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Transforming growth factor-β (TGF-β) induces the expression of chondrogenesis-related genes through TGF-β receptor II (TGFRII)–AKT–mTOR signaling in primary cultured mouse precartilaginous stem cells

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ABSTRACT

Precartilaginous stem cells (PSCs) are adult stem cells which could initiate chondrocytes and bone growth. In the current study, we purified PSCs from the neonate mice' perichondrial mesenchyme through immunomagnetic beads with the fibroblast growth factor receptor-3 (FGFR-3) antibody. Mouse PSCs were seeded and cultured, and their phenotype was confirmed by FGFR-3 over-expression. Transforming growth factor-β (TGF-β) was added to induce PSCs differentiation. TGF-β increased mRNA expression of chondrogenesis-related genes (collagen type II, Sox 9, and aggrecan) in the cultured PSCs, which was abolished by TGF-β receptor II (TGFRII) lentiviral shRNA depletion. TGF-β induced AKT activation in mouse PSCs, while the PI3K/AKT inhibitor (LY294002) and the AKT specific inhibitors (perifosine and MK-2206) largely suppressed TGF-β-induced collagen II, Sox 9, and aggrecan mRNA expression. Meanwhile, the mTOR complex 1 (mTORC1) blocker RAD001 or the mTORC1/2 dual inhibitor AZD-2014 also alleviated TGF-β-induced chondrogenesis-associated genes expression. Further, lentiviral shRNA depletion of SIN1 (a mTORC2 component) or mTOR inhibited TGF-β's effect in the mouse PSCs. In conclusion, our evidence suggests that TGF-β induces the expression of chondrogenesis-related genes through TGFRII–AKT–mTOR signaling in cultured mouse PSCs.

1. Introduction

The applications of chondrocytes in scientific researches and clinical applications are restrained due to the poor renew ability [1,2]. Precartilaginous stem cells (PSCs) are adult stem cells which could differentiate to chondrocytes and promote bone growth [3]. In 1999, Robinson et al. first separated PSCs from perichondrial mesenchyme (the ring of La Croix) of rat neonates by immunomagnetic beads conjugated with fibroblast growth factor receptor-3 (FGFR-3) antibody [3]. These PSCs have potential to differentiate directionally to chondrocytes and form cartilaginous tissues, and eventually promote bone growth [3,4]. Transforming growth factor-β (TGF-β) was tested to induce chondrocytes differentiation from stem cells [5–7], while its role in the PSCs and the underlying mechanisms are not studied.

TGF-β initiates signaling transduction by binding to type I and type II receptor serine/threonine kinases on the cell surface. TGF-β receptor II (TGFRII) then phosphorylate TGF-β receptor I (TGFRI) kinase domain, the latter then propagates the signal through phosphorylation of the Smad proteins [8]. The eight Smad proteins are divided into three types: the receptor-regulated Smad (R-Smad), the co-mediator Smad (Co-Smad), as well as the inhibitory Smad (I-Smad) [8]. The activated Smad complexes translocate into the nuclei and regulate the transcription of target genes [8,9]. Besides activating the traditional Smad-transcription pathway, TGF-β could also activate other signaling pathways (so called "non-Smad pathways") [10], which include the Erk/MAPK [11,12] pathway and the phosphoinositide 3-kinase (PI3K)/AKT(PKB)/mammalian target of rapamycin (mTOR) [13–16] pathway. These non-Smad pathways work together with Smad proteins, or independently, to regulate TGF-β's functions [8,9,11–15].

PI3K/AKT/mTOR cascade can be activated by a number of stimuli including growth factors, cytokines, nutrients and stresses [17]. This pathway plays major roles in cell growth, proliferation, survival, protein synthesis and differentiation [18–20]. Studies have shown that TGF-β activates the PI3K/AKT/mTOR signaling [13–16]. In the current study, we tested the potential role of this

Abbreviations: PSCs, precartilaginous stem cells; S6K, p70S6K1; FGFR-3, fibroblast growth factor receptor-3; TGF-β, transforming growth factor-β; TGFRII, TGF-β receptor II; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2.

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pathway in TGF-β-induced PSCs differentiation, and our results showed that AKT/mTOR activation is required for TGF-β-induced expression of chondrogenesis-related genes in cultured mouse PSCs.

2. Material and methods

2.1. Chemicals, reagents and antibodies

TGF-β, LY294002, perifosine, MK-2206, RAD001 and AZD-2014 were obtained from Selleck (Shanghai, China). Anti-AKT1, mTOR, SIN1, S6K, S6 and GAPDH antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other kinase antibodies used in this study were obtained from Cell Signaling Technology (Shanghai, China).

