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Conditional TGF-β_1 treatment increases stem cell-like cell population in myoblasts

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Abstract

The limitation in successfully acquiring large populations of stem cell has impeded their application. A new method based on the dedifferentiation of adult somatic cells to generate induced multipotent stem cells would allow us to obtain a large amount of autologous stem cells for regenerative medicine. The current work was proposed to induce a sub-population of cells with characteristics of muscle stem cells from myoblasts through conditional treatment of transforming growth factor (TGF)-β_1. Our results show that a lower concentration of TGF-β_1 is able to promote C2C12 myoblasts to express stem cell markers as well as to repress myogenic proteins, which involves a mechanism of dedifferentiation. Moreover, TGF-β_1 treatment promoted the proliferation-arrested C2C12 myoblasts to re-enter the S-phase. We also investigated the multi-differentiation potentials of the dedifferentiated cells. TGF-β_1 pre-treated C2C12 myoblasts were implanted into mice to repair dystrophic skeletal muscle or injured bone. In addition to the C2C12 myoblasts, similar effects of TGF-β_1 were also observed in the primary myoblasts of mice. Our results suggest that TGF-β_1 is effective as a molecular trigger for the dedifferentiation of skeletal muscle myoblasts and could be used to generate a large pool of progenitor cells that collectively behave as multipotent stem cell-like cells for regenerative medicine applications.

Keywords: TGF-β_1 • dedifferentiation • skeletal muscle • stem cell • multipotency

Introduction

The muscle healing process initiated by injury or disease, often leads to the excessive formation of fibrotic scar tissue; in this situation, stem cell-based therapies have been found to be beneficial for improved regeneration [1–5]. It is well accepted that adult mammalian tissues, such as skeletal muscle, contain somatic stem cells. Studies have shown that the transplantation of multipotent muscle stem cells (*i.e.* muscle derived stem cells/MDSCs) can produce improved cell survival, migration, engraftment and angiogenesis, when compared to the transplantation of myoblasts, which are lineage-determined myogenic cells [1–4]. However, the amount of muscle stem cells in normal or diseased skeletal muscle is usually very limited for cell isolation, and the *in vitro* culturing and propagation of isolated stem cells is difficult, thus requiring a long time period. Based on these facts, a possible induction of muscle stem cells from myoblasts *via* a mechanism of dedifferentiation would allow us to obtain a much larger quantity of autologous stem cells for use in regenerative medicine.

Dedifferentiation of terminally differentiated muscle cells (myofibres) in urodele amphibians is part of the representative mechanism of tissue and limb regeneration [6–8], but this process has not yet been proven in mammals. However, recent studies have identified agents that seem to induce the reprogramming of skeletal muscle of mammals *in vitro*. Terminally differentiated C2C12 myotubes can be induced to dedifferentiate into proliferating mononuclear cells through treatment with newt regeneration extract [9]. Defined synthetic molecules, such as reversine and myoseverin can induce the dedifferentiation of C2C12 myoblasts to more multipotent progenitor-type cells [10–12]. Moreover, the overexpression of the

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homeodomain transcription repressor muscle segment homeobox-1, which is considered to be related with cell dedifferentiation in mammals [8], promotes the dedifferentiation of murine myoblasts and even terminally differentiated myotubes [13].

In addition to some small molecules and the genetic modification of differentiated muscle cells, the possible correlation between the dedifferentiation of myoblasts and some specific growth factors has also been an important theory to investigate. Our previous study has demonstrated the dedifferentiation capacity of ciliary neurotrophic factor on skeletal muscle myoblasts *in vitro* [14]. Recent studies have also suggested that the multifunctional transforming growth factor (TGF)-β_1 may be related with dedifferentiation of some somatic cells, for example TGF-β_1 signalling is required during the dedifferentiation and regeneration processes after limb amputation in axolotls [15]. TGF-β_1 promotes the proliferation of human mesenchymal stem cells by inducing Smad3-dependent nuclear accumulation of β-catenin in mesenchymal stem cells [16]. Also, TGF-β_1 can stimulate the dedifferentiation of epithelial cells into malignant, invasive and metastatic fibroblastic cells [17–20]. Moreover, induction of epithelial–mesenchymal transition by TGF-β_1 treatment can stimulate cultured mammalian epithelial cells to adopt characteristics of stem cells [21].

Our previous studies have shown that TGF-β_1 can alter the muscle healing process in both phases of regeneration and fibrosis formation [22, 23]. It has also been documented that TGF-β_1 is capable of inhibiting myogenic differentiation of myoblasts [24–26], and lower concentrations of TGF-β1 are capable of maintaining the multipotency and self-renewal potential of stem cells [16, 27, 28]. Since TGF-β1 can maintain the undifferentiated status of both myoblasts and stem cells, along with its previously reported dedifferentiation capacity in various situations [15, 19, 21], it would be interesting to find out if TGF-β_1 can promote the dedifferentiation of myoblasts into muscle stem cells; that is, a reverse process of myogenic differentiation. In this study, we investigated whether TGF-β_1 treatment of myoblasts is effective in promoting cell dedifferentiation and enlarging the cell population with stem cell characteristics. Additionally, this study investigated the effect of TGF-β_1 treatment in cycle re-entry of myoblasts and the multi-differentiation potentials of TGF-β_1 pre-treated cells.

Materials and methods

Cell culture and TGF-β_1 treatment of cells

C2C12 myoblasts (purchased from American Type Culture Collection, Manassas, VA, USA), or primary myoblasts (isolated from mouse using the pre-plate technique) [29] were cultured in growth DMEM (Gibco BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 10% horse serum, 1% Penicillin–Streptomycin antibiotics and 0.5% chicken embryo extract, and incubated in 5% CO_2 at 37°C. Human recombinant TGF-β_1 (hrTGF-β_1, Sigma, St. Louis, MO, USA) of various concentrations (0, 0.1, 0.5 or 2.0 ng/ml) was applied to cells for 3 hrs, and cells were then continually cultured for different time periods in growth medium. Differentially treated cells were finally used for the cell cycling study (S-phase re-entry) and for detecting the expression of various stem cell marker proteins and myogenic cell marker proteins by immunocytochemistry or flow cytometry.

Immunofluorescent staining of cells and tissue sections

Cultured cells were fixed with 4% paraformaldehyde, while skeletal muscle cryo-sections were fixed with 4% formalin. After washing the samples with phosphate buffered saline, 10% horse serum was used to block nonspecific background for 1 hr. The following primary antibodies were applied: Sca-1 (BD Biosciences, San Jose, CA, USA – 557403, 1:200), myogenin (BD Biosciences – 556358, 1:200), p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA – 554085, 1:200), MyoD (Santa Cruz Biotechnology – 32758, 1:200), Pax7 (DSHB, Iowa City, IA, USA – Pax7-c, 1:100), dystrophin (Abcam, Cambridge, MA, USA – 15277, 1:200), myosin heavy chain (MHC, Sigma – m4376, 1:200). The secondary antibodies were Alexa Fluor 488 or 594 specific to various species (Invitrogen, Carlsbad, CA, USA – 1:400). 4′,6-diamidino-2-phenylindole (DAPi; Sigma) is use to fluorescent stain cell nuclei. Fluorescence microscopy (Leica Microsystemic, Inc., Bannockburn, IL, USA) was used to examine all of the immunofluorescence results and capture photographic images.

