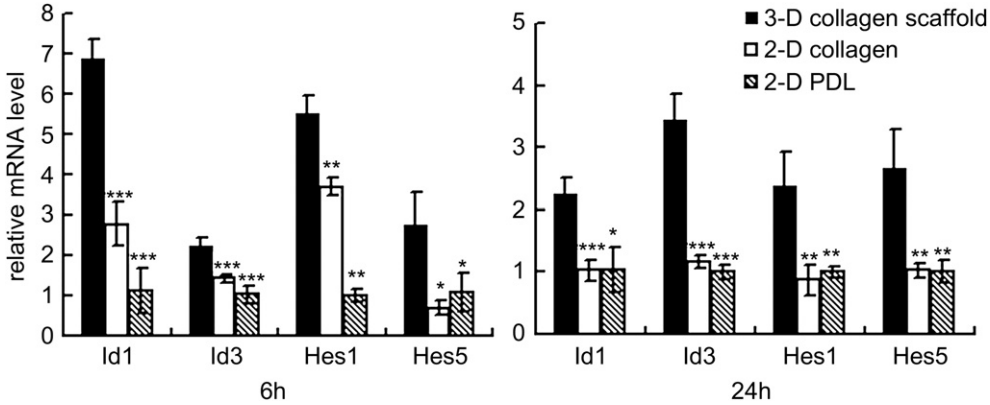

Fig. 2. The inhibitory bHLH were overexpressed by NPCs in 3-D collagen scaffolds. The expressions of inhibitory bHLH factors (*Id1, Id3, Hes1 and Hes5*) of NPCs in 3-D collagen scaffolds were higher than those on 2-D plates. The result is presented by fold change relative to the mRNA level of 2-D PDL group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

differences in the abilities of generating secondary colonies between NPCs differentiating on PDL-coating and collagen-coating 2-D plates (Fig. 3A). The linear regression was further used to estimate the abundance of clonogenic cells in the total population (Fig. 3B). In 3-D group, roughly one colony formed from every 28 cells or a frequency of 0.0355 colonies per cells plated ($R^2 = 0.98$). As to the NPCs cultured on collagen-coating 2-D culture plates, the incidence dropped to 0.0079 (roughly one colony per 126.6 cells, $R^2 = 0.99$), and similar to PDL-coating 2-D culture plates, the frequency of which stayed at 0.0106 or one colony per 94.8 cells ($R^2 = 0.99$). These results demonstrated that there are more undifferentiated NPCs maintained in the 3-D collagen scaffolds.

3.4. Self-renewal maintenance by mTOR inactivation

We have found that 3-D culture in collagen scaffolds may help to maintain the self-renewal status of NPCs. The mechanism was investigated. We have previously discovered that the inactivation of mTOR signal supported the self-renewal maintenance of NPCs ([25] and Fig. S1). In this experiment, the phosphorylation of mTOR reactor, p70S6K, was lower in 3-D culture than those in 2-D culture (Fig. 4A). Rapamycin, an inhibitor of mTOR signal, reduced the phosphorylation of p70S6K of the three groups to the same low level (Fig. 4B). Then we tested if rapamycin treatment could affect the self-renewal status. In 3-D collagen scaffolds, Id1 protein expressed lower than those in 2-D culture conditions (Fig. 4C), which was in accordance with the qPCR result (Fig. 2). The Id1 expression rose to a common high level under the treatment of rapamycin (Fig. 4C). As to the secondary colony formation assay, cells were plated at the density of 4cells/μL. In the absent of rapamycin, NPCs in 3-D collagen scaffolds formed 17.33 ± 1.15 colonies, and in 2-D culture yielded 8.50 ± 1.09 colonies on collagen-coating plates and 7.33 ± 0.58 colonies on PDL-coating plates (Fig. 4D). In the present of rapamycin, the number of colony formed of the 3-D, 2-D collagen and 2-D PDL group reached to a similar high level, with 17.92 ± 0.76, 18.17 ± 0.38 and 16.58 ± 0.76 respectively (Fig. 4D). These results indicated that mTOR signal participated in the self-renewal maintenance of NPCs in 3-D collagen scaffolds.

3.5. Up-regulation of REDD1 in 3-D-cultured NPCs

In numerous up-stream regulators of mTOR, growth factors gain more attention. However, excess doses of insulin could not further activate the phosphorylation of p70S6K of 3-D group (Fig. 5A). So we hypothesized that there could be other up-stream signals preventing the activation of mTOR. One of mTOR up-stream regulators—REDD1 has been reported to mediate the differentiation and development functions of mTOR [32,33]. In this study, REDD1 mRNA expression was found to be higher in 3-D group (Fig. 5B). At the same time, western blotting results demonstrated the REDD1 protein level was also higher in 3-D group than those in 2-D groups (Fig. 5C).