2.2. Precartilaginous stem cells isolation, purification, and culture

Similar to previously reported [3,4], the neonate C57BL/6J mice were provided by the animal center of authors institution. The tissues located around the perichondrial mesenchyme (the La Croix rings) were cut down and digested sequentially with Complete™ Trypsin Solution (Chemicon International Inc., CA) and 0.05% collagenase type one (Sigma Chemical Co., MO). After the cells were dispersed and suspended as single cell suspension in 0.1 M phosphate buffer saline (PBS), they were incubated with FGFR-3 antibody (c-15) (1:500, Santa Cruz Biotechnology Inc., Santa Cruz , CA) [3] and then purified by an immunomagnetic separation system (Miltenyi Biotech, Bergisch Gladbach, DE). The immuno-selected precartilaginous stem cells (PSCs) were then cultured in DMEM/F12 medium (Thermo Fisher Scientific Inc., CA), supplemented with 20% fetal calf serum (FCS, Gibco, Shanghai, China), 100 units/ml penicillin and streptomycin in a 5% CO_2/37 °C incubator. The detailed procedures have been described [3,4]. The medium was switched every 2 days. The protein and mRNA expression of FGFR-3 were tested by Western blots and RT-PCR to verify the phenotype of the mouse PSCs.

2.3. The MTT cell survival assay

The PSCs survival was assessed by the MTT (Sigma) assay. In brief, cells were collected and seeded in 96-well plates at a density of 1×10^5 cells/well in 200 ml of culture medium (containing 1% FBS). After treatment, 20 μl of MTT (5 mg/ml) solution was added to each well for 4 h at 37 °C, the cell viability was determined by measuring absorbance at 490 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA), OD value of treatment group was normalized to that of untreated control group.

2.4. FGFR-3 immunofluorescence

The purified PSCs were seeded into six-well-plates with 5×10^5 cells/well. After attachment, PSCs were fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were then permeabilized with 0.2% Triton X-100 solution for 5 min. Cells were then incubated with the rabbit-anti-FGFR-3 (1:200 dilution, sc-82, Santa Cruz) at 4 °C overnight. Next, detection of the bound primary antibodies was enabled by incubating cells with goat anti-rabbit IgG-Cy3 (Cellular Signaling Tech, Shanghai, China) for 1 h at 37 °C, the cells were then observed and images recorded under an Olympus fluorescence microscope (CX41, Olympus, Tokyo, Japan).

2.5. Protein isolation, Western blots and data quantification

After treatment, PSCs were washed twice with ice-cold PBS and then lysed using lysis buffer containing 1% Nonidet P-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 150 mmol/L sodium chloride and 10 mmol/L Tris–HCl (pH, 7.4). The lysates were collected and centrifuged. The concentration of the extracted protein was measured by bicinchoninic acid assay kit (Sigma). The extracted protein was boiled for 5 min in loading buffer. Samples (20 μg/well) were separated on 10% SDS–polyacrylamide gel, and after electro-blotting onto polyvinylidene fluoride (PVDF) membranes (Millipore, Shanghai, China), the blots were blocked with blocking solution [10% (w/v) milk in Tris-buffered solution plus Tween-20 (TBST)], incubated overnight at 4 °C with primary antibodies, and then incubated with HRP-conjugated anti-rabbit/mouse secondary antibodies. The detection was performed by Super-signal West Pico Enhanced Chemiluminescent (ECL) Substrate. The band intensity was quantified by ImageJ software (NIH) after normalization to the corresponding loading controls. And the quantification number was expressed as fold change vs. the band labeled with "1.00".