Real time-PCR study

Total RNA was obtained from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was then performed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The primers used for RT-PCR are shown in Table 1. Quantitative real time (RT)-PCR was performed on 25 ng of cDNA using the iQ SYBR Green Supermix (Bio-Rad) on the Bio-Rad MyiQ thermal cycler. The cycling parameters used for all primers were as follows: 95°C for 10 min.; PCR 40 cycles of: 30 sec. 95°C denaturation, 1 min. 55°C~60°C annealing, 30 sec. 72° extension were repeated. A DNA dissociation curve was performed for each sample, to ensure the purity of amplification products. All data were normalized to internal dye control and also normalized according to level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Correlation coefficient ($\geq$0.98) and amplification efficiencies (90–110%) were calculated by using the Bio-Rad iQ5 software.

BrdU immunochemical staining

C2C12 myoblasts were cultured in serum-free DMEM for 24 hrs to allow for proliferation-arrest and differentiation. Cells were then treated with various concentrations of hrTGF-β_1 (0, 0.1, 0.5 and 2.0 ng/ml) for 3 hrs and cultured for another 12 hrs with Bromodeoxyuridine (BrdU, BD Biosciences, San Jose, CA, USA) (1:1000) in growth medium. Cells were immunostained with an antibody to BrdU (Abcam – ab2284, 1:200) to observe the ratio of cells with newly synthesized DNA (S-phase cell cycle re-entry). The bound biotinylated BrdU antibody was detected using Streptavidin-conjugated Alexa-Fluor 488 (Invitrogen – S32354, 1:400).

Table 1 Primers for quantitative RT-PCR

Gene	GenBank accession	Primer sequence		Size (bp)
Sca-1 (Ly6a)	NM_010738	Forward	5′-aggaggcagcagttattgtgg-3′	114
		Reverse	5′-cgttgaccttagtacccagga-3′	
Pax7	NM_011039	Forward	5′-tctccaagattctgtgccgat-3′	132
		Reverse	5′-cggggttctctctcttatactcc-3′	
MyoD	NM_010866	Forward	5′-ggctacgacaccgcctacta-3′	204
		Reverse	5′-gttctgtgtcgcttagggat-3′	
GAPDH	NM_008084	Forward	5′-cctctggaaagctgtggcgt-3′	190
		Reverse	5′-ttggcaggtttctccaggcg-3′	

Propidium iodide (PI) staining and flow cytometry analysis of the cell cycle

For PI (Sigma) staining, cells were fixed by 75% ice cold ethanol for at least 4 hrs, and then stained with PI solution (0.05 mg/ml PI, 0.2 mg/ml RNase and 0.1% NaCitrate, Sigma) for 30 minutes at room temperature. Cell cycles of differentially treated muscle cells were then identified using a FACSCalibur flow-cytometer (Becton-Dickinson, San Jose, CA, USA).

In vivo myogenic differentiation assay

Two groups of cells, either pre-treated (0.5 ng/ml, 3 hrs) or non-treated with TGF-β_1 were injected separately (1×10^5 cells per group) into the gastrocnemius (GM) muscles of MDX/SCID mice, a dystrophic/immunodeficient mouse model (C57BL/10 ScSn-Dmdmdx crossed with C57BL/6J-Prkdcscid/SzJ, Jackson Laboratory, Bar Harbor, ME, USA). Muscle tissues were harvested for cryo-sectioning and histological studies 1 or 2 weeks after cell transplantation. The myogenic differentiation capacity was determined by measuring the number and minor axis diameters (the smallest diameter) of regenerating dystrophin positive myofibres using Northern Eclipse software (version 6.0, Empix Imaging Inc., Mississauga, ON, Canada). The measurement of cell engraftment was performed with ImageJ software (version 1.32j, National Institutes of Health, Bethesda, MD, USA). Freeform lines were drawn along the edge of the cell engraftment (Polygon Selections), which was judged by dystrophin positive myofibres, and the surface area inside the lined-up engraftment was analysed.

In vitro osteogenic differentiation assay

Osteogenic differentiation was induced by culturing C2C12 myoblasts (pre-treated or un-treated) in osteogenic medium [OM, normal medium supplemented with dexamethasone (0.1 μM), ascorbate-2-phosphate (50 μM) and β-glycerophosphate (10 mM) (all from Sigma)]. The medium was changed every 2 days. Osteogenesis was assessed by observation of alkaline phosphatase (ALP) activity 10 days after initial osteogenic induction. The Alkaline Phosphatase kit (Sigma-86c) was then used to detect ALP activity.

In vivo osteogenic differentiation assay

While under anesthesia, a 6-mm-diameter defect was created in the parietal bone of SCID mice without breaching the dura, and then a 7-mm Gelfoam disk impregnated with either 2×10^5 hrTGF-β_1 (0.5 ng/ml) pre-treated or non-treated C2C12 myoblasts was implanted into the defect. Bone healing was monitored radiographically using microCT (vivaCT40, Scanco Medical AG, Brüttisellen, Switzerland) at 8 weeks after surgery.

In vitro chondrogenic differentiation assay

Pellet culturing and chondrogenesis assay were performed, as described previously [30, 31]. 2.5×10^5 cells were placed in a 15-ml conical polypropylene tube and centrifuged at $600 \times g$ for 5 min. Cells at the bottom of the tube were then cultured in 1 ml of chondrogenic medium, which contains: high glucose DMEM supplemented with 1% ITSþPremix (BD Biosciences, Bedford, MA, USA), L-ascorbic acid-2-phosphate (0.1 mM, Sigma), dexamethasone (0.1 μM, Sigma), proline (400 mg/ml, Sigma) and bone morphogenetic protein 4 (BMP-4) (500 ng/ml, R&D Systems, Minneapolis, MN, USA). The pelleted cells were incubated at 37°C in 5% CO_2. The medium was changed every 3 days. Pellets were harvested 20 days later and embedded in paraffin. Chondrogenesis was confirmed by histological stain of Alcian blue to stain the highly sulphated proteoglycans that are characteristic of cartilaginous matrix. After that, sections were counterstained with nuclear fast red, which selectively stains cell nuclei.

In vitro adipogenic differentiation assay

The adipogenesis assay was conducted as described previously [31, 32]. Cells were plated in 6-well plates (1×10^5 cells per well) in control medium to allow attachment. Twenty-four hours later, the medium was replaced with adipogenic medium [high glucose DMEM supplemented with insulin (10 μM), dexamethasone (1 μM), isobutyl-methylxanthine (0.5 mM) and indomethacin (200 μM) (all from Sigma-Aldrich, St. Louis, MO, USA)]. Cultures were maintained for 10 days, and the medium was changed every 2 days. The cultures then were stained with Oil Red (Sigma), which indicates the intracellular lipid accumulation: after being fixed for 10 min. at room temperature in 10% neutral buffered formalin, the cells were incubated in Oil Red O reagent for 30 min. and washed three times with distilled water.

Population doubling analysis

Primary myoblasts were plated at 1000 cells per well in a collagen-coated 6-well plate and cultured in growth medium. Cells were treated with 0.5 ng/ml hrTGF-β_1 for 3 hrs and continually cultured for 48 hrs. TGF-β_1 treated cells and non-treated control cells were then harvested and counted for the different cell populations. The approximate population doubling time (PDT) was determined as follows: 2^n = cell number at harvest time/cell number initially plated; '*n*' refers to the number of doublings during the period of cell culture (48 hrs here), thus PDT = 48 hrs/*n*.

