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Control of mesenchymal cell fate via application of FGF-8b *in vitro*

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Abstract

In order to develop strategies to regenerate complex tissues in mammals, understanding the role of signaling in regeneration competent species and mammalian development is of critical importance. Fibroblast growth factor 8 (FGF-8) signaling has an essential role in limb morphogenesis and blastema outgrowth. Therefore, we aimed to study the effect of FGF-8b on the proliferation and differentiation of mesenchymal stem cells (MSCs), which have tremendous potential for therapeutic use of cell-based therapy. Rat adipose derived stem cells (ADSCs) and muscle progenitor cells (MPCs) were isolated and cultured in growth medium and various types of differentiation medium (osteogenic, chondrogenic, adipogenic, tenogenic, and myogenic medium) with or without FGF-8b supplementation. We found that FGF-8b induced robust proliferation regardless of culture medium. Genes related to limb development were upregulated in ADSCs by FGF-8b supplementation. Moreover, FGF-8b enhanced chondrogenic differentiation and

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CRediT authorship contribution statement

Takayoshi Otsuka: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Paulos Y. Mengsteab:** Conceptualization, Validation, Writing - original draft, Writing - review & editing. **Cato T. Laurencin:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

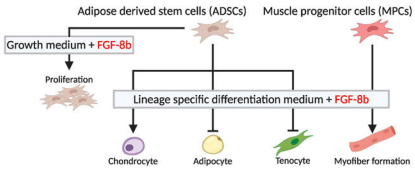
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102155.

suppressed adipogenic and tenogenic differentiation in ADSCs. Osteogenic differentiation was not affected by FGF-8b supplementation. FGF-8b was found to enhance myofiber formation in rat MPCs. Overall, this study provides foundational knowledge on the effect of FGF-8b in the proliferation and fate determination of MSCs and provides insight in its potential efficacy for musculoskeletal therapies.

Graphical Abstract



Keywords

Mesenchymal stem cells; Adipose-derived stem cells; Muscle progenitor cells; Fibroblast growth factor 8b; Differentiation

1. Introduction

Regeneration of complex tissues and organs, such as the limb, is well documented in lower vertebrates (e.g. fish and salamanders), which exhibit robust regeneration potential to regrow appendages after amputation (Brockes and Kumar, 2005; Tanaka, 2016). Mammals, in contrast, have a limited capacity for complex tissues and organ regeneration (Han et al., 2005; Laurencin and Nair, 2015). Therefore, understanding the limitation of regeneration in mammals and improving it remains a significant goal for therapeutic development. To develop strategies to regenerate complex tissues in mammals, and a limb one day, regenerative engineering approaches will be needed (Laurencin and Khan, 2012; Laurencin and Nair, 2016). Regenerative engineering is a convergence field whereby advanced material science, physical science, stem cell science, developmental biology and translational medicine with the goal of creating tools that regenerate or reconstruct functional tissues and organs (Ameer, 2020; Beachy et al., 2020; Bowers and Brown, 2019; Clegg et al., 2019; Heath, 2019; Ifegwu et al., 2018; James et al., 2016; Mengsteab et al., 2020; Moore and West, 2019; Nelson et al., 2018; Ogueri et al., 2019; Tang et al., 2019, 2020). As a part of this approach, developmental and regeneration biology will provide clues that can direct new engineering strategies for coordinated regeneration of tissues with limited regeneration capacity.

One of the unique and essential events during limb regeneration is blastema formation (McCusker et al., 2015; Stocum, 2017). The blastema is an undifferentiated heterogeneous cell population of lineage-restricted progenitor cells derived from connective tissue fibroblasts (Kragl et al., 2009; McCusker et al., 2016). Blastema formation requires an innervated wound epithelium, which functions to recruit blastema cells to the wound site and allows them to proliferate (McCusker et al., 2015; Stocum, 2017). In the absence of nerve signals, denervation of the appendage, regeneration fails to initiate or blastema size is

reduced depending on the timing of denervation (Singer, 1978). Thus, it appears that nerve signaling is required to maintain cells in an undifferentiated and proliferative state until the final pattern and size of the missing limb is restored (Farkas and Monaghan, 2017; Satoh et al., 2018).