2.6. Total RNA isolation and real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was prepared by RNA-TRIZOL extraction (Gibco). Concentration and purity of the extracted RNA were measured spectrophotometrically at A260 and A280. Real time-reverse transcription–polymerase chain reaction (real-time PCR) was performed by using TOYOBO ReverTra Ace RT-PCR kit according to the manufacturer's instructions. Primers were F:5′-GTGGGAGCGA CAACTTTACC-3′/R:5′-GAGAACGAAACCAGGGCTACT-3′ for *Sox9*; F:5′-aCAAGAGCAAGGGAAGAAGCA-3′/R:5′-TGGACAGTAGACGGAG GAAAG-3′ for *Collagen type II*; F:5′-AGAATCCATAACTGCCCCAAC-3′/ R:5′-GTCACGCCCTCCACTAACTCT-3′ for *Aggrecan*; F: 5′-GAAGGTG AAGGTCGGAGTC-3′/R: 5′-GAAGATGGTGATGGGATTTC-3′ for *GAPDH* and F:5′-CGCTTTGCTGAGGTCTATAAGGC-3′/R, 5′-GATATTGGA GCTCTTGAGGTCCCT-3′ for *TGFRII*. A typical reaction (50 μL) contained 1/50 of reverse transcription–generated cDNA and 200 nM of primer in 1 × SYBR Green RealTime Master Mix (Toyobo, Shanghai, China) buffer. The PCR reactions were carried out on a Bio-Rad IQ5 multicolor detection system by using 2 μg of synthesized cDNA under the following conditions: 95 °C for 5 min, 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. All real-time PCRs were performed at least in triplicate. The TNFα mRNA expression level was expressed as the fold change vs. control group.

2.7. Target protein shRNA-knockdown through lentiviral infection

Four different non-overlapping lentiviral shRNAs against same targeted protein (mTOR, SIN1 or TGFRII) were designed, synthesized and verified by the Shanghai Kaiji Biotech (Shanghai, China). The pre-experiments were performed to test the knocking-down efficiency of these four shRNAs. Of which, two most efficient ones were selected for further experiments. PSCs were seeded in a six-well plate in the growth medium. The lentiviral shRNAs were added to the cells (15 μl/ml), after 12 h, the medium was replaced by fresh growth medium, and cells were further cultured for additional 48 h. The expression of target protein (mTOR, SIN1 or TGFRII) and the equal loading in infected cells were always detected by Western blots. Control cells were infected with same amount of lentiviral scramble non-sense shRNA (labeled as "sc-shRNA") (Shanghai Kaiji Biotech).

2.8. Data analysis

Data were collected using a minimum of three experiments and used to calculate the mean ± S.D. Statistical differences were analyzed by one-way *ANOVA* followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 18). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Mouse precartilaginous stem cells (PSCs) isolation, purification and verification

Using the methods described above, we successfully isolated and purified precartilaginous stem cells (PSCs) from perichondrial mesenchyme of the neonate mice. Morphological images in Fig. 1A showed PSCs at day 1 and day 4 of culture. Fibroblast growth factor receptor-3 (FGFR-3) was recognized as a marker for PSCs. Thus, we tested its expression in the cultured PSCs. The immunofluorescence image in Fig. 1B demonstrated FGFR-3's expression in PSCs' plasma membrane. Further, RT-PCR and Western blot results confirmed mRNA and protein expression of FGFR-3 in the cultured PSCs (Fig. 1C and D). Note that the cells left after immunomagnetic separation were negative for FGFR-3 expression (Fig. 1C and D).

3.2. TGF-β receptor-II is required for TGF-β-induced expression of chondrogenesis-related genes in primary cultured mouse PSCs

In the current study, we tested the potential role of TGF-β on PSCs differentiation. We found that the mRNA expression of chondrogenesis-related genes, including Sox 9, collagen II and aggrecan [4], was significantly upregulated after TGF-β stimulation in primary cultured PSCs (Fig. 2C–E). We then explored the involvement of TGF-β receptor-II (TGFRII) in the process. The lentiviral

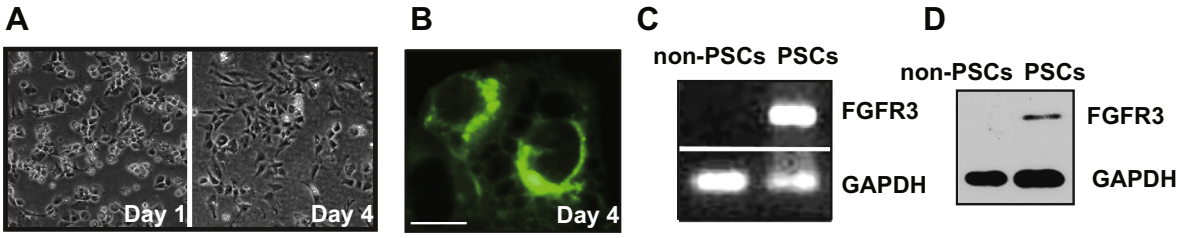

Fig. 1. Mouse precartilaginous stem cells (PSCs) isolation, purification and verification. The morphology of mouse precartilaginous stem cells (PSCs) at day 1 and day 4 of culture was shown (A).Immunofluorescence microscopy (B), Western blots (C) and RT-PCR (D) results showed the specific expression of FGFR-3 in cultured PSCs (day 4), while the cells left after immunomagnetic separation were negative for FGFR-3, GAPDH was tested as the loading control (C and D). Magnification: 1:200 (A). Bar = 15 μm (B). Experiments in this figure were repeated three times, and similar results were obtained.