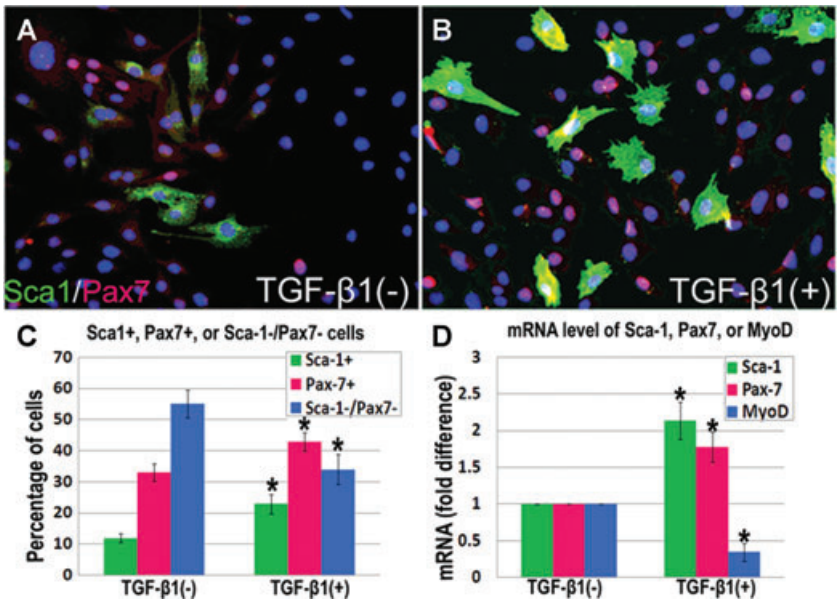

Fig. 1 Activation of stem cell markers in C2C12 myoblasts treated with TGF-β_1. Conditional TGF-β_1 treatment (0.5 ng/ml) induced myoblasts to express more stem cell markers: Sca-1 (green) or Pax7 (red) (**A** and **B**). Cell nuclei were shown with DAPI staining (blue). Statistical comparison of the percentage of the three cell populations (Sca-1$^+$, Pax7$^+$ and Sca-1$^-$/ Pax7$^-$) in the TGF-β_1 treated and non-treated cells is shown (**C**). Quantitative RT-PCR study showed that, mRNA levels of Sca-1 and Pax7 were up-regulated, while mRNA level of MyoD was down-regulated in TGF-β_1 pre-treated cells (**D**). '*' in the chart indicates the value being significantly different with the control cells (TGF-β_1^-).

Statistical analysis

At least three samples obtained from each patient were pooled for statistical analysis. All of the results from this study are expressed as the mean standard error (S.E.). The differences between two means were considered to be statistically significant if *P*-value is <0.05. A Student's t-test was used to determine whether there were statistically significant differences between two means, when two groups were analysed. For analyses with multiple groups, the ANOVA test was used in conjunction with a *post hoc* test (Dunnett as error protection), to determine whether particular differences between two means were statistically significant.

Results

Conditional TGF-β_1 treatment of C2C12 myoblasts increased the ratio of cells with positive stem cell markers *in vitro*

In addition to the effect of TGF-β_1 repressing myogenic differentiation [24–26], our previous studies have shown that extended exposure of myoblasts to high concentrations of TGF-β_1 (*i.e.* 5 ng/ml) was able to promote their transdifferentiation to myofibroblasts [23, 33]. In this experiment, we further discover that a controlled exposure to TGF-β_1, such as a transient treatment of myoblasts with lower concentrations of TGF-β_1 (*i.e.* 0.5 ng/ml), can in fact promote the expression of stem cell markers and reduce the expression of myogenic markers (Fig. 1).

C2C12 myoblasts were treated with 0.5 ng/ml of hrTGF-β_1 for 3 hrs and then cultured for another 5 hrs in growth medium without TGF-β_1. Cells were then fixed and immunostained with antibodies against Sca-1 and Pax7. Sca-1 is a stem cell marker of MDSCs and multipotent side population [1, 34]; while the transcription factor Pax7 is expressed mostly in satellite cells which are also often considered as muscle stem cells [35–37]. Three sub-populations of cells were observed in cultured cells: Sca-1 positive (Sca-1$^+$/Pax7$^-$), Pax7 positive (Sca-1$^-$/Pax7$^+$) and both Sca-1 and Pax7 negative (Sca-1$^-$/Pax7$^-$) cells (Fig. 1A and B). Results showed that TGF-β_1 treatment changed the ratio of the 3 sub-populations (Sca-1$^+$/Pax7$^-$: Sca-1$^-$/Pax7$^+$: Sca-1$^-$/Pax7$^-$) from 12%:33%:55% to 23%:43%:34% (Fig. 1C). Also, results of quantitative RT-PCR showed that expression of stem cell related genes (Sca-1 and Pax7) were up-regulated within the TGF-β_1 pre-treated cells, while the myogenic related genes (MyoD) were down-regulated (Fig. 1D).

TGF-β_1 treatment activates S-phase re-entry of the proliferation-arrested C2C12 myoblasts *in vitro*

When differentiation of myoblasts is initiated, a group of cells accumulate in the G1-phase of the cell cycle, and then exit the cell cycle upon entering the G0-phase and halt proliferation [38]. For the proliferation-arrested cells in the differentiating process, cell cycle re-entry could indicate an escape from the myogenic differentiation fate and could be a result of cell dedifferentiation [39–41]. We studied the effect of TGF-β_1 treatment on S-phase cell cycle re-entry of proliferation-arrested C2C12 myoblasts by both BrdU staining and PI staining/flow cytometry analysis.

C2C12 myoblasts were cultured in serum-free DMEM for 24 hrs to allow for proliferation arrest and differentiation. Cells were then transiently treated with hrTGF-β_1 (0, 0.1, 0.5 and 2.0 ng/ml) before being cultured with BrdU in the growth medium for 12 hrs. The ratio of cells with newly synthesized DNA (BrdU$^+$) can indicate if and what dosage of TGF-β_1 treatment can induce

