Tissue Eng Regen Med (2024) 21(2):309–318
https://doi.org/10.1007/s13770-023-00598-x

Online ISSN 2212-5469 TERM

ORIGINAL ARTICLE

mTOR Plays an Important Role in the Stemness of Human Fetal Cartilage Progenitor Cells (hFCPCs)

Him-Cha Shin[1] · Jiyoung Kim[2] · So Ra Park[1] · Byung Hyune Choi[2]

Received: 18 August 2023 / Revised: 18 August 2023 / Accepted: 7 September 2023 / Published online: 9 October 2023
© Korean Tissue Engineering and Regenerative Medicine Society 2023

Abstract

BACKGROUND: Mammalian target of rapamycin (mTOR) is known to regulate self-renewal ability and potency of embryonic stem cells (ESCs) and adult stem cells in opposite manners. However, its effects vary even among adult stem cells and are not reported in fetal stem/progenitor cells. This study investigated the role of mTOR in the function of human fetal cartilage-derived progenitor cells (hFCPCs).

METHODS: mTOR activity in hFCPCs was first examined via the level of phosphor-mTOR until passage 19, together with doubling time of cells and senescence-associated b-galactosidase (SA-bGal). Then, the effect of 100 nM rapamycin, the inhibitor of mTOR, was investigated on self-renewal ability, proliferation rate and osteogenic/adipogenic potential of hFCPCs *in vitro*. Expression of stemness genes (Oct-4, Sox2 and Nanog) and cell cycle regulators (CDK4 and Cyclin D1) was measured at mRNA or protein levels.

RESULTS: mTOR activity was maintained constantly at high levels in hFCPCs until passage 19, while their proliferation rate was decreasing from 48 h at passage 13 to 70 h at passage 9 and senescent cells were observed at passage 18 ($8.3 \pm 1.2\%$) and 19 ($15.6 \pm 1.9\%$). Inhibition of mTOR in hFCPCs impaired their colony forming frequency (CFU-F) by 4 folds, while showing no change in their doubling time and expression of CDK4 and Cyclin D1. Upon mTOR inhibition, Oct4 expression decreased by 2 folds and 4 folds at the mRNA and protein levels, respectively, while that of Sox2 and Nanog did not change significantly. Finally, mTOR inhibition reduced osteogenic and adipogenic differentiation of hFCPCs *in vitro*.

CONCLUSION: This study has shown that mTOR plays an important role in the self-renewal ability of hFCPCS but not in their proliferation, The effect of mTOR appears to be associated with Oct-4 expression and important in the osteogenic and adipogenic differentiation ability of hFCPCs.

Keywords Human fetal cartilage progenitor cells (hFCPCs) · Mammalian target of rapamycin (mTOR) · Stemness · Rapamycin

✉ Byung Hyune Choi
bryan@inha.ac.kr

[1] Department of Physiology and Biophysics, Inha University College of Medicine, 100 Inha-ro, Michuhol-gu, Incheon 22212, South Korea

[2] Department of Biomedical Sciences, Inha University College of Medicine, 100 Inha-ro, Michuhol-gu, Incheon 22212, South Korea

1 Introduction

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase and belongs to the phosphoinositide 3-kinase (PI3K) kinase family. mTOR is activated by various extracellular cues such as growth factor, nutrient state, and amino acid levels and involved in diverse cellular events including protein synthesis, metabolism, autophagy and cell growth [1, 2]. mTOR consists of two complex, mTOR

complex1 (mTORC1) and mTOR complex2 (mTORC2). mTORC1 and mTORC2 is distinguished by their adaptor proteins such as regulatory-associated protein of mTOR (Raptor) and rapamycin-insensitive companion of mTOR (Rictor), respectively [3, 4]. Besides, mTORC1 is directly inhibited by rapamycin but mTORC2 is insensitive to it [1]. mTORC1 positively regulates cell proliferation and growth by promoting protein and lipid synthesis through its downstream target of p70 ribosomal protein S6 kinase (S6k1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). mTORC1 also regulates mitochondria function and autophagy [5]. The biological activity of mTORC2 is much revealed but it is known to regulate cell survival and cytoskeleton function by phosphorylating serum/glucocorticoid regulated kinase 1 (SGK1) and protein kinase C (PKC) [6, 7].

mTOR plays important roles in the regulation of various stem cell function like cell proliferation, self-renewal and differentiation [8]. Homozygous mutation of mTOR impairs cell proliferation in developing mouse embryo and embryonic stem cells (ESCs) *in vitro* [9]. Inhibition of mTOR also impairs self-renewal ability and proliferation of human ESCs, while enhances their endodermal and mesodermal differentiation potential [10]. At the molecular level, mTOR enhances expression of pluripotency genes and represses expression of genes inhibiting cell growth and development, which are essential for the long-term undifferentiated growth and maintenance of pluripotency in human ESCs [10]. However, there is also a conflicting report that activation of mTOR by withdrawal of leukemia inhibitor factor (LIF) causes down-regulation of pluripotency genes of Oct-4, Sox2 and Nanog [11].

The role of mTOR seems somewhat different in adult stem cells than in ESCs [8]. mTOR plays an important role in homeostasis of hematopoietic stem cells (HSCs) [12]. mTOR activity is relatively low in HSCs than in hematopoietic progenitor cells (HPCs) [13, 14], but increases in HSCs along with ages [15]. Activation of mTOR by deletion of its negative regulators like PTEN, TSC1 and PML drives them from quiescence into proliferation but subsequently reduces hematopoiesis and self-renewals of HSCs, which eventually leads to their exhaustion [13, 14]. Similarly, conditional deletion of TSC1 in HSCs of young mice mimicks the phenotype of HSCs of old mice, while inhibition of mTOR by rapamycin increases life span and self-renewal ability of HSCs in old mice [15]. Similar results are also reported in epidermal stem cells and mammary stem cells where activation of mTOR causes cellular senescence and exhaustion of stem cell pools [13], Mohapatra et al. In human mesenchymal stem cells (MSCs), inhibition of mTOR activity by rapamycin also increases their self-renewal ability, differentiation potential and regenerative potential *in vitro* [17].