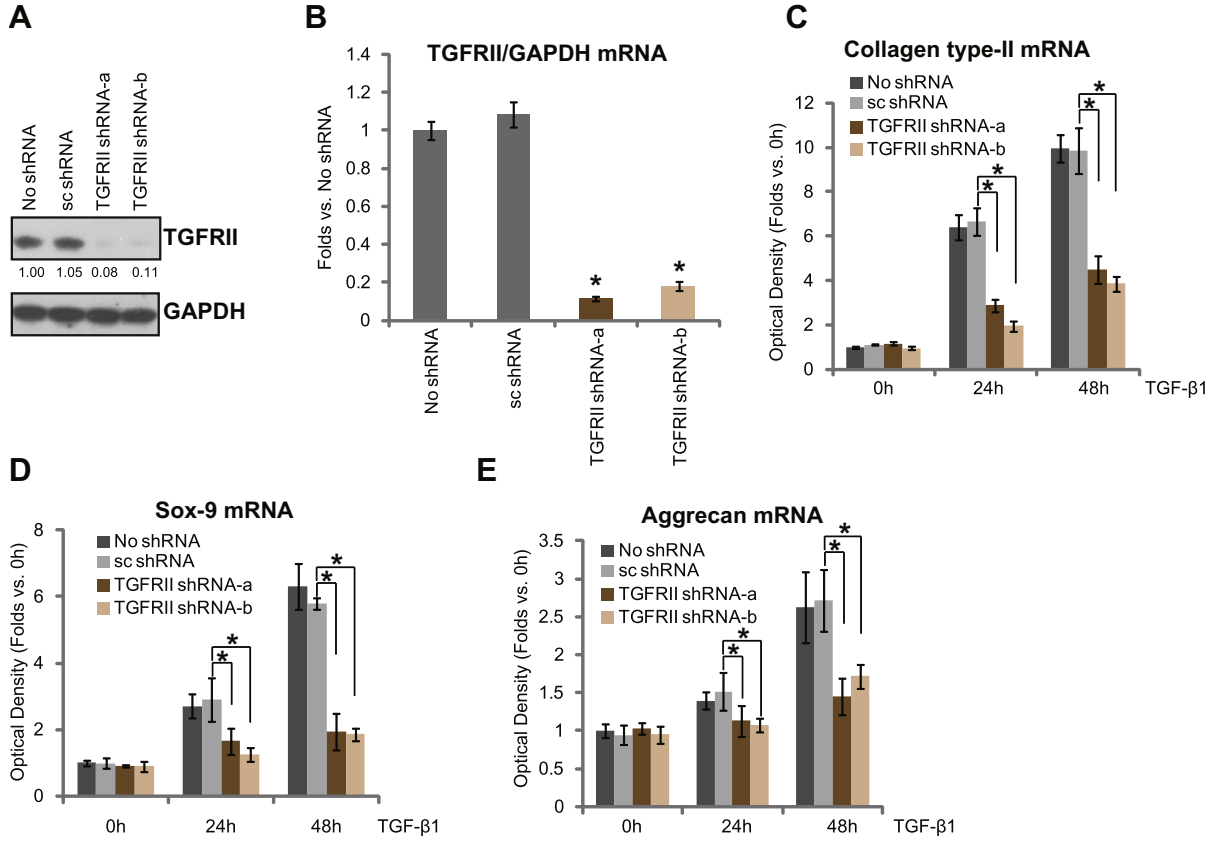

Fig. 2. TGF-β receptor-II is required for TGF-β-induced expression of chondrogenesis-related genes in primary cultured mouse PSCs. TGFRII lentiviral shRNAs (-a/-b) or scramble lentiviral shRNA (15 μl/ml each) were added to PSCs (day 4) for 48 h. Afterwards, the protein and mRNA expression of TGFRII and GAPDH were tested by Western blots (A) and Real-time PCR (B), respectively. TGFRII protein expression was quantified. Above PSCs (infected with TGFRII lentiviral shRNAs or scramble lentiviral shRNA) and the parental PSCs were treated with TGF-β (25 ng/ml) for 24 and 48 h, mRNA expression of collagen II (C), Sox 9 (D) and aggrecan (E) was tested by real time-PCR, GAPDH was also tested as a internal control and was equivalent among different groups (not shown). Experiments in this figure were repeated three times, and similar results were obtained. *$p < 0.05$ vs. scramble shRNA group (B–E).