Fibroblast growth factors (FGFs) play multiple roles in regeneration such as the control of cell migration, proliferation, and differentiation (Maddaluno et al., 2017). Among the FGFs, FGF-2 and FGF-8 were found to be expressed in axolotl dorsal root ganglia (DRG) and are sufficient to induce blastema formation (Makanae et al., 2014; Satoh et al., 2011). Grafting of an FGF-2 + FGF-8-soaked bead in wounded skin results in an ectopic blastema in the absence of nerves, suggesting that these growth factors are neurotropic in nature (Satoh et al., 2011). In addition, the essential role of FGF-8 signaling has been demonstrated in axolotl limb regeneration as well as amniote limb development (Crossley et al., 1996; Lewandoski et al., 2000; Nacu et al., 2016; Satoh et al., 2016).

Although blastema formation is a key event during complex tissue regeneration, induction of blastema formation in mammals remains challenging. To model blastema induction, mesenchymal stem cells (MSCs) are an attractive cell source for therapeutic approaches because of their undifferentiated state and its use in point-of-care therapies (Chamberlain et al., 2007; Pittenger et al., 1999; Yu et al., 2020). Some studies have demonstrated the potential use of MSCs for limb regeneration (Masaki and Ide, 2007; Taghiyar et al., 2017). FGF signaling regulates limb bud development and the differentiation into the musculoskeletal tissue (Ornitz and Marie, 2015; Yun et al., 2010). FGF-2 has been suggested to be a potent mitogenic growth factor that stimulates the proliferation of MSCs (Ahn et al., 2009; Zaragosi et al., 2006). FGF-2 has also exhibited biphasic effects on multilineage differentiation of MSCs (Hanada et al., 1997; Kim et al., 2015; Lai et al., 2011; Neubauer et al., 2004; Solchaga et al., 2005). On the other hand, little is known about the effect of FGF-8 on MSCs maintenance and fate determination.

Herein, we sought to understand the role of FGF-8 on the fate of MSCs. Thus, in this study, we aimed to provide foundational knowledge on the effect of FGF-8 on the proliferation and differentiation of MSCs *in vitro*. We demonstrated that FGF-8b consistently enhanced cell proliferation under multi-lineage differentiation conditions. In addition, FGF-8 signaling successfully recapitulates gene expression related to limb development. Finally, we described the diverse effects of FGF-8b in the differentiation of MSCs, suggesting its therapeutic potential for the regeneration of musculoskeletal tissues.

2. Material and methods

2.1. Reagents

Recombinant growth factors were obtained from R&D Systems (Minneapolis, Minnesota): human FGF-2 (233-FB), FGF-8b (423-F8), transforming growth factor-β3 (TGF-β3, 243-B3), platelet-derived growth factor-BB (PDGF-BB, 220-BB), and mouse growth differentiation factor 6 (GDF-6, 855-G6). Dexamethasone (D4902), L-ascorbic acid (A4544), beta-glycerophosphate (G9422), 3-isobutyl-1-methylxanthine (IBMX, I7018), recombinant human Insulin (rh-insulin, I2643), indomethacin (I8280), and ITS (I3146) were

obtained from Millipore Sigma (St. Louis, Missouri). Fetal bovine serum (FBS, 16000044), horse serum (16050122), penicillin-streptomycin (pen/strep, 15140122), and sodium pyruvate (11360070) were obtained from Gibco, Thermo Fisher Scientific (Waltham, Massachusetts).