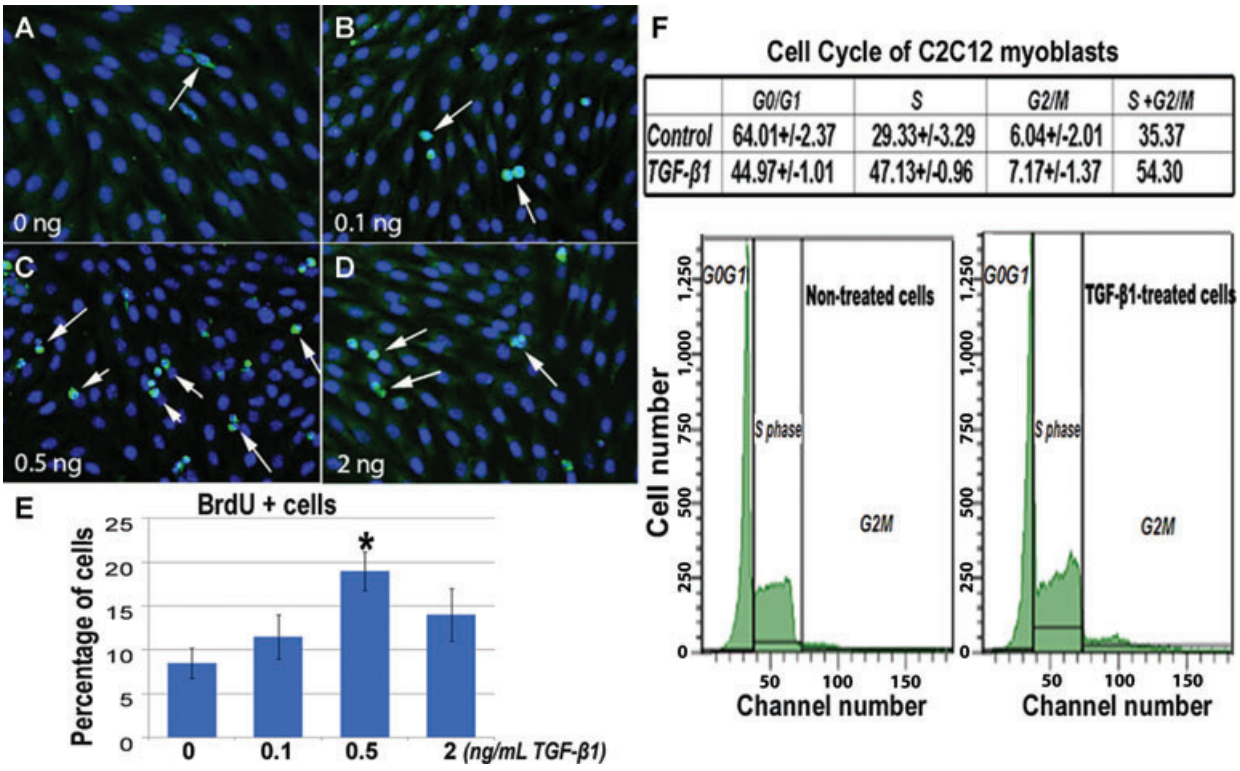

Fig. 2 S-phase re-entry of proliferation-arrested C2C12 myoblasts by TGF-β_1 treatment. C2C12 myoblasts pre-treated with hrTGF-β_1 (0.1, 0.5 and 2 ng/ml, 3 hrs) showed increased BrdU incorporation after incubation with BrdU. BrdU: green; DAPI: blue (**A–E**). PI staining/flow cytometry analysis shows that more cells were present in the S-phase in the cells pre-treated with TGF-β_1 (0.5 ng/ml, 3 hrs) (**F**). '*' indicates the value being significantly different with the control cells (0 ng/ml of TGF-β_1).

S-phase re-entry of proliferation-arrested C2C12 myoblasts. Results showed that TGF-β_1 treatment, especially at 0.5 ng/ml, resulted in a greater number of $BrdU^+$ cells than the control non-treated groups (Fig. 2A–E), indicating that S-phase re-entry occurred after treatment. In another experiment, proliferation-arrested C2C12 myoblasts were treated with hrTGF-β_1 (0.5 ng/ml) and then cultured for another 5 hrs in growth medium before being harvested for PI staining. Compared to control groups, more cells were detected in S-phase (47.13% *versus* 29.33%) in the TGF-β_1 treated groups (Fig. 2F), indicating the S-phase re-entry of some cells after TGF-β_1 treatment. The promoted cell cycle re-entry by TGF-β_1 stimulation may also indicate improved capabilities of self-renewal and long-term proliferation, which are similar to the characteristics of muscle stem cells [42].

TGF-β_1 treatment reduces the expression of MyoD and p21 in the proliferation-arrested C2C12 myoblasts *in vitro*

To further investigate the mechanism of S-phase re-entry of the proliferation-arrested C2C12 myoblasts induced by TGF-β_1, we tested the expression of MyoD and p21, two important factors regulating myoblast growth and differentiation [43–45]. C2C12 myoblasts were cultured in serum-free DMEM for 24 hrs, and then treated with or without hrTGF-β_1 (0.5 ng/ml) for 3 hrs. Cells were fixed 5 hrs later for detecting the expression of MyoD and p21. MyoD is a myogenic regulator [46] and it can induce cell-cycle arrest during differentiation of the myoblast by directly activating the expression of p21 and retinoblastoma protein (Rb) [47, 48]. p21 and its downstream target Rb regulate cell cycle withdrawal [49]. p21 also represses cells from entering the S-phase by inhibiting cyclin D1-dependent kinase (Cdk) [38]; thus, decreased expression of the cell cycle inhibitor p21 could indicate an increased potential of S-phase re-entry. In fact, our results have shown that the TGF-β_1 treated group features a significantly lower ratio of cells expressing p21 (Red) and MyoD (Green) (Fig. 3D-F, G) compared to the non-treated control cells (Fig. 3A-C, G). Therefore, this result further verifies that TGF-β_1 treatment can promote the S-phase re-entry of the proliferation-arrested myoblasts and reverse the myogenic differentiation process, which may be related with the dedifferentiation of these cells.

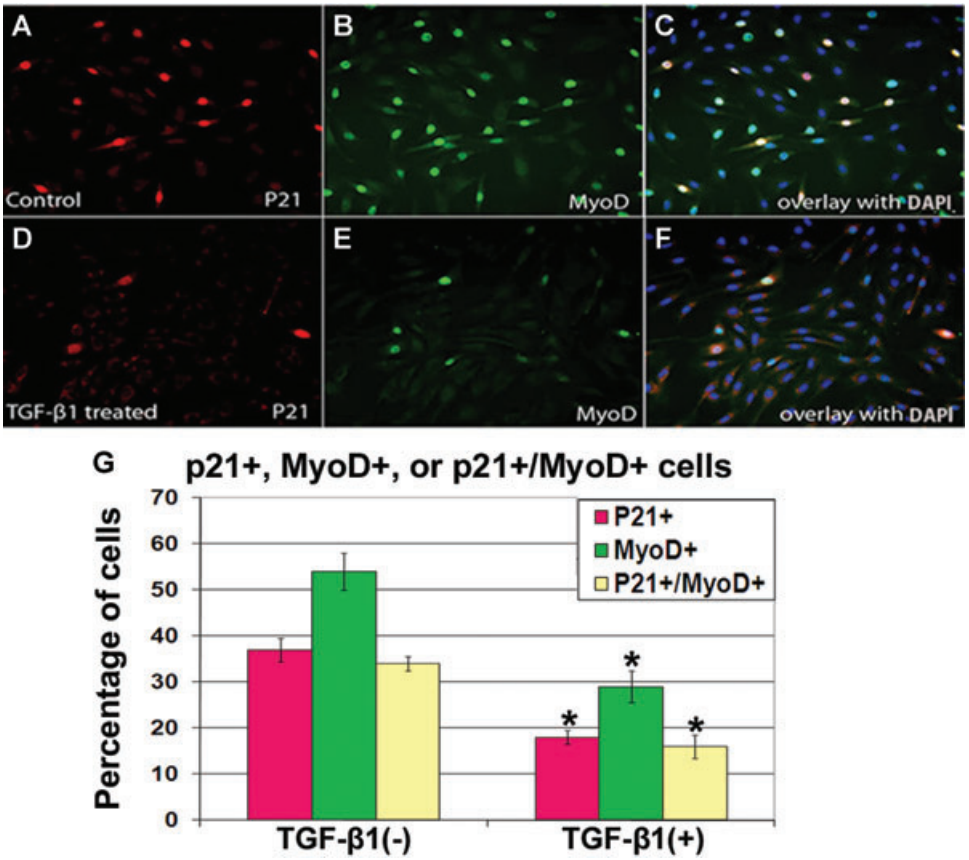

Fig. 3 p21 and MyoD expression in proliferation-arrested C2C12 myoblasts with or without TGF-β_1 treatment. p21 (red) and MyoD (green) were highly expressed in proliferation-arrested control C2C12 myoblasts (**A–C**), while their expression were both greatly reduced after hrTGF-β_1 pre-treatment (0.5 ng/ml) (**D–F**). DAPI: blue. Statistical quantitation of the percentage of the cells positive for p21, MyoD or both of the proteins in the TGF-β_1 treated and non-treated cells was conducted (**G**). '*' indicates the value being significantly different with the control cells (TGF-β_1^-).