We have previously reported human fetal cartilage-derived progenitor cells (hFCPCs) of gestation age 12–14 (GA 12-14) have stem cell properties and can expand up to 28–30 passages without significant changes in the doubling time when cultured in an optimized culture medium [18, 19]. Similar to MSCs, they also show immune-privilege and immune-modulatory activity and have anti-inflammatory effect on interleukin-1β(IL-1β)-treated synoviocytes *in vitro* [20, 21]. Stem cells or progenitor cells from fetal tissues are reported in various sources such as bone marrow, lung, blood, kidney, brain, liver and cartilage [18, 22–26]. They have high telomerase activity and often express some of the pluripotent genes [18, 22, 23], and show higher stem cell property than adult stem cells [24, 25]. Although they share some of ESCs properties, they are committed cells and pre-terminally differentiated into specific lineages therefore presumed to be more similar to adult stem cells [27]. However, the role of mTOR in fetal stem/progenitor cells are not understood yet. In this study, we investigated mTOR activity and its role in the proliferation, self-renewal and differentiation abilities of hFCPCs.

2 Materials and methods

2.1 Culture of hFCPCs

hFCPCs were kindly provided by professor Byoung-Hyun Min at Cell Therapy Center, Ajou University School of Medicine. Cells were originally isolated from human fetal cartilage tissue of gestation age 12 with the approval of institutional review board (IRB) of Ajou University (AJIRB-MED-SMP-10-268). hFCPCs at passage 2 were cultured at 8×10^3 cells/cm^2 in minimal essential medium alpha (α-MEM; Gibco, Carlsbad, CA) supplemented with 1% fetal bovine serum (FBS; ATCC, Manassas, VA), 100 U/mL penicillin (Gibco) and 100 μg/mL streptomycin (Gibco). Culture medium was replenished every 2 days and cells were harvested for subculture using 0.05% trypsin–EDTA (Gibco) at 80% confluency. The proliferation rate of cells was calculated at each passage by the following formula: Doubling time = (t2-t1)×log2/(logN2–logN1), where N2 is cell number at harvest (t2), N1 is cell number at seeding (t1), and t2-t1 is culture time in hours (hr).

2.2 Cellular senescence assay

hFCPCs at passages 17, 18 and 19 were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde (Sigma, St-Louis, MO, USA) for 10 min at room temperature. Then, samples were incubated with senescence-associated β-galactosidase (SA-β-gal) staining

solution (Cell signaling, Beverly, MA) for 12 h at 37 °C. SA-β-gal positive cells shown in blue color were counted under an inverted microscope (Eclipse TS100; Nikon, Tokyo, Japan) and the percentage of SA-β-gal positive cells among the total cell number was calculated.

2.3 Colony-forming unit-fibroblasts (CFU-F) assay

hFCPCs at passage 5 were cultured in α-MEM as above at a density of 30 cells/60 mm dish (Nunc, New York, NY) in the presence of 0, 0.1, 1, 10, 50, and 100 nM rapamycin (Invitrogen, Carlsbad, CA). After 14 days, cells were washed with PBS, fixed for 15 min with 4% paraformaldehyde (Sigma) in PBS, and stained for 30 min with 0.5% crystal violet (Sigma). Colonies with larger than 3 mm in diameter were counted and the colony forming efficiency was calculated by the percentage to the seeding cell number.

2.4 WST-1 assay

FCPCs at passage 5 were seeded at 5×10^3 cells in 96 well plate and cultured for 10 days in the presence of 0, 0.1, 1, 10, 50, and 100 nM rapamycin. Cells were treated with 10% (v/v) WST-1 solution (EZ-CYTOX; DoGenBio, Seoul, Korea) and incubated for 1 h at 37 °C. The absorbance was measured at 450 nm using a microplate reader.

2.5 Quantitative reverse-transcriptase PCR (q-RT-PCR)

Total RNA was extracted from cells by TRIZOL reagent (Invitrogen), and its concentration was quantified using Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) at 260 nm. Total RNA of 1 μg was used to synthesize cDNA using a reverse transcription kit (ELPIS Biotech, Daejeon, Korea) and T100™ Thermal Cycler (Bio-Rad, Hercules, CA). The reaction was carried out sequentially for 10 min at 25 °C, for 60 min at 42 °C, and for 10 min at 75 °C. For quantitative real-time PCR, 1X SYBR Green Reaction Mix (Toyobo, Osaka, Japan) was used with 1 μg cDNA and 10 pM of each primer. Target genes and primer sequences used are as follow: Oct-4, 5'-ATGGGGAAGGTGAAGGTCG-3' and 5'-TAAAAGCAGCCCTGGTGACC-3', Sox2, 5'-AGGGG-GAAAGTAGTTTGCTGCCT-3' and 5'-TGCCGCC GCCG ATGATTGTT-3', Nanog, 5'-CCTTGGCTGC CGTCTCTGGCT-3' and 5'-AGC AAAGCCTCCCAATC CCAA-3', CDK4, 5'-AGGCTTGCCAGTGGAGACCA TAAA-3' and 5'-CCAGGACCAGCTCCATGTG-3', and GAPDH, 5'-ATGGG GAAGGTGAAGGTCG-3' and 5'-TAAAAGCAGCCCTGGTGACC-3'. The reaction was performed for 30–40 cycles of 95 °C for 10 s and 60 °C for 30 s using CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Ct values normalized to that of GAPDH were presented in histograms.

2.6 Western blotting

Cells were lysed in RIPA buffer (Sigma) containing protease inhibitor cocktail for 30 min on ice. Cell lysate was harvested by centrifugation at 12,000 rpm for 20 min at 4 °C and protein concentration was determined using Bio-Rad Protein Assay Kit I (Bio-Rad) at 595 nm. Ten micrograms of lysate were separated on a 8% ~ 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. Protein bands were transferred to polyvinylidene difluoride (PVDF; Bio-Rad) membranes using Trans-Blot SD Semi Dry Transfer Cell (Bio-Rad) at 22 V for 1 h. Membranes were blocked with 5% non-fat skin milk (Blocking-Grade Blocker; Bio-Rad) in 1X Tris-buffered saline-Tween 20 (TBST; 10 mM Tris–HCl, 150 mM NaCl, and 0.05% Tween-20) at room temperature for 1 h. Membranes were then incubated with primary antibodies at 4 °C overnight and subsequently with horseradish peroxidase (HRP)-conjugated anti-IgG secondary antibody for 1 h at 4 °C. Specific bands were visualized using an ECL detection kit (ELPIS Biotech). The intensity of bands was quantitated by Image J software and normalized to that of β-actin to present in histograms. Primary antibodies used are as follow: anti-mTOR (phospho S2448) (1:10,000; ab109268, abcam), anti-S6K1 (phospho T389) (1:10,000; ab126818, abcam), anti-Oct-4 (1:5,000; ab18976, abcam), anti-Sox2 (1:5,000; ab97959, abcam) and anti-β-actin (1:1,000; sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibody used was goat anti-rabbit IgG H&L (HRP) (1:10,000; ab6721, abcam).