2.2. Adipose derived stem cells (ADSCs) and muscle progenitor cells (MPCs) isolation and culture

ADSCs and MPCs were isolated from Sprague-Dawley rats (age: 16–18 weeks, weight: 450–550 g). Rats were euthanized with CO_2 inhalation followed by neck dislocation. Inguinal fat pads were harvested and minced into small pieces. An equal volume of collagenase type I (0.2%, w/v) (Gibco) in Hank's balanced salt solution was added to the fat tissue and agitated at 37 °C for 90 min. The cell suspension was filtered through a 70 μm filter for the removal of solid aggregates. The cells were plated in 10 cm dishes containing DMEM-F12 with 10% FBS and 1% pen/strep (growth medium; GM), then placed in a 37 °C–5% CO_2 incubator. The media was changed after 72 h. The cells were passaged at 80–90% confluence. The GM was changed every two to three days and cells were expanded to passage 3 for this study.

For MPCs isolation, gastrocnemius muscles were harvested and minced into small pieces. The muscle tissues were digested in a mix of collagenase type I (0.2%, w/v) and dispase II (0.4%, w/v) (Sigma) in DMEM-F12 at 37 °C for 120 min. The cell suspension was filtered through a 100 μm filter for the removal of solid aggregates. The cells were plated in 10 cm dishes containing DMEM-F12 with 20% FBS, 25 ng/mL of FGF-2 and 1% pen/strep. To remove faster growing non-muscle cells, the supernatant containing non-adhered MPCs were transferred to Matrigel (Corning, Glendale, Arizona) coated 10 cm dishes after 24 h. The cells were passaged after 80% confluence. The culture media was changed every three to four days and the cells were expanded to passage 2 for this study.

2.3. Flow cytometry

Rat ADSCs and MPCs were characterized by flow cytometry (MACSQuant Analyzer 10; Miltenyi Biotec, Bergisch Gladbach, Germany). Rat ADSCs, passage three, and MPCs, passage two, (5×10^5 cells per sample) were collected and washed thrice in sterile PBS containing 1% FBS, then incubated on ice with fluorescein isothiocyanate (FITC)-conjugated anti-rat CD90, CD29 (integrin beta 1), CD45, CD11b (BD BioSciences, San Jose, California), and CD31 (abcam, Cambridge, Massachusetts) at a concentration of 10 μg/mL for 30 min in the dark at 4 °C. Unlabeled cells were used as controls. FlowJo software (Treestar, Inc., Ashland, Oregon) was used to create the histograms.

2.4. MTS assay

The metabolic activity and proliferation of cells were assessed using the CellTiter® 96 AQueous nonradioactive cell proliferation assay (MTS assay; Promega, Madison, Wisconsin) following the manufacturer's protocol. Briefly, cells were washed with PBS, then MTS reagent in a ratio of 5:1 (media: MTS) added to each well. The plates were incubated for 1 h at 37 °C. The absorbance of the resulting solution was read at 490 nm using a microplate reader.

2.5. DNA quantification

DNA was isolated and quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Carlsbad, California) following the manufacturer's instructions. Briefly, cell lysates were collected and mixed with the Quant-iT PicoGreen reagent, measured via spectrophotometry at 535 nm with excitation at 485 nm, and DNA content was quantified using a standard curve.

2.6. Multi-lineage differentiation induction

Rat ADSCs at passage 3 or MPCs at passage 2 were used to verify the differentiation capacity. Cells were seeded in 6 well plates at 1.5×10^5 cells/well or 24 well plates at 3.0×10^4 cells/well and grown to at least 80% confluence before being cultured in the differentiation medium.

To induce osteogenic differentiation, rat ADSC were cultured up to 21 days in Growth medium (GM) supplemented with 100 nM dexamethasone, 50 μg/mL L-ascorbic acid, and 10 mM beta-glycerophosphate (Osteogenic medium). Mineralization of the extracellular matrix was visualized by staining with Alizarin Red S on day 21. The cells were fixed in 10% formalin for 10 min, and then incubated for 20 min in 1% Alizarin Red S (pH 4.2). After qualitative analysis by microscopy, the stain was eluted by 10% cetylpyridinium chloride for 15 min and analyzed at 450 nm absorbance.