3.6. Self-renewal maintenance by REDD1 high expression

We further examined whether the inactivation of mTOR signal in 3-D collagen scaffolds was caused by the high expression of REDD1. As shown in Fig. 6A, the REDD1 RNAi lentivirus effectively repressed the expression of REDD1 (Fig. 6A). The silence of REDD1 released the repression of the p70S6K phosphorylation, and the activation level reached to the similar level among the 2-D and 3-D groups (Fig. 6B). The reduced Id1 protein expression was observed by REDD1 knocking-down, and the Id1 expression of the 3-D group was similar to those of the two 2-D groups (Fig. 6C). As to the colony form assay, cells were plated at the density of 4cells/μL. NPCs infected by control lentivirus formed more colonies (13.17 ± 2.56 colonies) in 3-D group than those in 2-D groups (7.25 ± 0.66 colonies in 2-D collagen group and 4.08 ± 0.88 colonies in 2-D PDL group). Infecting with REDD1 RNAi lentivirus reduced the number of colonies formed in 3-D group (3.25 ± 0.50 colonies), resulting in a similar colonies number among the 3-D and 2-D groups (3.33 ± 0.38 colonies in 2-D collagen group and 2.92 ± 0.52 colonies

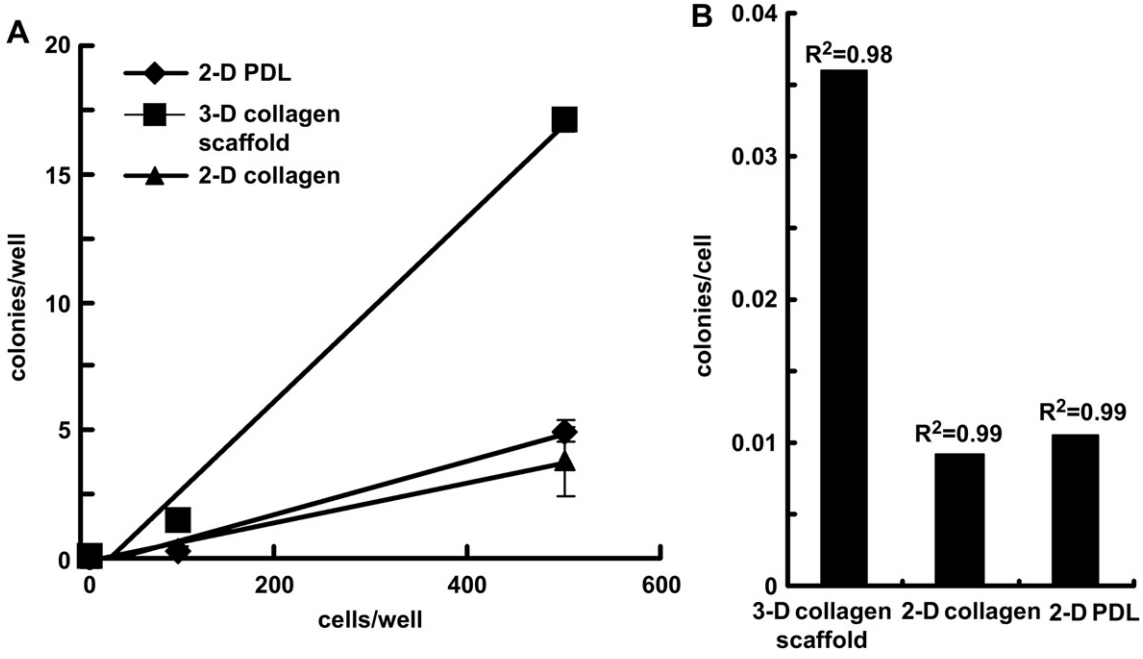

Fig. 3. 3-D collagen scaffolds tended to keep the self-renewal of NPCs in a differentiating medium. (A) A gradient dilution of NPCs differentiated in 3-D scaffolds and on 2-D plates for 7 days to re-proliferate in growth factor containing medium, colonies were formed in a density-dependent manner. At all cell densities, NPCs in 3-D collagen scaffolds produced more colonies than those in 2-D culture did. (B) linear regression analysis of data shown in (A) demonstrated the relative efficiency of colony formation of NPCs differentiated in different culture conditions.