2.7 Lineage differentiation

hFCPCs at passage 5 were seeded at 5×10^4 cells/well in 6 well plates and cultured in adipogenic or osteogeic medium in presence of 0 or 100 nM rapamycin. Adipogenic medium consisted of low glucose Dulbeco's modified Eagle's medium (DMEM-LG; Gibco) with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μM dexamethasone (Sigma), 500 nM 3-isobutyl-1-methylxanthine (IBMX; Sigma), 0.05 mg/mL human insulin (Sigma), and 100 μM indomethacin (Sigma). After 2 weeks, cells were stained with oil red O dye (Sigma). Positive stains were extracted in 100% isopropanol and quantified at 500 nm using a microplate reader. Osteogenic differentiation was performed using StemPro® Osteogenesis Differentiation Kit (Gibco) consisting of Osteocyte/Chondrocyte Differentiation Basal Medium with 10% Osteogenesis Supplement, 100 U/mL penicillin and 100 μg/mL

streptomycin. After 2 weeks, cells were stained with alizarin red S dye (Sigma). Positive stains were extracted in 100 nM cetylpyridinium chloride (Sigma) and quantified at 570 nm using a microplate reader.

2.8 Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's host hoc test using SPSS software (IBM, Armonk, NY). The data was presented as mean $\pm$ standard deviation (SD) from 3 independent experiments (n = 3). Statistical significance was assessed by $*p < 0.05$ and $**p < 0.01$.

3 Results

3.1 mTOR activity was maintained at constant levels along with the long-term passages of hFCPCs

Changes in the mTOR activity was first investigated in hFCPCs along with passages until passage 19 by Western blot analysis for phospho-mTOR at serine 2448 (p-mTOR) and phospho-S6k at threonine 389 (p-S6k), the downstream molecule of mTOR. When examined at passages 3, 5, 7, 17, 18 and 19, the amount of p-mTOR and p-S6k was very high at passage 3 and did not change significantly until passage 19 (Fig. 1A). When the proliferation rate of cells was compared, the doubling time was maintained constantly around 48 h until passage 13, and gradually increased thereafter from 50 h at passage 14 to 70 h at passage 19 (Fig. 1B). The percentage of senescent cells positive for SA-β-gal staining was 0% at passage 17, 8.3 $\pm$ 1.2% at passage 18, and 15.6 $\pm$ 1.9% at passage 19 (Fig. 1C). These results suggest that mTOR activity is relatively high and maintained at constant levels along with passages in hFCPCs regardless of proliferation delay and appearance of senescent cells at late passages.

3.2 Inhibition of mTOR impaired self-renewal ability of hFCPCs without affecting their proliferation rate

To investigate the role of mTOR in the self-renewal ability of hFCPCs, rapamycin was treated at 0, 0.1, 1, 10, 50 and 100 nM during CFU-F assay (Fig. 2). Inhibition of mTOR activity by rapamycin resulted in a dose-dependent decrease in the colony forming ability of hFCPCs. The CFU-F frequency of hFCPCs was reduced approximately 4 folds by 100 nM rapamycin when compared with that of the untreated control. For comparison, the colony forming ability of human bone marrow MSCs did not affected by 100 nM rapamycin (data not shown).

Then, we investigated whether the reduction in the CFU-F frequency by rapamycin was due to its cytotoxicity or inhibition of cell proliferation. In the WST-1 assay, treatment of rapamycin at 0, 0.1, 1, 10, 50 and 100 nM for 10 days did not affect cell viability or proliferation rate of hFCPCs (Fig. 3A). Treatment of 100 nM rapamycin did not affect doubling time of hFCPCs measured at passages 4, 5 and 6 (Fig. 3B). Finally, treatment of rapamycin for 10 days did not affect the expression of cell-cycle related gene of CDK4 and cyclin D1 in hFCPCs (Fig. 3C). These result suggest that mTOR is necessary for self-renewal ability of hFCPCs but not for cell viability or proliferation ability.

3.3 Inhibition of mTOR reduces Oct-4 expression of hFCPCs

To understand the underlying mechanism of decrease in the self-renewal ability of hFCPCs by inhibition of mTOR, we then examined the effect of rapamycin treatment on the expression of pluripotency genes in hFCPCs. In qRT-PCR analysis, rapamycin at 100 nM reduced the expression of Oct-4 ($p < 0.05$) by approximately 2 folds but did not affect that of Sox2 and Nanog in hFCPCs (Fig. 4A). The decrease in the Oct-4 expression by 100 nM rapamycin was also confirmed at the protein level by Western blot analysis (approximately fourfold reduction, $p < 0.01$), where it did not change the amount of Sox2 protein (Fig. 4B).

The inhibition of mTOR activity by rapamycin was confirmed by reduced amount of p-S6k ($p < 0.01$) and β-actin was used as an internal control. It appears that mTOR activity is important in the expression of Oct-4 but not Sox2 and Nanog.