TGF-β_1 treatment improves the myogenic differentiation capacities of C2C12 myoblasts – dedifferentiated cells form myotubes and repair dystrophic muscle

To further characterize the stem cell-like cells induced by TGF-β_1 treatment of C2C12 myoblasts, we investigated the multi-differentiation potentials of the cells, which include myogenic differentiation and osteogenic differentiation. The result of *in vitro* myogenesis assay showed that C2C12 myoblasts pre-treated with 0.5 ng/ml of hrTGF-β_1 formed the highest number of myotubes 5 days after myogenic induction (serum deprivation), compared to other groups of cells (0, 0.1 or 5 ng/ml of hrTGF-β_1) (Fig. 4A–C). To study the myogenic differentiation potential of the dedifferentiated cells *in vivo*, C2C12 myoblasts were pre-treated with 0.5 ng/ml of hrTGF-β_1 for 3 hrs prior to transplantation. The same number (1×10^5) of treated or non-treated cells was injected separately into the GM muscles of MDX/SCID mice. Muscle tissues were harvested for histological studies 1 or 2 weeks after cell transplantation. Previous studies showed that the myogenic marker myogenin is not detectable in non-regenerating normal muscle, but expressed in differentiating myoblasts and newly formed myofibers in the regenerating muscle of mice [50, 51]. In the current study we detected that the TGF-β_1 pre-treated cells resulted in larger-sized myofibres and fewer myogenin-positive mononuclear myoblasts or newly formed myofibers than the control cells at 1 week after transplantation, thus indicating a faster myogenic differentiation (Fig. 4D–G). Myogenin-positive cells were hardly detectable in both groups at 2 weeks after transplantation, but the TGF-β_1 treated group continued to show larger myofibres and improved engraftment compared to the control group (Fig. 4H–L).

This result demonstrates that myogenic differentiation potential of TGF-β_1 pre-treated myoblasts was improved compared to control myoblasts. In fact, many *in vivo* studies also indicated that the transplantation of muscle stem cells resulted in better muscle regeneration than myoblasts, which was due to the increased proliferation, antioxidant ability, cell survival, cell migration, and thus improved engraftment of the muscle stem cells [1, 4, 52, 53]. Therefore, the promoted *in vivo* myogenic differentiation potential by TGF-β_1 pre-treatment could be further confirmation that TGF-β_1 treatment increased the population of stem cell-like cells in C2C12 myoblasts.