To induce adipogenic differentiation, rat ADSC were cultured for 14 days in GM supplemented with 0.5 mM IBMX, 1 μM dexamethasone, 10 μg/mL rh-insulin, and 100 μM indomethacin (Adipogenic medium). Adipogenic differentiation was confirmed by staining with Oil-Red O at 14 day. The cultures were fixed in 10% formalin for 10 min, and then incubated for 20 min in Oil Red O solution. After qualitative analysis by microscopy, the stain was eluted by 100% isopropanol for 10 min and analyzed at 510 nm absorbance.

To induce chondrogenic differentiation, rat ADSCs were cultured in GM supplemented with 1% ITS, 100 nM dexamethasone, 50 μg/mL ascorbate-2-phosphate, 0.9 mM sodium pyruvate, and 10 ng/mL TGF-β3 (Chondrogenic medium). For micromass culture, a 20 μL drop, containing 5.0×10^5 cells, was carefully placed in the center of each well of a 24-well plate. The cell drops were incubated for 2 h at 37 °C and 5% CO_2, subsequently CM was carefully added to each well. The micromass pellets were then cultured for 21 days in CM. The pellets were fixed in 10% formalin for 24 h, embedded in paraffin, cut into 5 μm sections and stained with standard hematoxylin/eosin and 1% alcian blue (pH 1.0)/0.1% nuclear fast red.

To induce tenogenic differentiation, rat ADSCs were cultured for 14 days in GM supplemented with 50 ng/mL GDF-6, and 10 ng/mL PDGF-BB (Tenogenic medium) (Norelli et al., 2018).

To induce myogenic differentiation, rat ADSCs or MPCs were cultured for 14 days in DMEM-F12 supplemented with 5% horse serum and 1% pen/strep (Myogenic medium).

2.7. Immunofluorescence

Cells were rinsed with PBS, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and permeabilized with 0.1% Triton X 100 for 10 min at room temperature. Then, cells were blocked by 1% bovine serum albumin (Sigma) and incubated with primary antibodies overnight at 4 °C. Thereafter, cells were rinsed thrice with PBS and incubated with secondary antibody for 2 h at room temperature in the dark. Primary antibodies used were anti-SCXA (1:250, abcam), anti-tenomodulin (1:100, abcam), anti-Tenascin C (1:200, Novus Biologicals, Littleton, Colorado), anti-Pax7 (1:200, LSBio, Seattle, Washington), anti-MYOD (1:200, LSBio), and anti-Desmin (1:100, Invitrogen). DAPI (Invitrogen) was used as nuclear staining. As for secondary antibodies, Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) and Goat Anti-mouse IgG H&L (Alexa Fluor® 594) were used. All stained samples were examined under a Leica DMi8 inverted microscope (Leica Microsystems, Wetzlar, Germany).

2.8. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Alameda, California) according to the manufacturer's instructions. 1–4 μg of total RNA was reverse-transcribed to cDNA by using RNA to cDNA EcoDry Premix (Clontech, Palo Alto, California) in a total volume of 20 μL. Taqman predesigned primers (Thermo Fisher Scientific) (Supplementary Table 1) were used for qRT-PCR, and the signal was detected by an iQ5™ real-time PCR machine (Bio-Rad, Hercules, California). The threshold cycle values of target genes were standardized against GAPDH expression and normalized to the expression in the control culture. The fold change in expression was calculated using the ΔΔCt comparative threshold cycle method. All qRT-PCRs were run in triplicate.