3.4 Inhibition of mTOR reduced adipogenic and osteogenic differentiation abilities of hFCPCs

Previously, inhibition of mTOR by rapamycin increased osteogenic differentiation ability of MSCs cultured in the presence of fibroblast growth factor-2 (FGF-2), while it did not show any effect of their adipogenic differentiation [24]. We then examined the effect of mTOR inhibition by rapamycin on the adipogenic and osteogenic differentiation of hFCPCs (Fig. 5). hFCPCs were cultured in the adipogenic or osteogenic induction medium for 14 days with or without 100 nM rapamycin. In the result, 100 nM rapamycin reduced adipogenic differentiation of hFCPCs by approximately 2 folds in the Oil red O staining (Fig. 5A) and osteogenic differentiation of hFCPCs by

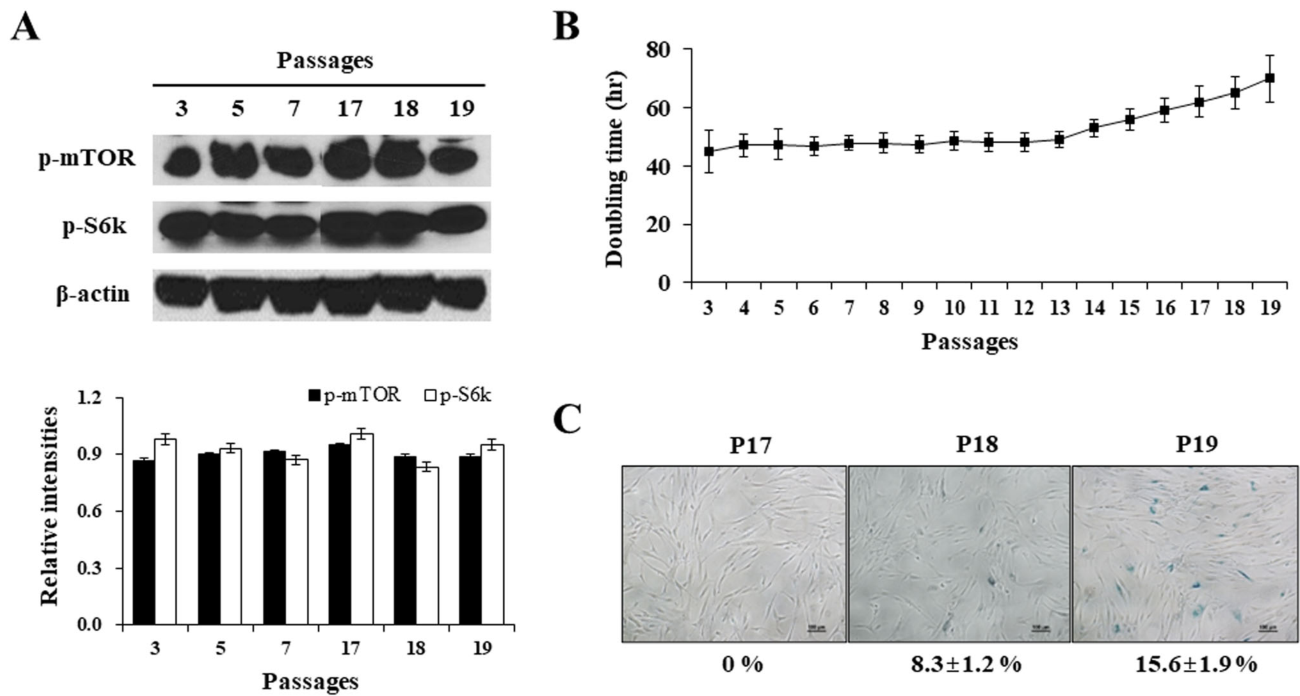

Fig. 1 mTOR activity in hFCPCs during the long-term passages *in vitro*. hFCPCs were cultured until passage 19. **A** Phosphorylation of mTOR (p-mTOR) and S6k (p-S6k) was assessed by Western blotting at passages 3, 5, 7, 17, 18, and 19. β-actin was used as an internal control. The band intensities were quantitated using Image J software. The normalized data for p-mTOR and p-S6k against that of β-actin is presented in the histogram (n = 3). **B** The proliferation rate of hFCPCs was assessed by the doubling times (hr) at all passages from 3 to 19 (n = 3). **C** Senescence of hFCPCs were examined by senescent associated β-galactosidase staining at late passages of 17, 18 and 19. The number of positive cells was calculated and noted at the bottom of each image (n = 3). Scale bar = 100 μm. All data are presented by mean ± SD from 3 independent experiments (n = 3)

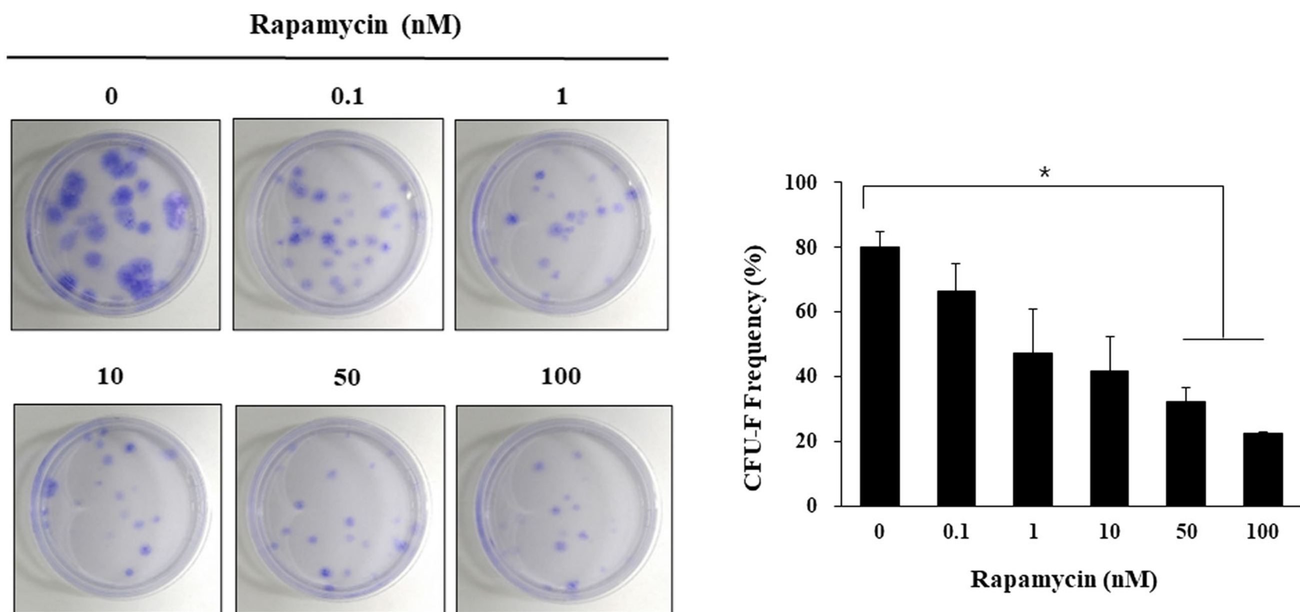

Fig. 2 Effect of rapamycin, an mTOR inhibitor, on the colony forming ability (CFU-F) of hFCPCs. The CFU-F assay was performed with hFCPCs at passage 3 in the presence of rapamycin at 0, 0.1, 1, 10, 50, and 100 nM. The number of colonies larger than 3 mm in diameter was counted and presented by mean ± SD from 3 independent experiments (n = 3). $*p < 0.05$