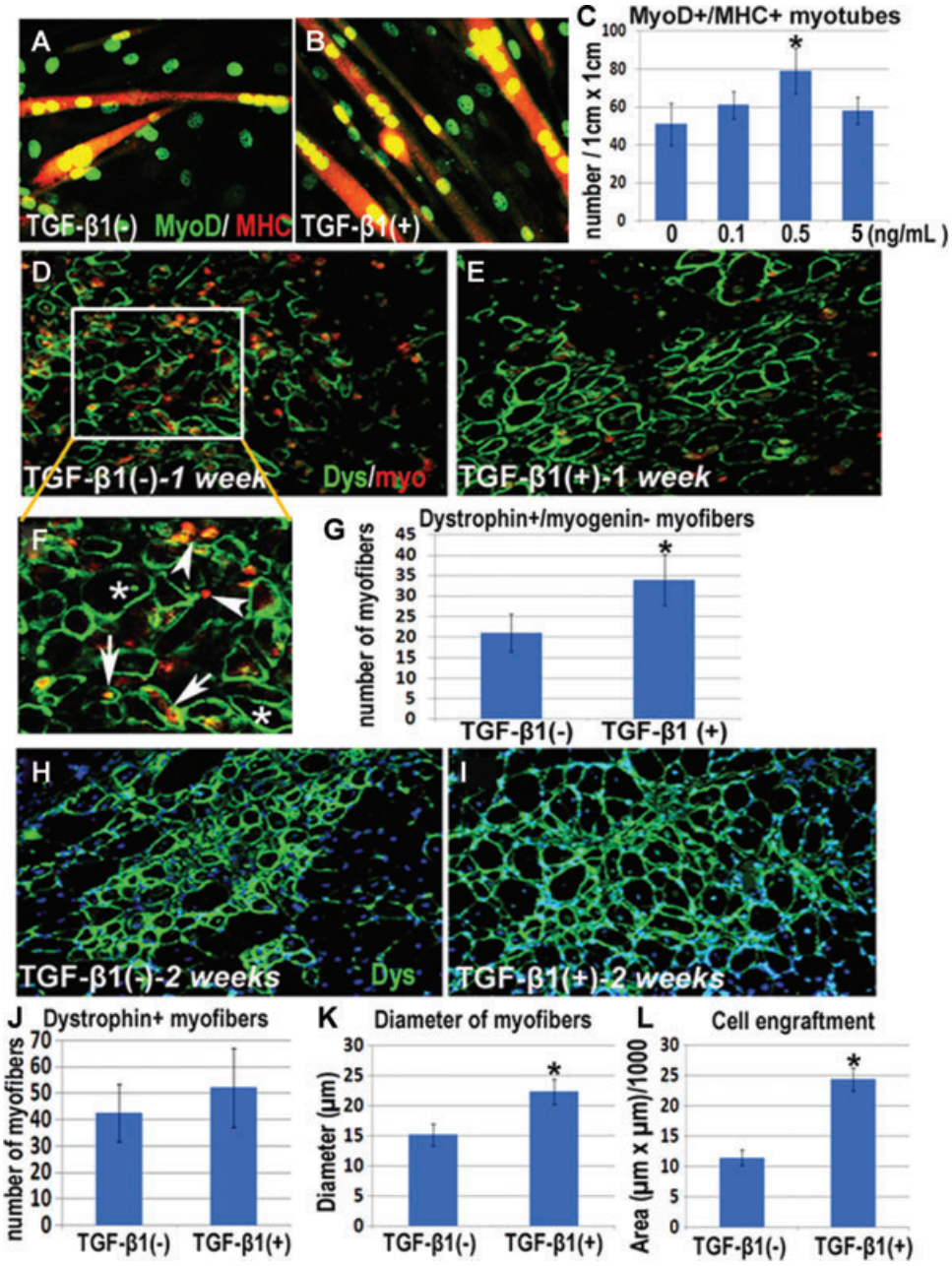

Fig. 4 Improved efficiency of TGF-β_1 pre-treated C2C12 myoblasts in *in vitro* myotube formation and in muscle regeneration after transplantation into the skeletal muscle of MDX mice. *In vitro* myotube formation study showed that hrTGF-β_1 pre-treated cells (0.5 ng/ml) formed the highest number of myotubes [MyoD$^+$/ myosin heavy chain (MHC)$^+$] (**A–C**). Dystrophin (green) and myogenin (red) were immunostained on muscle sections 1 week (**D** and **E**) or 2 weeks (**H** and **I**) after transplantation of control cells and hrTGF-β_1 (0.5 ng/ml) pre-treated cells. DAPI was shown as blue (**H** and **I**). The amplified image showed the localization of myogenin protein in either myoblasts (**F,** arrowheads) or newly formed myofibres/myotubes (**F,** arrows), but not in the relatively-mature larger myofibres (**F,** stars). Statistical quantitation of the number of dystrophin+/myogenin- myofibres (**G,** 1 week; **J**, 2 weeks), myofibre diameter (μm) (**K,** 2 weeks) or the cell engraftment (μm^2) (**L,** 2 weeks) of the samples was included. '*' in the chart indicates the value being significantly different with the control cells (TGF-β_1^-).

TGF-β_1 treatment improves the osteogenic differentiation capacities of C2C12 myoblasts – dedifferentiated cells repair skull defect in mice

We also investigated the osteogenic potential of the dedifferentiated cells, in order to confirm that the cells can differentiate into other cell lineages. hrTGF-β_1 pre-treated (0.5 ng/ml, 3 hrs) and non-treated C2C12 myoblasts were cultured in osteogenic medium for 4 days before being fixed for detecting the activity of ALP, an early marker of osteogenesis [54]. Results showed that the TGF-β_1 pre-treated cells demonstrated a higher ALP signal, indicating a stronger osteogenic potential of dedifferentiated cells compared to the control cells (Fig. 5A, B and E) *in vitro*. *In vivo,* we created a 6-mm-diameter defect in the parietal bone of SCID mice without breaching the dura, and then a 7 mm Gelfoam disk impregnated with either 2×10^5 hrTGF-β_1 pre-treated (0.5 ng/ml, 3 hrs) or non-treated C2C12 myoblasts was transplanted into the

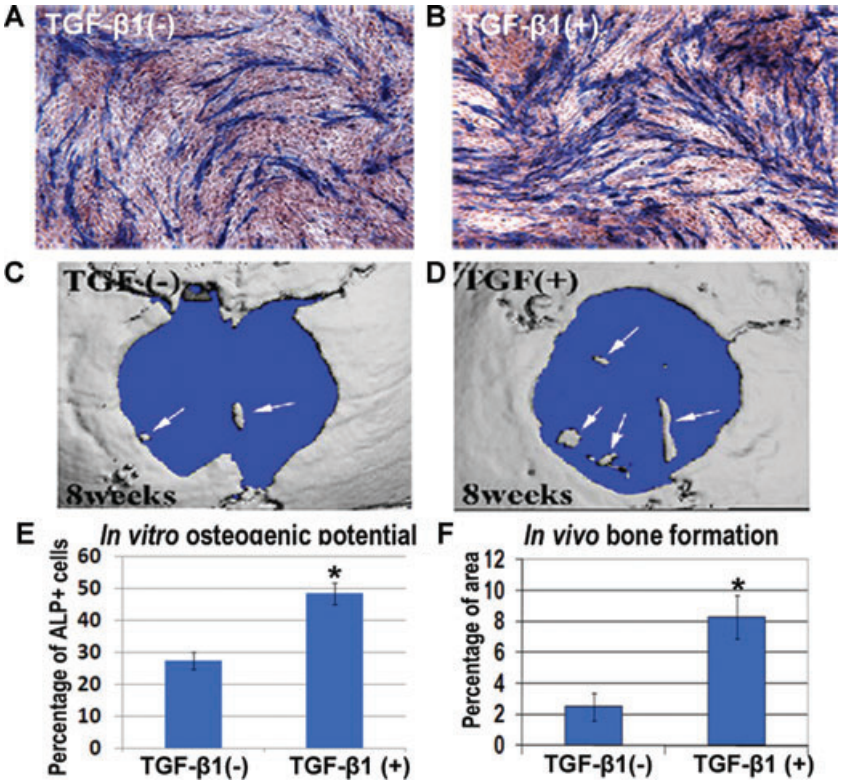

Fig. 5 Osteogenic potential of TGF-β_1 pre-treated C2C12 myoblasts *in vitro* and their efficiency in repair skull defect *in vivo*. *In vitro* osteogenic differentiation was compared between C2C12 control cells (**A**) and cells pre-treated with hrTGF-β_1 (0.5 ng/ml) (**B**), by measuring the percentage of cells positive with ALP signal. The efficiency of bone tissue formation *in vivo* was compared between C2C12 control cells (**C**) and cells pre-treated with TGF-β_1 (**D**). Four mice in each group (control cells and TGF-β_1 treated cells) were included in this study. Some small bone tissue was observed in the defect area at 8 weeks after surgery by micro CT, and TGF-β_1 pre-treated C2C12 myoblasts demonstrated a higher efficiency in repairing injured bone (**D**) compared to the control cells (**C**). Statistical quantitation of ALP^+ cells *in vitro* (**E**) and bone tissue formation *in vivo* was included (**F**). '*' indicates the value being significantly different with the control cells (TGF-β_1^-).

defect. Bone healing was monitored by microCT (vivaCT40, Scanco Medical AG). Small bone tissues were observed in the defect area as shown by micro CT at 8 weeks after surgery (arrows, Fig. 5C and D), and the TGF-β_1 pre-treated C2C12 obviously formed more bone tissue than the control cells (Fig. 5C, D and F).

TGF-β_1 treatment also increases the number of stem cell-like cells in primary myoblasts

To further verify our results acquired with C2C12 myoblasts, the effects of TGF-β_1 were also studied on primary myoblasts that were isolated from mice using the preplate technique [29]. The immunostaining studies shows that pre-treatment of myoblasts with TGF-β_1 resulted in elevated populations of both $Pax7^+/p21^-$ cells (Fig. 6A–C) and $Sca\text{-}1^+/CD34^+$ cells (both are surface markers of MDSCs) (Fig. 6D and E). A population doubling analysis was also conducted. Primary myoblasts were cultured with serum-free DMEM for 24 hrs, and then were treated with 0.5 ng/ml of hrTGF-β_1 for 3 hrs. Treated cells and control cells were then re-plated and cultured in growth medium for 48 hrs to compare their proliferation rates with population doubling analysis. Results showed that TGF-β_1 pre-treated primary myoblasts had a shorter PDT compared to the control cells (Fig. 6F), indicating an increased proliferating potential of the stem cell-like cells. TGF-β_1 pre-treated primary myoblasts were also transplanted into the MDX/SCID mice as it was done with C2C12 myoblasts, and improved myofibre formation was observed 2 weeks after cell transplantation (Fig. 6G–I). In addition to the improved myogenic potential, results of the *in vitro* chondrogenesis assay (Fig. 6J–L) and adipogenesis assay (Fig. 6M–O) indicated that primary myoblasts pre-treated with TGF-β_1 also had improved chondrogenic and adipogenic potentials. So that, these results acquired with the primary myoblasts were shown to be similar to those with C2C12 myoblasts.