2.9. Statistical analysis

GraphPad Prism 7 (GraphPad Software; San Diego, California) was used for statistical analysis and graph design. Results were expressed as the mean values ± standard deviation. Comparisons between two groups were performed with the unpaired Student's t-test. Comparisons of more than two groups were done by one-way ANOVA with Dunnett's post-hoc test. Differences were considered significant if the p-value was < 0.05. Statistical significance was shown with * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

3. Results

3.1. Rat ADSCs and MPCs characterization

Isolated ADSCs showed a spindle-shaped and flat polygonal morphology (Supplementary Fig. 1A). ADSCs at passage 3 were analyzed for expression of MSC specific cell-surface markers (Supplementary Fig. 1B–F). Flow cytometry analysis showed that ADSCs were positive for the MSC surface markers CD90 (98.4% ± 2.8) and CD29 (99.2% ± 1.4), and negative for the hematopoietic stem cell surface markers CD11b (1.0% ± 0.2), CD45 (0.4% ± 0.3) and endothelial surface marker CD31 (0.8% ± 0.2). Rat ADSCs were successfully differentiated into multiple lineages such as bone, fat, and cartilage (Supplementary Fig. 1G–H). Primary rat MPCs at passage 2 were characterized by immunofluorescence and flow

cytometry analysis. MPCs were positive for a satellite stem cell marker (Pax7), and myogenic markers (MyoD and Desmin) (Supplementary Fig. 2A–C). Flow cytometry analysis showed that MPCs were positive for CD29 (99.9% ± 0.1), and negative for CD45 (0.1% ± 0.1) and CD31 (0.2% ± 0.1) (Supplementary Fig. 2D–F).

3.2. FGF-8b increases proliferation in cultured rat ADSCs and MPCs

The ability of FGF-8b to promote proliferation of rat ADSCs was evaluated by metabolic and total DNA content analysis (Fig. 1). The metabolic activity of rat ADSCs was followed for 14 days, and metabolic activity was enhanced at day 3 of culture when GM was supplemented with 100 ng/mL of FGF-8b (Fig. 1A). At days 7 and 14, the metabolic activity of rat ADSCs supplemented with 100 ng/mL of FGF-8b continued to be upregulated in comparison to GM alone (Fig. 1A). The effects of lower concentrations of FGF-8b (10 ng/mL and 50 ng/mL) were only seen at day 14 (Fig. 1A). Total DNA content was surveyed as a surrogate for cell count, and an increase in total DNA content of rat ADSCs was observed at day 7 and 14 when the GM was supplemented with 50 or 100 ng/mL of FGF-8b (Fig. 1B). Furthermore, the mitogenic potential of rat ADSCs were evaluated in osteogenic, chondrogenic, adipogenic, tenogenic, and myogenic mediums. In all medium conditions, the supplementation of FGF-8b at 100 ng/mL was found to significantly increase DNA content (Fig. 1C). Finally, the total DNA was quantified in MPCs, and it was found that total DNA was significantly increased when MPCs were cultured in myogenic medium supplemented with 100 ng/mL of FGF-8b (Fig. 1D).

3.3. FGF-8b regulates expression of FGF signaling pathway components in ADSCs

Following 7 days of culture, targets of the FGF-8 signaling pathway from the receptor to downstream signaling targets was evaluated. Endogenous mRNA expression of FGF-8 was not detected by qRT-PCR in growth medium with or without FGF-8b supplementation. FGF-8b supplementation significantly enhanced the mRNA expression of FGF-2, fibroblast growth factor rector 1 (FGFR-1), and FGFR-2 (Fig. 2A). No significant effect on the mRNA expression of FGFR-3 and FGFR-4 were found (Fig. 2A). Next, the mRNA expression of downstream targets of FGF signaling were assessed to determine the effect of FGF-8b supplementation on intracellular signaling. Several downstream targets of FGF signaling were found to be upregulated when ADSCs were supplemented with FGF-8b, suggesting that exogenous FGF-8b supplementation effects signal transduction through the canonical pathway (Fig. 2B). Finally, FGF-8b supplementation (100 ng/mL) was found to upregulate the expression of an osteogenic (Runx2) and chondrogenic marker of differentiation (Sox9), while adipogenic (Pparg) and tenogenic (Scx) markers were down regulated (Fig. 2C). Of note, myogenic marker (Myod1) was only detected in FGF-8b supplementation (Supplementary Table 2).