TGFRII-shRNAs were utilized to knockdown TGFRII in cultured PSCs. Note that we utilized two different shRNAs (TGFRII siRNA-a, and TGFRII siRNA-b) against non-overlapping gene sequence of mouse TGFRII, aiming to exclude off-target effects (same for all the shRNA experiments in the paper). Western blots and Real-time PCR results showed that protein and mRNA expression of TGFRII were dramatically down-regulated in PSCs by both lentiviral TGFRII-shRNAs, while cells infected with scramble-shRNA virus showed equivalent TGFRII expression as the parental cells (Fig. 2A and B). Significantly, real-time PCR results clearly showed that TGFRII was required for TGF-β-induced chondrogenesis genes expression in PSCs, as mRNA expression of collagen II, Sox 9, and aggrecan by TGF-β was largely inhibited by the two TGFRII lentiviral shRNAs in the PSCs (Fig. 2C–E). Thus, TGFRII is required for TGF-β-induced chondrogenesis in primary cultured mouse PSCs.

3.3. AKT activation is important for TGF-β-induced chondrogenesis-related genes expression in primary cultured mouse PSCs

Next we explored the cellular signaling mechanisms of TGF-β-induced chondrogenesis-related genes expression. We first examined the effect of TGF-β on AKT activation in cultured PSCs. Western blot results in Fig. 3A clearly demonstrated AKT activation in TGF-β-treated PSCs, which was reflected by upregulation of phosphorylated-AKT at both Ser 473 and Thr 308 (Fig. 3A). To explore the role of AKT in TGF-β-induced chondrogenesis genes expression, AKT inhibitors were applied. As shown in Fig. 3B, the PI3K/AKT inhibitor LY294002, as well as two specific AKT inhibitors, perifosine [21] and MK-2206 [22], largely inhibited TGF-β-induced AKT activation (Ser 473 and Thr 308 phosphorylation) (Fig. 3B). Significantly, TGF-β-induced mRNA expression of chondrogenesis genes (collagen II, Sox 9, and aggrecan) was also inhibited by above AKT inhibitors (Fig. 3C–E), indicating that AKT activation is important for TGF-β-induced expression of chondrogenesis in mouse PSCs. Note that these AKT inhibitors had no significant effect on mouse PSCs viability (Fig. 3F).

3.4. Activation of mTORC1 and mTORC2 is required for TGF-β-induced chondrogenesis-related genes expression in primary cultured mouse PSCs

There are at least two mTOR complexes, namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). As shown in Fig. 4A, TGF-β induced significant mTORC1 activation in cultured PSCs, which was shown by upregulation of phosphorylation of p70S6K1 (S6K) and 4E-BP1. Results in Fig. 3A have already demonstrated that TGF-β induced AKT Ser 473 phosphorylation, indicating mTORC2 activation by TGF-β in mouse PSCs. Significantly, the mTORC1 inhibitor RAD001 (RAD) and the mTORC1/2 dual inhibitor AZD-2014 (AZD) suppressed TGF-β-induced collagen II and Sox 9 mRNA expression in mouse PSCs (Fig. 4C and D), suggesting that both mTORC1 and mTORC2 are involved in TGF-β-induced chondrogenesis in PSCs. Both RAD001 and AZD-2014 blocked TGF-β-induced mTORC1 activation (S6K phosphorylation), while only AZD-2014 inhibited TGF-β-induced AKT Ser 473 phosphorylation (the indicator of mTORC2 activation, Fig. 4B). To further investigate the role mTORC1 and mTORC2 in TGF-β's effect, the shRNA strategy was utilized to knockdown SIN1 (a mTORC2 component) or mTOR