Discussion

The source of muscle 'precursors' during the muscle healing process has been suggested include activated satellite cells, locally released muscle stem cells, circulating haematopointic and bone marrow cells. However, the natural muscle healing process from injuries or diseases (*i.e.* Duchenne muscular dystrophy/DMD) often leads to the excessive formation of fibrotic scar tissue, in which case stem cell-based therapies can be helpful for improved regeneration [2, 5, 55, 56]. Compared to the time-consuming isolation of a limited number of muscle stem cells and *in vitro* propagation, the induction of stem cells from homogenous myoblasts could be a much more efficient way to obtain enough cells with multi-differentiation potentials for transplantation and tissue

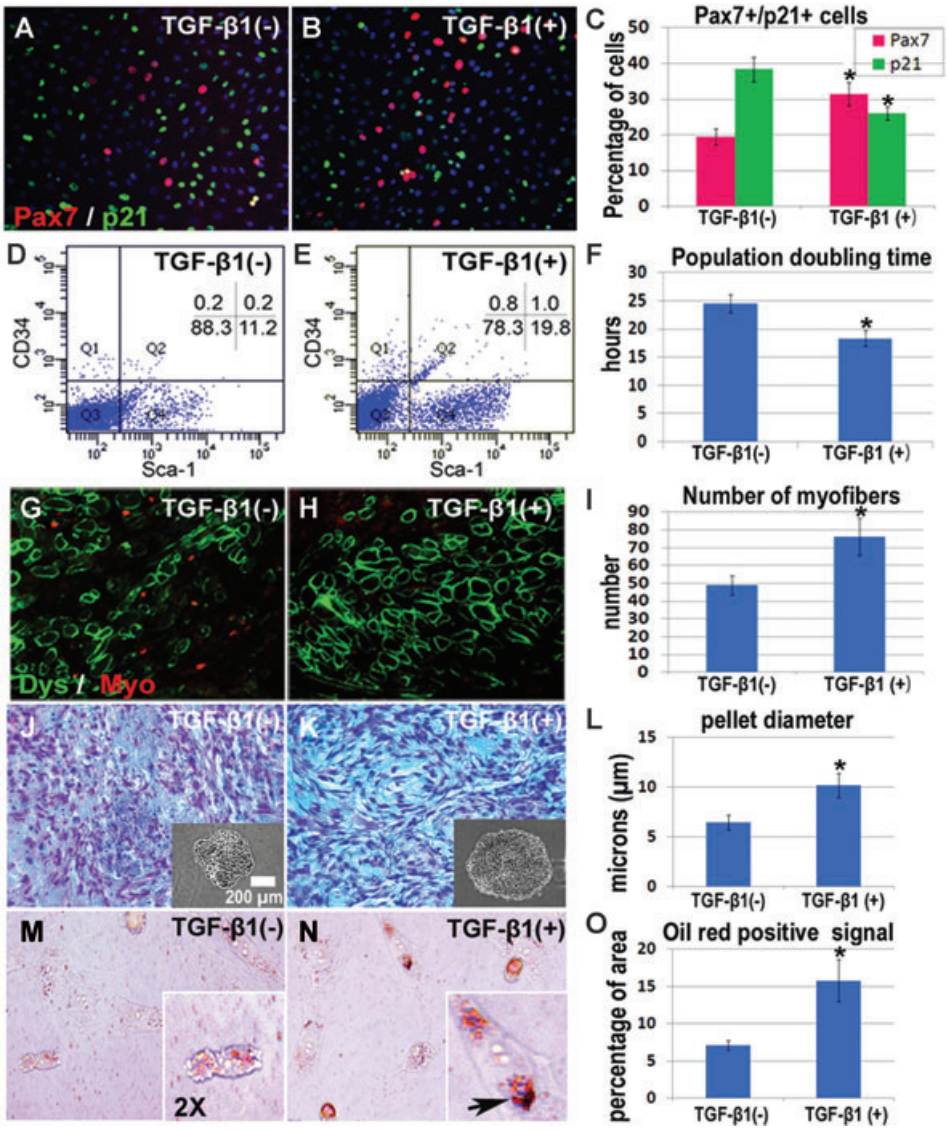

Fig. 6 Effects of TGF-β_1 treatment on isolated primary myoblasts. Immunostaining analysis (**A–C**) and flow cytometry study (**D** and **E**) showed elevated populations of both Pax7$^+$/p21$^-$ cells and Sca-1$^+$/CD34$^+$ cells with TGF-β_1 treatment (**A–E**). With population doubling analysis, TGF-β_1 pre-treated primary myoblasts had a shorter PDT compared to the control cells (**F**). Cell transplantation into MDX/SCID mice also demonstrated improved myogenic potential of TGF-β_1 pre-treated cells (**H**) compared to the control cells (**G**) (2 weeks after transplantation). Dys: dystrophin; myo: myogenin. Statistical quantitation of the number of dystrophin$^+$ myofibres of the samples was included (**I**). Results of the chondrogenesis assay showed that TGF-β_1 pre-treated cells produced pellets with better characteristic of cartilaginous matrix (**K,** Alcian blue staining and nuclear fast red counterstaining) and larger diameter (**K**, attached sub-image), compared to that of control cells (**J**). Result of adipogenesis assay showed that TGF-β_1 pre-treated cells formed more adipocyte-like cells with positive Oil Red staining (lipid droplets) (**N**) compared to control cells (**M**). 2× magnified sub-images were also attached (**M** and **N**; arrow indicates lipid droplets). Statistical quantitation of pellet diameter (**L**) and Oil Red positive signal (**O**) was included. '*' indicates the value being significantly different with the control cells (TGF-β_1^-).

engineering [57]. In the current experiment, we discovered that after conditional TGF-β_1 stimulation *in vitro*, myoblasts demonstrated an increased expression of stem cell markers and an increased ability for S-phase re-entry. Moreover, these stem cell-like cells generated by the TGF-β_1 pre-treatment, which may not be identical to endogenous muscle stem cells but carry many characteristics of muscle stem cells, were shown to have improved regenerative capacities *in vivo* in effectively repairing both dystrophic muscle and injured bone. In addition to the specific cell line of C2C12 myoblasts, similar effects of TGF-β_1 were also observed with primary myoblasts isolated from mice (Fig. 6). This suggests that TGF-β_1 is generally functional in increasing the number of stem cell-like cells when applied to skeletal muscle myoblasts. The representative mechanism suggested for our results is proposed as Figure 7.