3.4. FGF-8b enhances chondrogenic differentiation while suppressing tenogenic and adipogenic differentiation

The effect of FGF-8b on the osteogenic, chondrogenic, adipogenic, and tenogenic differentiation of rADSCs was evaluated. First, we investigated osteogenic differentiation by assessing the extent of calcium deposition and the expression of osteogenic markers of rADSCs with and without FGF-8b supplementation. We found that FGF-8b supplementation

did not significantly affect the calcium deposition of rADSCs both qualitatively and quantitatively (Fig. 3A–C). A trend towards the upregulation of osteogenic markers (Runx2 and Sp7) was found (Fig. 3D). The results suggest that FGF-8b supplementation does not affect osteogenic differentiation *in vitro*.

FGF-8b supplementation was found to enhance chondrogenic differentiation of micromass cultures of rat ADSCs. After 21 days of culture, the size of micromass cultures of rat ADSCs were qualitatively larger (Fig. 4A). FGF-8b increased the cellularity of micromass cultures as observed in sections stained with hematoxylin & eosin (Fig. 4B and C). Additionally, the intensity of alcian blue staining was enhanced by FGF-8b supplementation, which is indicative of increased proteoglycan content within the extracellular matrix (Fig. 4D and E). FGF-8b supplementation significantly enhanced the transcription of aggrecan at day 21, and a trend towards enhanced sox9 expression was observed (Fig. 4F). These results suggest that FGF-8b enhances micromass size through the proliferation of rat ADSCs and contributes to the development of a chondrogenic environment and transcriptome.

FGF-8b supplementation to adipogenic medium significantly inhibits rat ADSC adipogenesis when assessed at day 14. FGF-8b downregulated the formation of lipids as observed by oil red O staining (Fig. 5A and B). The accumulation and distribution of lipids in ADSCs was more robust in adipogenic medium alone (Fig. 5A), whereas the presence of lipids was sparse in the FGF-8b condition (Fig. 5B). This was confirmed by colorimetric analysis of extracted oil red O from stained cultures (Fig. 5C). Transcriptionally, FGF-8b was found to significantly downregulate the adipogenic markers PPARg, fabp4, and adipoq (Fig. 5D). These results suggest the exogenous administration of FGF-8b has a profound inhibitory effect on adipogenesis.

FGF-8b supplementation to tenogenic medium resulted in enhanced proliferation of ADSCs and inhibition of tenogenic differentiation. Immunostaining shows anisotropic organization of the confluent ADSC cultures (Fig. 6A–D). FGF-8b supplementation resulted in higher cellularity compared to tenogenic medium alone, as shown by nuclear staining (Fig. 6A–D). The signal intensity of scleraxis and tenomodulin did not differ in either condition and was weak overall (Fig. 6A–D). Tenascin C staining was more robust in tenogenic medium alone (Fig. 6E and F). FGF-8b was found to downregulate the expression of tenogenic markers (Scx, Tnmd, and Tnc) and tendon extracellular matrix proteins (Collagen 1 and 3, Fig. 6G). These results suggest that supplementation of FGF-8b in tenogenic medium leads to the proliferation of ADSCs rather than differentiation.

3.5. FGF-8b enhances myofiber formation of rat MPCs

Although the ability of ADSCs to differentiate towards myogenic lineage has been reported, the gold standard culture system for robust myogenic differentiation of ADSCs is still missing (Di Rocco et al., 2006). The low level of muscle specific proteins was assessed by immunofluorescence staining of MyoD and Desmin after 14 days of culture (Supplementary Fig. 3). The effect of FGF-8b on myogenic differentiation was evaluated with rat MPCs.

FGF-8b was found to enhance myofiber formation of MPCs as determined by immunostaining and qRT-PCR analysis. FGF-8b was found to enhance the presence of

Desmin, the main intermediate filament of skeletal muscle (Fig. 7A, B). To determine the progression of myogenesis, qRT-PCR analysis was conducted. Data indicated that an early marker of myogenesis (Myf5) was unchanged due to FGF-8b supplementation (Fig. 7C). However, mature myogenic markers (Myod1 & Myog) and slow skeletal muscle gene expression (Tnnt1) were upregulated in MPCs (Fig. 7C). These data suggest that FGF-8b supplementation to myogenic medium significantly contribute to MPC maturation.