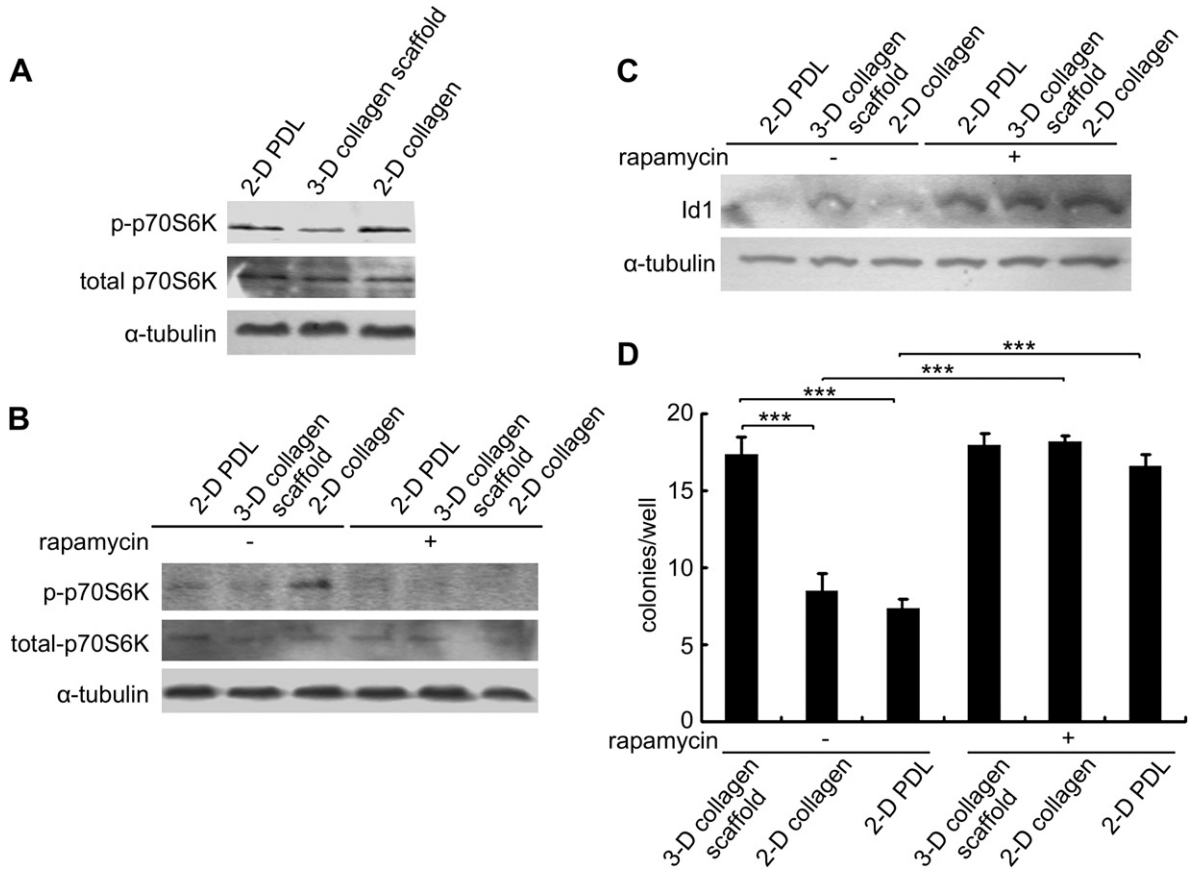

Fig. 4. Low mTOR activity supported the self-renewal of NPCs. (A) The phosphorylation of p70S6K in 3-D group was lower than those in 2-D groups. (B) rapamycin decreased the phosphorylation of p70S6K of 3-D and 2-D groups to a common level. (C) Id protein expressions of 3-D and 2-D groups were lifted by rapamycin to a common level. (D) rapamycin enhanced the clone formation efficiency of 3-D and 2-D groups to a common level.

in 2-D PDL group). (Fig. 6D). These results demonstrated that the silence of REDD1 could abrogate the self-renewal maintenance of NPCs in 3-D collagen scaffolds through activation of mTOR pathway.

4. Discussions

The 3-D culture system provided cells with cell–cell interaction, cell–matrix interaction and 3-D physical microenvironments close to *in vivo* microenvironments. Thus, 3-D culture is a useful culture system for researching the self-renewal and differentiation mechanisms of stem cells. In this study we have studied the properties of NPCs in 3-D collagen scaffolds, and further elucidated the regulatory mechanism of NPC self-renewal and differentiation in 3-D collagen scaffolds. REDD1-mTOR pathway was found to participate in regulating the self-renewal and differentiation of NPCs.