In the skeletal muscle, although both Sca-1$^+$ cells (MDSCs) and Pax7$^+$ cells (satellite cells) can be multipotent muscle stem cells, they are thought to be two distinct populations of muscle precursor cells [1, 36]. Sca-1$^+$ cells are in fact of more 'stemness' compared to Pax7$^+$ cells [1, 36]. Pax7 is essential for renewal and maintenance of satellite cells by maintaining proliferation and multipotency, and preventing precocious differentiation [36, 58]. Satellite cells, usually latent in normal adult muscle, express Pax7 but not MyoD; when satellite cells are activated to proliferate and migrate in the muscle regeneration process, they co-express Pax7 and MyoD [36]. The proliferated cells then gradually lose Pax7,

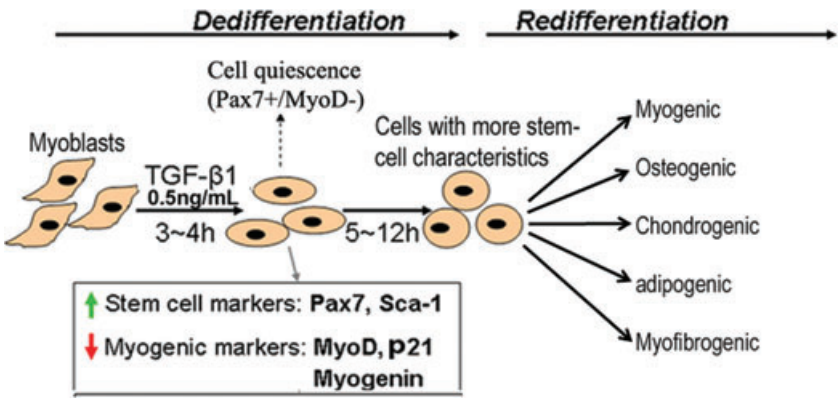

Fig. 7 A potential process of dedifferentiation and redifferentiation of muscle cells. Based on this study, we are proposing that a transient/lower concentration of hrTGF-β_1 (0.5 ng/ml) treatment can promote the dedifferentiation of some of the myoblasts into stem cell-like cells, which can then redifferentiate into multiple cell lineages. There is also the possibility of cell quiescence demonstrated by increased Pax7 expression and decreased MyoD expression in some of the TGF-β_1 treated cells.

but maintain MyoD expression and differentiate as myogenic cells [36]. As shown in our results, TGF-β_1 generated a new balance among the different cell populations, with more stem cells (Sca-1^+ or $Pax7^+$ cells) and fewer differentiating myogenic cells ($MyoD^+$ cells), indicating that TGF-β_1 treatment modified the characteristics of myoblasts towards that of muscle stem cells.

Our results also indicate that a lower concentration (0.5 ng/ml) of transient TGF-β_1 treatment of myoblasts could generate a very different result compared to that of a higher concentration (over 5 ng/ml) given constantly [24, 26]. The effects of high concentrations of TGF-β_1 could be complicated. For example, TGF-β_1 can significantly inhibit myotube formation in a dose-dependent fashion [24, 26]. Similar to the role of TGF-β_1 in epithelial–mesenchymal transition, a process whereby fully differentiated epithelial cells transform to a mesenchymal phenotype giving rise to fibroblasts and myofibroblasts [59, 60], our previous studies also showed that continuous exposure to TGF-β1 caused myoblasts to transdifferentiate into myofibroblastic cells *in vitro* and *in vivo* [23]. Also, a high concentration of TGF-β_1 (5 ng/ml) was shown to induce Runx2 activation which may coordinate with BMP-2 signalling in promoting osteogenic potential of C2C12 [61]. However, previous studies documented that lower concentrations (*i.e.* 0.1 ng/ml) of TGF-β1 are capable of maintaining the multipotency and self-renewal potential of stem cells [16, 27, 28]. Our current results further reveal that myoblasts transiently treated with lower concentrations of TGF-β_1 did not transdifferentiate into myofibroblasts, but rather could transition into stem cell-like cells with positive stem cell markers and multi-differentiation potency. We suggest that the contrasting results of TGF-β_1 treatment of myoblasts could be due to the fact that higher concentrations of TGF-β_1 may promote myoblast dedifferentiation into stem cell-like cells first, and then subsequently promote the redifferentiation of these cells into myofibroblasts. The lower concentrations of transient TGF-β_1 treatment could only be effective in accomplishing the dedifferentiation process, but not the redifferentiation process (Fig. 7). It is also noted that a transient exposure to TGF-β_1 (3–5 hrs) is important for maintaining the activation of stem cell markers because an extended exposure (8–24 hrs) of myoblasts to TGF-β_1 may increase the risk of the redifferentiation of the induced stem cell-like cells towards fibrotic cells [23].

The heterogeneity of the myoblasts may result in a varying response of the cells to TGF-β_1 treatment. Besides cell dedifferentiation, it is possible that cell quiescence was also induced by TGF-β_1 pre-treatment (Fig. 7). Quiescent satellite cells express the satellite cell marker Pax7 and activated satellite cells co-express Pax7 and MyoD [36, 37, 62]. Increased Pax7 expression and decreased MyoD expression in the TGF-β_1 pre-treated myoblasts may indicate that some of the cells had transitioned from $Pax7^+/MyoD^+$ activated status to $Pax7^+/MyoD^-$ quiescent status. The cells with increased Pax7 expression after TGF-β_1 pre-treatment may include both proliferating stem-like cells and quiescent cells (Fig. 7). Previous cell cycling studies had shown that serum-deprivation induces growth arrest of cells in the form of quiescence (G0-phase), and cells can then be promoted to re-enter the S-phase in a synchronized fashion [63]. However, a myoblast represents a specific cell lineage. Besides cell quiescence, myoblasts differentiate and fuse to form myotubes with serum deprivation. In cell-cycle related experiments, 24 hrs of serum deprivation was conducted to promote cell differentiation, but avoid possible fusion. At least 50% of the myoblasts were $MyoD^+$, whereas over 70% of these $MyoD^+$ cells were also $p21^+$ (Fig. 3A–C). These populations of cells should be differentiating ($MyoD^+/p21+$), but not quiescent cells ($MyoD^-$) [64]. The differentiating cells, quiescent cells and other cells accumulated at G1-phase may act differently when re-entering the S-phase. Our results showed that compared to the control cells, TGF-β_1 treatment reduced the number of cells positive with MyoD and p21 (Fig. 3) as well as increased the number of cells re-entering the S-phase (Fig. 2). p21 is a negative regulator of cell cycle, and its absence alone can even determine the S-phase re-entry of terminal differentiated myotubes [65]. The TGF-β_1-induced down-regulation of p21 in many differentiating cells could be a further verification of the increased potential of S-phase re-entry.

Evidence now suggests that adult myoblasts and even myofibres can undergo dedifferentiation with various manipulations and become more stem cell-like *in vitro* [10, 11, 13, 14]. However, the dedifferentiation process of muscle cells in mammals *in vivo* was an extremely controversial topic for years. Dedifferentiation of muscle cells and 'blastema' formation in urodele amphibians are necessary for limb regeneration, which has not been observed in adult mammals yet. In foetal mice, however, the early stages of

digit regeneration from amputation may apply similar mechanism to that of urodele amphibians, in which a signalling pathway of muscle segment homeobox-1 and BMP-4 is involved [66]. Our previous study on muscle healing indicated that endogenous expression of TGF-β_1 (early-phase, day 2 to day 5 after muscle injury) and stem cell proliferation (days 2 to 6 after muscle injury) seems to have a correlated time course [23]. Meanwhile, it was shown that TGF-β_1 signalling is critical for the dedifferentiation and regeneration processes after limb amputation in axolotls [15], which may reflect a possibly similar mechanism in the wound healing process of mammals. It is possible that the dedifferentiated muscle cells after muscle injury may be one source of the 'precursors' to eventually accelerate the regeneration process, and TGF-β_1 may be related with the process.

In summary, this study has revealed the effect of TGF-β_1 pretreatment in enlarging the population of stem cell-like cells in myoblasts. This method could lead to possible therapeutic applications to increase the population of autologous stem cells for stem cell-based therapy.

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