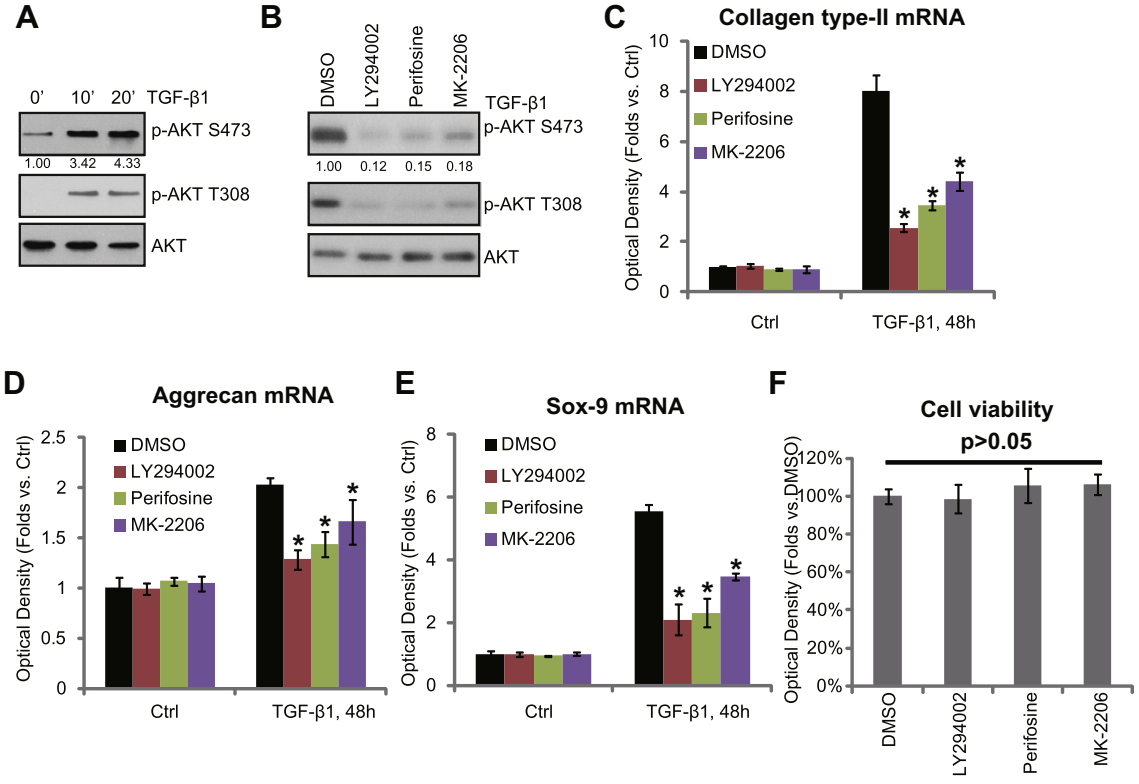

Fig. 3. AKT activation is important for TGF-β-induced chondrogenesis-related genes expression in primary cultured mouse PSCs Mouse PSCs were treated with TGF-β (25 ng/ml) for indicated time, phospho-AKT (Ser 473 and Thr 308) and regular AKT were tested by Western blots, phospho-AKT at Ser 473 was quantified (A). Mouse PSCs were pre-treated with LY294002 (500 nM), perifosine (2.5 μM) or MK-2206 (5 μM) for 1 h, followed by TGF-β (25 ng/ml) stimulation, cells were further cultured, after 20 min, phospho-AKT (Ser 473 and Thr 308) and regular AKT were tested by Western blots, phospho-AKT at Ser 473 was quantified (C); 48 h later, mRNA expression of collagen II (C), Sox 9 (D) and aggrecan (E) was tested by real time-PCR, GAPDH was also tested as a internal control and was equivalent among different groups (not shown). The effect of above AKT inhibitors on PSCs viability was tested by MTT assay (48 h treatment) (F). Experiments in this figure were repeated three times, and similar results were obtained. *$p < 0.05$ vs. TGF-β only group (C–E).