Collagen is the major class of insoluble fibrous protein in extracellular matrix (ECM). As a natural material, collagen is well developed for 3-D culture. NPCs derived from spinal cord cultured on electrospun aligned collagen nanofibrous scaffolds exhibited a strong proliferation [34,35]. It was also reported that collagen hydrogel could promote the neurogenesis or gilogenesis of NPCs [36,37]. However in our experiments, the fewer neuronal differentiation (Fig. 1G), the higher expression of Hes and Id (Figs. 2 and 4C), and the higher level of clone formation efficiency (Fig. 3) all indicated that the self-renewal of NPCs was maintained in the 3-D collagen scaffolds. Unlike hydrogel, the collagen scaffolds in our experiments were sponge-like scaffolds with an average pore size of 100 μm (Fig. 1B). NPCs culture in the collagen scaffold tended to aggregate (Fig. 1C,D). The physical microenvironments offered by our collagen scaffolds were also different from those offered by hydrogel. These may act as a cause of the different behaviors of NPCs in our 3-D collagen scaffolds.

We have previously found that mTOR play a role in the neurogenesis and self-renewal of NPCs in 2-D *in vitro* culture ([25] Fig. S1). In this study, lower mTOR activity was proved to support the high clone formation efficiency and higher Id protein expression of NPCs in 3-D collagen scaffolds (Fig. 4). These results suggested that mTOR inhibition is also vital to the self-renewal of 3-D cultured NPCs. We have found that the inactivation of mTOR was attributed to REDD1 overexpression in 3-D culture. REDD1 was initially identified as a stress-responsive gene. It blocked the activation of mTOR through the tuberous sclerosis complex (TSC1/TSC2) [38–40]. REDD1 elevation was also found to be essential to neuron death in Parkinson's disease [27,41]. It was reported that REDD1 protected dividing cells from apoptosis, but induced the apoptosis of non-dividing cells, like neurons [42]. In our study, we found higher expression of REDD1 in NPCs and few neurons differentiated in 3-D culture (Figs. 1G and 5), but the total cell number in 3-D scaffolds did not changed during the detected period (Fig. S2). This suggested that the REDD1 overexpression may not induced apoptosis of NPCs, but functions in the self-renewal of NPCs.

Recently, REDD1 was found to play a role in the developmental processes. Ellisen *et al.* found that during mouse embryonic development, the expression pattern of REDD1 paralleled that of