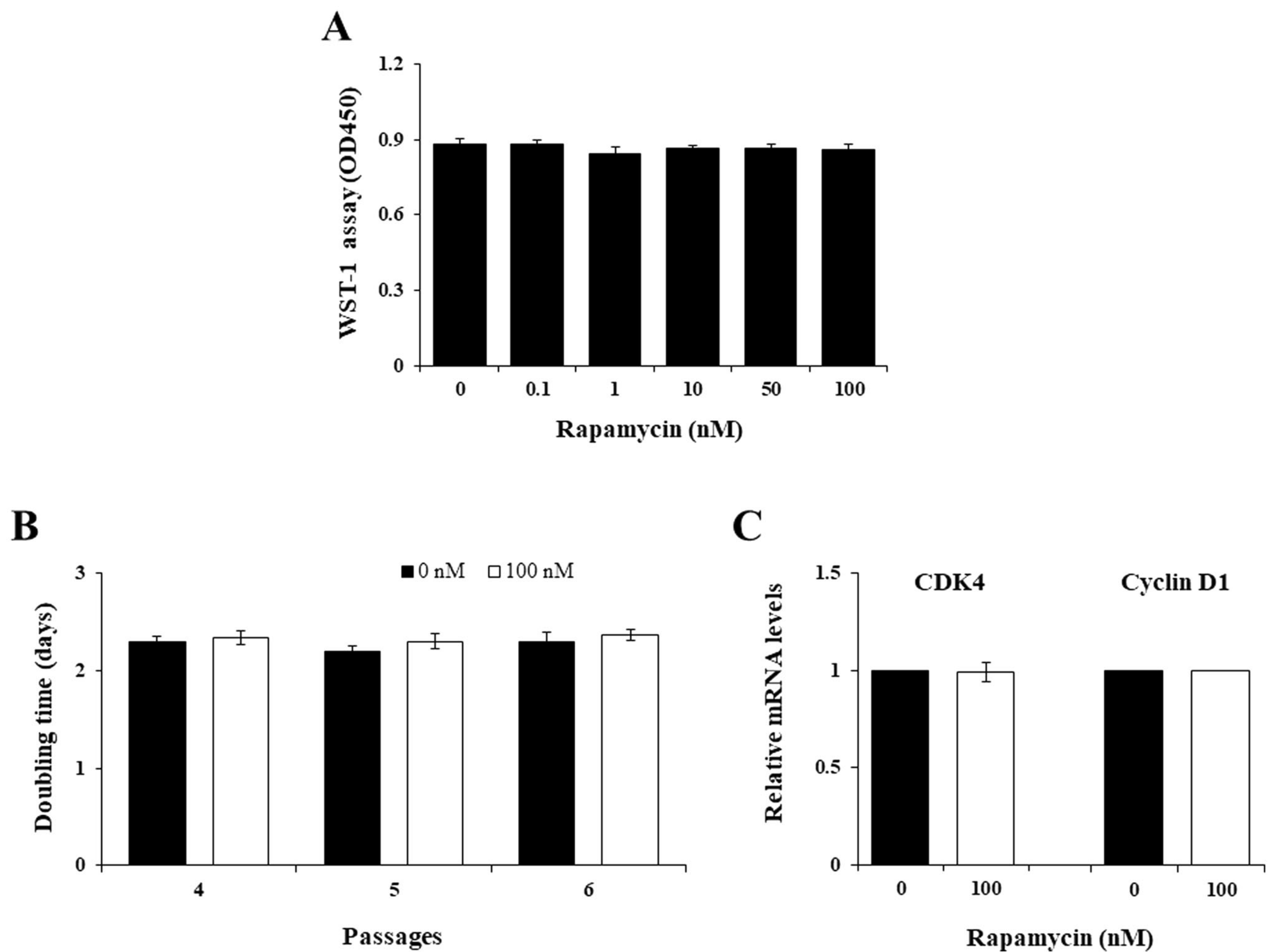

Fig. 3 Effect of rapamycin on the viability and proliferation of hFCPCs. **A** hFCPCs at passage 3 were cultured in the presence of rapamycin at 0, 0.1, 1, 10, 50 and 100 nM. After 10 days, WST-1 assay was performed to evaluate cell viability. **B** hFCPCs at passage 3 were cultured with 0 or 100 nM rapamycin until passage 6. Proliferation rate of cells was determined by the doubling times (days) at each passage. **C** Effect of rapamycin treatment at 100 nM on the expression of cell cycle related genes of CDK4 and cyclin D1 was examined by RT-PCR. Relative values were shown in the histogram. All data are presented by mean ± SD from 3 independent experiments (n = 3)

approximately 3.6 folds in the Alizarin red S staining (Fig. 5B). This result suggests that mTOR is important in both adipogenic and osteogenic abilities of hFCPCs.

4 Discussoin

In this study, we investigated the role of mTOR in self-renewal, proliferation and differentiation abilities of hFCPCs. We found that mTOR activity is relatively high in hFCPCs and constantly maintained along with the long-term culture until passage 19. Since approximately 15.6% of hFCPCs are senescent cells with the increase of doubling time from 48 h at passage 13 to 72 h at passage 19, the constant mTOR activity until passage 19 indicates that it is not involved in the replicative senescence of hFCPCs. This result is different from previous reports that persistent mTOR activity is associated with premature senescence and impaired self-renewal ability in adult stem cells eventually depleting its pool *in vivo* [13–16]. Activation of mTOR is known to increase cell proliferation transiently while reducing self-renewal ability of HSCs [14], but it is not clear if mTOR differentially regulates cell proliferation and self-renewal of adult stem cells. In human neural stem cells (NSCs), inhibition of mTOR by rapamycin reduces neuronal differentiation without affecting their proliferation [28]. However, in another report, mTOR activity is low in NSCs of aged mice and its activation increases NSCs proliferation and neurogenesis [29].