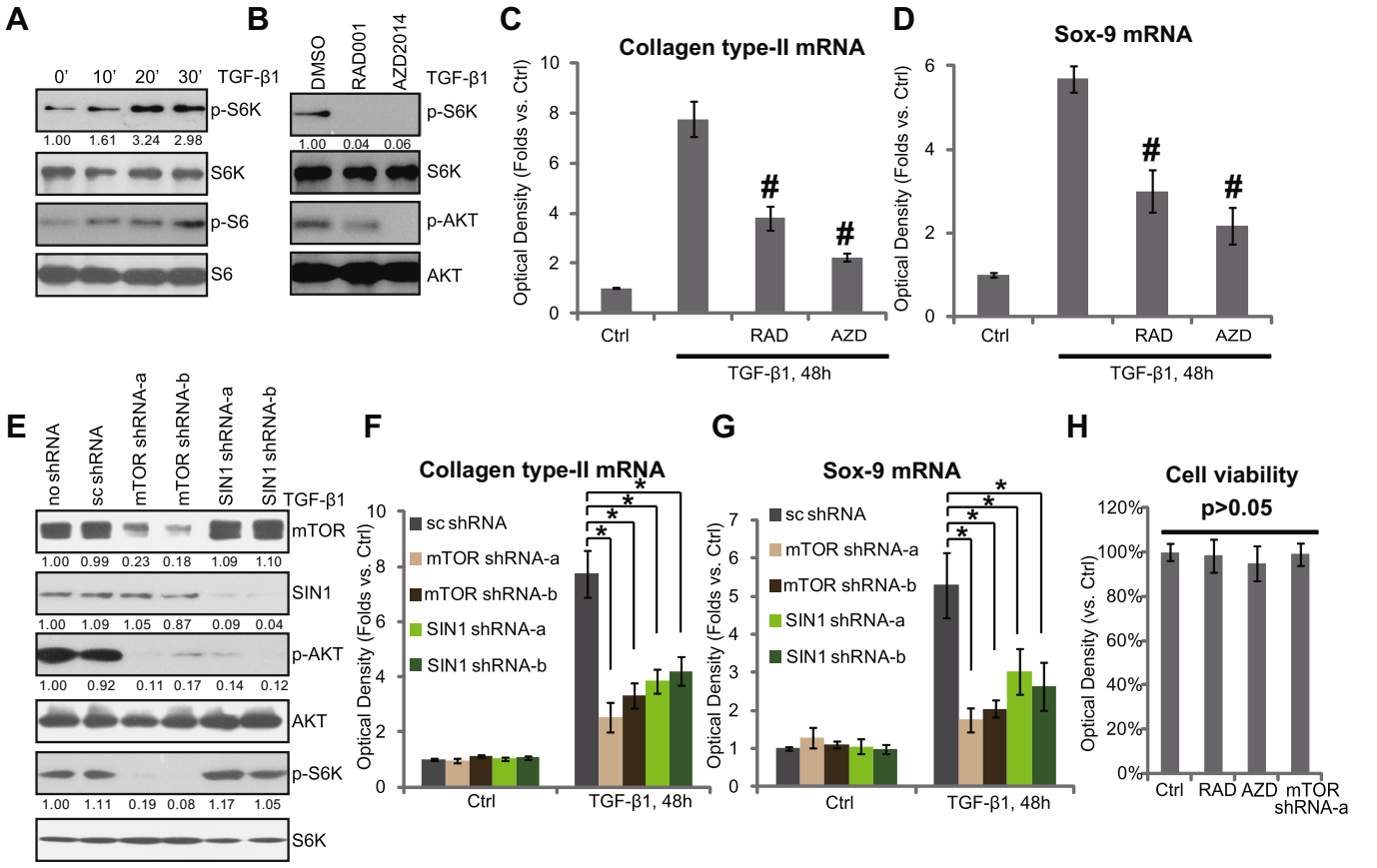

Fig. 4. Activation of mTORC1 and mTORC2 are required for TGF-β-induced chondrogenesis-related genes expression in primary cultured mouse PSCs Mouse PSCs were treated with TGF-β (25 ng/ml) for indicated time, p-S6K (Thr 389), regular S6K, p-S6 (Ser 235/236) and regular S6 were tested by Western blots, p-S6K was quantified (A). Mouse PSCs were pre-treated with RAD001 (100 nM) or AZD-2014 (AZD, 0.25 μM) for 1 h, followed by TGF-β (25 ng/ml) stimulation, cells were further cultured, after 30 min, p-S6K (Thr 389), regular S6K, p-AKT (Ser 473) and regular AKT were tested by Western blots, p-S6K was quantified (B); mRNA expression of collagen II (C) and Sox 9 (D) was tested by real-time PCR 48 h after TGF-β stimulation. SIN1 lentiviral shRNAs (-a/-b), mTOR lentiviral shRNAs (-a/-b) or scramble-shRNA (15 μl/ml each) were added to mouse PSCs (day 4) for 48 h, afterwards, the expression of SIN1 and mTOR was tested by Western blots (E), above cells were either left untreated, or treated with TGF-β (25 ng/ml), AKT/S6K phosphorylation (20 min after stimulation) (E), as well as collagen II (F) or Sox 9 (G) mRNA expression (48 h after stimulation) were tested. Indicated proteins were quantified in (E). The effect of RAD001 (100 nM, 48 h), AZD-2014 (AZD, 0.25 μM, 48 h) or mTOR lentiviral shRNA-a (48 h) on viability of mouse PSCs was tested by MTT assay (F). Experiments in this figure were repeated three times, and similar results were obtained. $^{\#}p < 0.05$ vs. TGF-β only group (C and D). $^{*}p < 0.05$ (F and G).