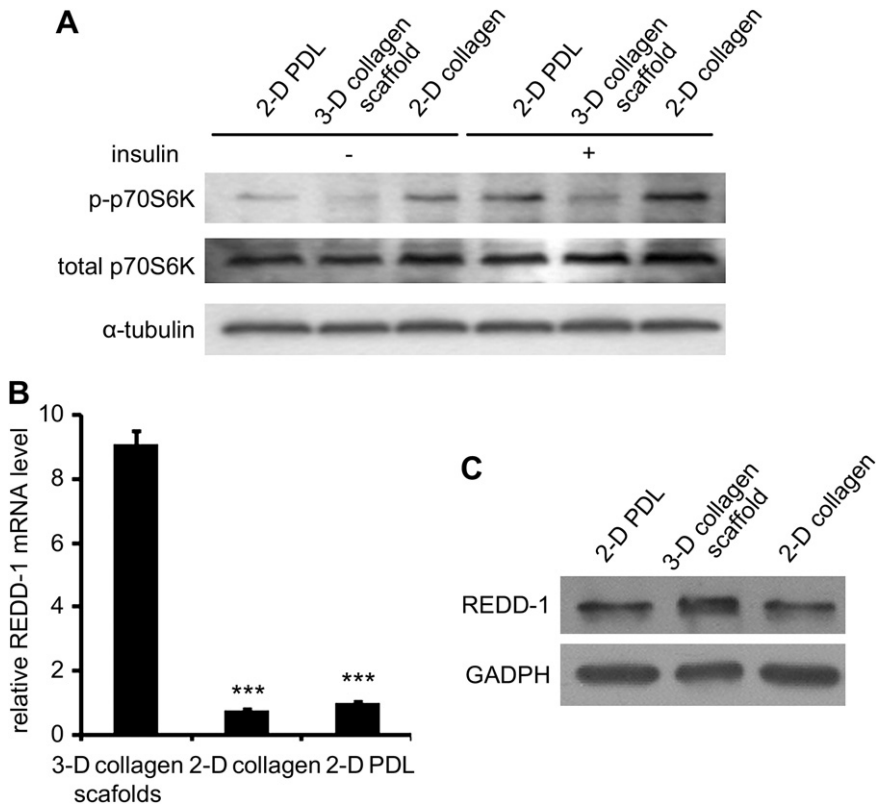

Fig. 5. REDD1 was overexpressed in 3-D cultured cells. (A) insulin could not lift the phosphorylation of p70S6K of 3-D and 2-D groups to a common level. (B-C) mRNA (B) and protein (C) expression of REDD1 both upregulated in 3-D group.

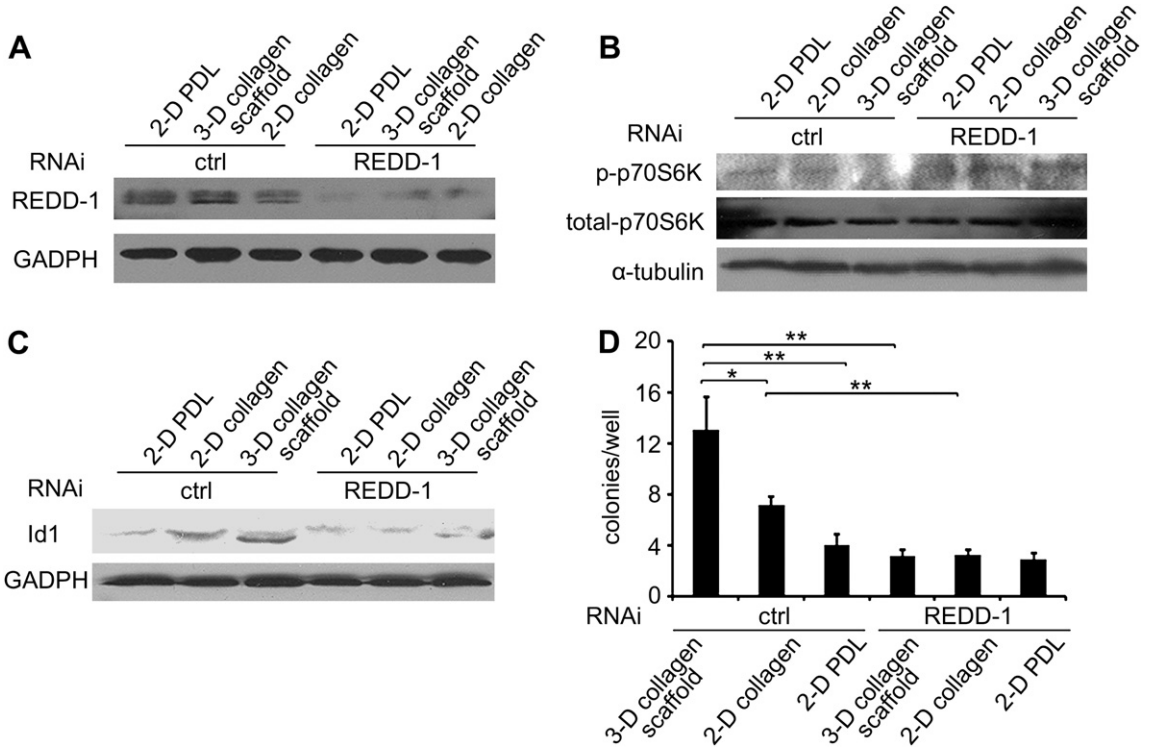

Fig. 6. REDD1 was vital to the stemness maintenance of 3-D- cultured NPCs. (A) REDD1 RNAi lentivirus effectively reduced the expression of REDD1. (B) REDD1 knocking-down induced the phosphorylation of p70S6K of 3-D and 2-D groups to a similar level. (C) REDD1 knocking-down suppressed the Id expression of 3-D and 2-D group to a similar level. (D) REDD1 knocking-down attenuated the clone formation efficiency of 3-D and 2-D to a similar level.