Inhibition of mTOR is known to increase life span of many animal species such as mice, yeast, Drosophila and even C. elegans [15, 30–33] and enhance stem cell property

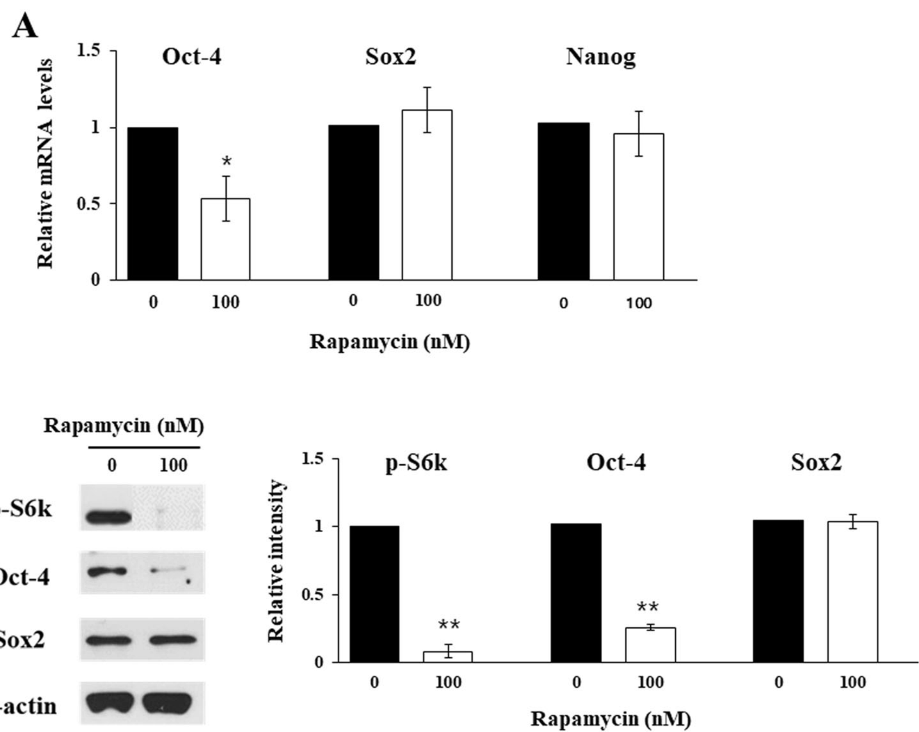

Fig. 4 Effect of rapamycin on the expression of pluripotency genes in hFCPCs. hFCPCs at passage 5 were cultured for 14 days with or without 100 nM rapamycin. **A** mRNA levels of Oct4, Sox2 and Nanog were examined by qRT-PCR. Relative mRNA values are shown in the histogram. **B** Protein levels of p-S6k, Oct-4 and Sox2 were examined by Western blot analysis. Band intensities were obtained by Image J software and normalized values by that of β-actin were presented in the histogram. All data are presented by mean ± SD from 3 independent experiments (n = 3). $*p < 0.05$ and $**p < 0.01$

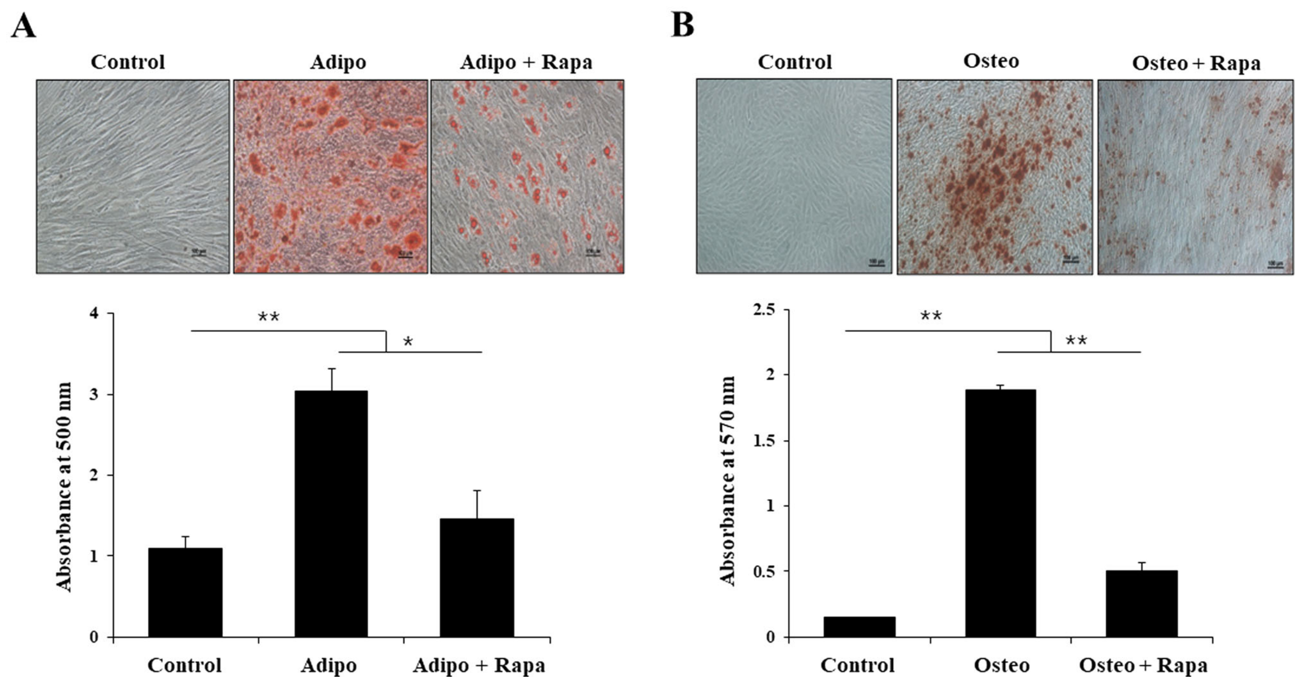

Fig. 5 Effect of rapamycin on the differentiation ability of hFCPCs. hFCPCs at passage 5 were induced to differentiate into adipogenic and osteogenic lineages with or without 100 nM rapamycin for 14 days. **A** Adipogenic differentiation of hFCPCs were examined by oil red O staining. Lipid droplets were extracted and the signal intensity at 500 nm was presented in the histogram. **B** Osteogenic differentiation of hFCPCs were examined by alizarin red S staining. Calcium deposits were extracted and the signal intensity at 570 nm was presented in the histogram. Scale bars = 100 μm. All data are presented by mean ± SD from 3 independent experiments (n = 3). $*p < 0.05$ and $**p < 0.01$