in mouse PSCs. Again, we utilized two non-overlapping lentiviral shRNAs (-a/-b) against same target gene to rule out possible off-target effects. As expected, SIN1 shRNAs only affected TGF-β-induced mTORC2 activation (AKT Ser 473 phosphorylation), while mTOR depletion abolished TGF-β-induced both mTORC1 and mTORC2 activation (p-S6K1) (Fig. 4E). Notably, SIN1 shRNA-knockdowns or mTOR shRNA-knockdowns inhibited TGF-β-induced collagen II and Sox 9 mRNA expression in mouse PSCs (Fig. 4F and G). RAD001, AZD-2014 and mTOR lentiviral shRNA-a had no significant effect on mouse PSCs survival (Fig. 4H). Based on these data, we suggest that activation of mTORC1 and mTORC2 are required for TGF-β-induced chondrogenesis-related genes expression in mouse PSCs.

4. Discussions

In the current study, we successfully isolated, purified and cultured PSCs from the perichondrial mesenchyme (the ring of La Croix) of neonate mice through immunomagnetic beads (containing FGFR-3 antibody).These FGFR-3 expressing mouse PSCs were response to TGF-β, and showed increased mRNA expression of chondrogenesis-related genes (collagen type II, Sox 9, and aggrecan) and activation of AKT/mTOR signaling after TGF-β stimulation. By using both pharmacological and shRNA strategies, our evidence suggests that TGFRII–AKT–mTOR signaling cascade is important for TGF-β-induced chondrogenesis genes expression in primary cultured mouse PSCs.

It is now well established that TGF-β could activate the non-Smad pathways [10]. Of these non-Smad signaling cascades, AKT signaling plays an important role in regulating TGF-β's functions [13–16]. In the current study, we found that TGF-β induced significant AKT activation in primary culture mouse PSCs, while the PI3K/AKT inhibitors (LY294002, perifosine and MK-2206) suppressed TGF-β-induced collagen II, Sox 9, and aggrecan mRNA expression. Thus, activation of AKT, a non-Smad pathway, might be required for TGF-β-induced chondrogenesis in mouse PSCs.

MTOR kinase forms at least two distinct multi-protein complexes, including mTOR complex 1 (mTORC1) and mTORC2. MTORC1, the rapamycin-targeting complex is composed of mTOR, Raptor and PRAS40 [23]. MTORC2, on the other hand, is composed of mTOR, Rictor, SIN1, mLST8 and Protor [24–26]. Activated mTORC1 phosphorylates its downstream targets including p70S6K1 (S6 K) and 4E-BP1 to promote protein translation and cell growth [23]. When mTORC2 is activated, it phosphorylates AKT at Ser 473 to increase its enzymatic activity [27]. In the current study, we found that TGF-β activated both mTORC1 and mTORC2 in primary cultured mouse PSCs, which is important for the chondrogenesis genes expression.

Rapamycin and rapamycin analogs (rapalogs, i.e., RAD001) inhibits mTOR kinase activity by binding to the FKBP-12 (FK506-binding protein of 12 kDa), the latter forms a complex with C-terminus of mTOR [28]. It interferes exclusively with the kinase activity of the mTORC1, but not mTORC2. Several mechanisms of potential resistance to rapalogs have been identified, including a negative feed-back loop activation of AKT [29] and Erk [30–32]. Recently, ATP-competitive kinase inhibitors of mTOR, or the second generation mTOR inhibitors (i.e., AZD-2014, AZD-8045 and OSI-027) have been developed [32,33]. These kinase inhibitors not only block mTORC1 activation, but also abolish mTORC2 activity [32,34]. Here, we found that RAD001 as well as the mTORC1/2 dual inhibitor AZD-2014 suppressed TGF-β-induced chondrogenesis genes expression. Further, shRNAs-knockdown of SIN1 (a mTORC2 component) or mTOR also alleviated TGF-β's effect in mouse PSCs. Thus, we suggest that both mTORC1 and mTORC2 activation might be involved in TGF-β-induced chondrogenesis of mouse PSCs.

In summary, we successfully purified and cultured FGFR3-expressing PSCs from neonate C57BL/6J mice, and TGF-β induced the expression of chondrogenesis-related genes through TGFRII–AKT–mTOR signaling in the primary mouse PSCs.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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