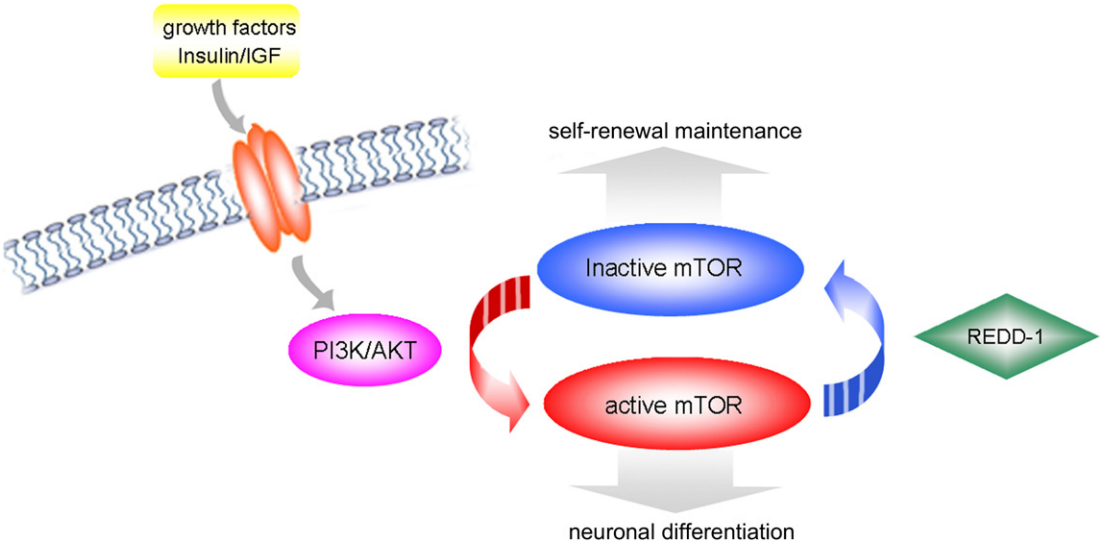

Fig. 7. The diagram of the vital position of mTOR in regulating the differentiation and self-renewal of NPCs.

p63 which is essential to epithelial differentiation, and REDD1 was upregulated by p63 [43]. REDD1 was necessary to retinoic acid (RA)-induced myeloid differentiation, and REDD1 was upregulated by RA [44]. REDD1 was also found to mediate the inhibition of mTOR by Plzf during the stemness maintenance of SPCs, and REDD1 was upregulated by Plzf [32]. Moreover, during neural differentiation, the involvement of REDD1 has been detected. *In vivo*, neuronal differentiation and neuron migration were delayed by REDD1 overexpression [33]. In our study, REDD1 mediated the stemness maintenance of 3-D cultured NPCs (Figs. 5 and 6), which further confirmed that REDD1 functioned in developmental process. However, the upstream regulators of REDD1 in regulating the self-renewal of NPCs need further exploration.

As we all know, the differentiation and self-renewal of NPCs is modulated by several signal pathways. mTOR pathway was newly identified as an important participant in regulating the differentiation and self-renewal of NPCs. In general, mTOR is investigated as an effecter of extracellular growth factor signals, especially insulin/IGF signals. Bateman *et al.* released that TOR functioned in the temporal development of *Drosophila* under the control of insulin receptor [22]. We have previously reported mTOR's respond to insulin stimulation supported the neuronal differentiation of 2-D-cultured NPCs [25]. In 3-D culture, a negative regulator of mTOR, REDD1 was identified to support the mTOR inactivation and self-renewal of NPCs (Figs. 5 and 6). From these results, we speculated that mTOR underwent dual regulation in controlling the self-renewal and differentiation of NPCs. Insulin and other growth factors induced mTOR activity and functioned as a positive regulator to differentiation and as a negative regulator to self-renewal. REDD1, depressing mTOR activity, functioned just opposite to growth factors (Fig. 7). As to the profound mechanisms, which signals take charge of or interact with REDD1-mTOR signal to maintain the self-renewal statement of NPCs, need to be further investigated.

5. Conclusion

NPCs were cultured in 3-D collagen scaffolds and on 2-D PDL- and collagen-coating plates. We have found that the neuronal differentiation of NPCs cultured in 3-D scaffolds was decreased compared with those cultured on 2-D plating. In the differentiation medium, NPCs cultured in 3-D scaffolds maintained higher secondary cloning efficiency compared with NPCs cultured on 2-D plates. We further investigated the mechanisms controlling the self-renewal and differentiation of NPCs in 3-D culture. The inactivated mTOR was found to support the self-renewal of NPCs cultured in 3-D scaffolds. The mTOR inactivation was attributing to the overexpression of REDD1 by NPCs cultured in 3-D scaffolds, and REDD1 knocking-down abolished the self-renewal maintenance of NPCs in 3-D scaffolds.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2012.11.063.

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