of HSCs and MSCs [17]. In contrast, mTOR is known to play an important role in the proliferation and self-renewal ability of ESCs [9, 10]. It is also reported that down-regulation of mTOR activity is required for the reprograming of somatic cells to gain stemness [34], which further supports the opposite roles of mTOR in the self-renewal ability of adult stem cells versus ESCs. In this study, inhibition of mTOR by rapamycin significantly reduced self-renewal ability of hFCPCs without decreasing their proliferation rate. Therefore, like ESCs, mTOR might be necessary for the self-renewal ability of hFCPCs.

However, the situation looks more complicated than we can think from these results. We have previously shown that self-renewal ability of hFCPCs decreases gradually along with the passages [19], while mTOR activity is maintained high consistently in this study. Therefore, there could be other mechanism(s) regulating self-renewal ability of hFCPCs in addition to mTOR. It is also reported that dual inhibition of mTORC1/2 by AZD8055 suppresses clonogenic function of hematopoietic progenitor cells (HPCs), while enhances generation of HSCs [35]. Known that HSCs are more primitive cells than HPCs, this result is inconsistent with the other reports mentioned above, which suggests that the role of mTOR could be cell-type specific or different even among different adult stem/progenitor cells. Considering that mTOR is one of key regulators of homeostasis and involved in a variety of cellular events, it could exert diverse and sometimes conflicting effects on different types of stem cells under different context.

In adult stem cells, activation of mTOR is known to increase ROS generation in HSCs [14], while inhibition of mTOR decreases ROS generation in MSCs [17] or increases expression of mitochondrial superoxide dismutase (MnSOS) leading to inhibition of ROS generation and oxidative stress in epithelial stem cells [36]. Either activation or inhibition of mTOR leads to increased expression of cell cycle inhibitors in HSCs [15] or pluripotency genes in MSCs [17], but their correlation with ROS generation is not clearly revealed yet. These effect of mTOR in adult stem cells must be different in ESCs or hFCPCs where mTOR plays an import role in the self-renewal ability. Inhibition of mTOR in human ESCs is known to suppress expression of ESCs markers such as Oct-4, Sox2, and Nanog [10]. Our result shows that inhibition of mTOR by rapamycin decreases Oct-4 expression but not Sox2 and Nanog, while efficiently inhibits self-renewal ability of hFCPCs. Therefore, gene expression network regulating self-renewal ability of hFCPCs is different from that of ESCs, being sufficient only with Oct-4. The specific mechanism of Oct-4 action in hFCPCs is not clear at present and needs further investigation.

mTOR is important in maintaining pluripotency and blocking spontaneous commitment of human ESCs into mesodermal and endodermal, but not ectodermal lineages [10, 37]. In contrast, inhibition of mTOR by rapamycin enhances osteogenic differentiation potential but not adipogenic potential of MSCs [17]. Another study showed that knockout of Raptor, the adaptor of mTORC1, promotes osteogenic potential and reduces adipogenic potential in MSCs, whereas knockout of Rictor, the adaptor of mTORC2, leads completely opposite results [38]. These findings suggest that mTOR is not merely involved in the self-renewal ability, though in opposite ways, in ESCs and MSCs, but regulates their differentiation potential in a lineage-specific manner. In this study, inhibition of mTOR by rapamycin reduced both osteogenic and adipogenic potential of hFCPCs. It is a different result from that of MSCs, but similar to that of ESCs because both ESCs and hFCPCs lost their stemness and potency upon mTOR inhibition. However, hFCPCs are already committed cells to the chondrogenic lineage and would not differentiate into osteogenic and adipogenic lineages spontaneously upon mTOR inhibition. We found that mTOR inhibition did not affect chondrogenic differentiation of hFCPCs significant (data not shown) and speculate that it impaired the plasticity of hFCPCs to osteogenic and adipogenic lineages upon forced differentiation condition.

In conclusion, this study showed that inhibition of mTOR impairs self-renewal ability of hFCPC without affecting their proliferation rate. This phenomenon seems correlated with reduced expression of Oct-4 but not Sox2 and Nanog among the key stemness genes, and loss of their plasticity to osteogenic and adipogenic lineages. These findings are somewhat different from previous results shown in adult stem cells or ESCs and further studies are needed to investigate underlying mechanism(s).

Acknowledgement This research was supported by the research grant from Inha University (63000-1).

Data availability The datasets generated during or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no potential conflicts of interest.

Ethical statement This study was conducted in accordance with the institutional review board (IRB) of Ajou University Medical Center (AJIRB-MED-SMP-10–268). This study does not contain any experiment with animal subjects.

References

1. Weichhart T. mTOR as regulator of lifespan, aging, and cellular senescence: a mini-review. Gerontol. 2018;64:12–34.

2. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol. 2011;12:21–35.
3. Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, et al. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell. 2002;110:177–89.
4. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol. 2004;14:1296–302.
5. García-Martínez JM, Moran J, Clarke RG, Gray A, Cosulich SC, Chresta CM, et al. Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR). Biochem J. 2009;421:29–42.
6. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell. 2012;149:274–93.
7. Jacinto E, Loewith R, Schmidt A, Lin S, Rüegg MA, Hall A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 2004;6:1122–8.
8. Meng D, Frank AR, Jewell JL. mTOR signaling in stem and progenitor cells. Development. 2018;145:152595.
9. Murakami M, Ichisaka T, Maeda M, Oshiro N, Hara K, Edenhofer F, et al. mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. Mol Cell Biol. 2004;24:6710–8.
10. Zhou J, Su P, Wang L, Chen J, Zimmermann M, Genbacev O, et al. mTOR supports long-term self-renewal and suppresses mesoderm and endoderm activities of human embryonic stem cells. Proc Natl Acad Sci USA. 2009;106:7840–5.
11. Cherepkova MY, Sineva GS, Pospelov VA. Leukemia inhibitory factor (LIF) withdrawal activates mTOR signaling pathway in mouse embryonic stem cells through the MEK/ERK/TSC2 pathway. Cell Death Dis. 2016;7:e2050.
12. Wu F, Chen Z, Liu J, Hou Y. The Akt–mTOR network at the interface of hematopoietic stem cell homeostasis. Exp Hematol. 2021;103:15–23.
13. Gan B, DePinho RA. mTORC1 signaling governs hematopoietic stem cell quiescence. Cell Cycle. 2009;8:1003–6.
14. Chen C, Liu Y, Liu R, Ikenoue T, Guan KL, Liu Y, et al. TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species. J Exp Med. 2008;205:2397–408.
15. Chen C, Liu Y, Liu Y, Zheng P. mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. Sci Signal. 2009;2:75.
16. Castilho RM, Squarize CH, Chodosh LA, Williams BO, Gutkind JS. mTOR mediates Wnt-induced epidermal stem cell exhaustion and aging. Cell Stem Cell. 2009;5:279–89.
17. Gharibi B, Farzadi S, Ghuman M, Hughes FJ. Inhibition of Akt/mTOR attenuates age-related changes in mesenchymal stem cells. Stem Cells. 2014;32:2256–66.
18. Choi WH, Kim HR, Lee SJ, Jeong N, Park SR, Choi BH, et al. Fetal cartilage-derived cells have stem cell properties and are a highly potent cell source for cartilage regeneration. Cell Transplant. 2016;25:449–61.
19. Kim HR, Kim J, Park SR, Min BH, Choi BH. Characterization of human fetal cartilage progenitor cells during long-term expansion in a Xeno-free medium. Tissue Eng Regen Med. 2018;15:649–59.
20. Lee SJ, Kim J, Choi WH, Park SR, Choi BH, Min BH. Immunophenotype and immune-modulatory activities of human fetal cartilage-derived progenitor cells. Cell Transplant. 2019;28:932–42.
21. Kim J, Tran AN, Lee JY, Park SH, Park SR, Min BH, et al. Human fetal cartilage-derived progenitor cells exhibit anti-inflammatory effect on IL-1β-mediated osteoarthritis phenotypes in vitro. Tissue Eng Regen Med. 2020;19:1237–50.
22. Guillot PV, Gotherstrom C, Chan J, Kurata H, Fisk NM. Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. Stem Cells. 2007;25:646–54.
23. Kita K, Gauglitz GG, Phan TT, Herndon DN, Jeschke MG. Isolation and characterization of mesenchymal stem cells from the sub-amniotic human umbilical cord lining membrane. Stem Cells Dev. 2010;19:491–502.
24. Krattinger N, Applegate LA, Biver E, Pioletti DP, Caverzasio J. Regulation of proliferation and differentiation of human fetal bone cells. Eur Cell Mater. 2011;21:46–58.
25. Mirmalek-Sani SH, Tare RS, Morgan SM, Roach HI, Wilson DI, Hanley NA, et al. Characterization and multi-potentiality of human fetal femur-derived cells: implications for skeletal tissue regeneration. Stem Cells. 2006;24:1042–53.
26. O'Donoghue K, Fisk NM. Fetal stem cells. Best Pract Res Clin Obstet Gynaecol. 2004;18:853–75.
27. Laurent A, Hirt-Burri N, Scaletta C, Michetti M, de Buys RA, Raffoul W, et al. Holistic approach of Swiss fetal progenitor cell banking: Optimizing safe and sustainable substrates for regenerative medicine and biotechnology. Front Bioeng Biotechnol. 2020;8:557758.
28. Lee JE, Lim MS, Park JH, Park CH, Koh HC. S6K promotes dopaminergic neuronal differentiation through PI3K/Akt/mTOR-dependent signaling pathways in human neural stem cells. Mol Neurobiol. 2016;53:3771–82.
29. Romine J, Gao X, Xu XM, So KF, Chen J. The proliferation of amplifying neural progenitor cells is impaired in the aging brain and restored by the mTOR pathway activation. Neurobiol Aging. 2015;36:1716–26.
30. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009;460:392–5.
31. Kaeberlein M, Powers RW 3rd, Steffen KK, Westman EA, Hu D, Dang N, et al. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science. 2005;310:1193–6.
32. Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr Biol. 2004;14:885–90.
33. Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Müller F. Influence of TOR kinase on lifespan in C. elegans. Nature. 2003;426:620.
34. Wang S, Xia P, Ye B, Huang G, Liu J, Fan Z. Transient activation of autophagy via Sox2-mediated suppression of mTOR is an important early step in reprogramming to pluripotency. Cell Stem Cell. 2013;13:617–25.
35. Yang A, Xiao X, Zhao M, LaRue AC, Schulte BA, et al. Differential responses of hematopoietic stem and progenitor cells to mTOR inhibition. Stem Cells Int. 2015;2015:561404.
36. Iglesias-Bartolome R, Patel V, Cotrim A, Leelahavanichkul K, Molinolo AA, Mitchell JB, et al. mTOR inhibition prevents epithelial stem cell senescence and protects from radiation-induced mucositis. Cell Stem Cell. 2012;11:401–14.
37. Lee KW, Yook JY, Son MY, Kim MJ, Koo DB, Han YM, et al. Rapamycin promotes the osteoblastic differentiation of human embryonic stem cells by blocking the mTOR pathway and stimulating the BMP/Smad pathway. Stem Cells Dev. 2010;19:557–68.
38. Martin SK, Fitter S, Dutta AK, Matthews MP, Walkley CR, Hall MN, et al. Brief report: the differential roles of mTORC1 and mTORC2 in mesenchymal stem cell differentiation. Stem Cells. 2015;33:1359–65.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.