4. Discussion

This study describes the fundamental effects of FGF-8b exogenous supplementation on the differentiation potential of ADSCs and MPCs. Here we show that FGF-8b supplementation enhances ADSCs and MPCs proliferation in all differentiation medium conditions assessed. The proliferative effect of the FGF family was expected as several studies have demonstrated this result. The present study also demonstrates that FGF-8b supplementation can also upregulate or downregulate differentiation markers of ADSCs and MPCs. Of note, we demonstrate that FGF-8b supplementation 1) activates the FGF signaling pathway, 2) inhibits adipogenic and tenogenic differentiation, and 3) enhances chondrogenic and myogenic differentiation. FGF-8b did not affect osteogenic differentiation. Overall, this study builds a foundation for studying the response of ADSCs and MPCs to exogenous FGF-8b, a key growth factor in limb development and regeneration.

4.1. Exogenous FGF-8 recapitulate FGF signaling response during limb development

FGF signaling has an essential role in limb development (Teven et al., 2014). During limb morphogenesis, FGF-8 (among other FGFs) is expressed in apical ectodermal ridge (AER), while the limb bud mesenchyme expresses FGF-2 and FGF-10 (Martin, 1998; Sun et al., 2000). Furthermore, Fgfr1 and Fgfr2 are expressed in limb mesenchyme during limb development (Martin, 1998; Xu et al., 1998; Yu and Ornitz, 2008). Dusp6 and spry4 are known as negative regulators of FGF signaling and are induced by FGF-8 signaling during limb development (Kawakami et al., 2003; Li et al., 2007; Minowada et al., 1999). At later stages of limb morphogenesis, the expression of FGF-8 is restricted to the anterior AER which interacts with sonic hedgehog (Shh) to make an anteroposterior pattern. Fgf-dependent etv4, etv5, and twist1 interact with Shh signaling to control anterior-posterior patterning of the limb (Mao et al., 2009; Zhang et al., 2010). Our results recapitulate the same upregulated expression pattern of FGF receptors (Fgfr1 and Fgfr2) and downstream genes such as etv4, etv5, dusp6, spry4, and twist1 in rat ADSCs (Fig. 2A, B). Exogenous Fgf-8b did not induce FGF-8 expression in ADSCs, analogous to limb mesenchyme. This is consistent with the restricted expression of FGF-8 in the AER and its absence in limb mesenchyme during limb development. Instead, supplementation of FGF-8 induced FGF-2 expression (Fig. 2A), which leads to enhanced proliferation in MSCs (Ahn et al., 2009; Zaragosi et al., 2006).

The role of FGF signaling seems to be conserved in development and regeneration (Maddaluno et al., 2017; Yokoyama, 2008). Common FGFs are expressed in both the developmental and regeneration process (e.g. FGF-4, FGF-8, and FGF-10) (Christensen et al., 2002; Han et al., 2001), and FGFs treatment enhances regeneration response (Makanae

et al., 2014; Nacu et al., 2016; Yokoyama et al., 2001). The interaction of FGF-8 and Shh is also observed in axolotl limb regeneration for successful pattern formation (Nacu et al., 2016). However, there is an interesting difference in salamander limb. Unlike anurans and amniotes, AER-specific FGFs such as FGF-8 were expressed in salamander mesenchyme (Nacu et al., 2016; Purushothaman et al., 2019). Thus, it is of interest to understand whether the AER-specific FGFs expression in MSCs enhance the regeneration response in mammals. Our exogenous treatment of FGF-8b did not trigger endogenous FGF-8 expression in ADSCs. Further study focused on the endogenous effect of AER-specific FGFs might provide new insight in mammalian regeneration.

4.2. FGF-8 signaling demonstrates different MSC-fate decision compared to FGF-2

FGFs exert multifaceted effects through the activation of four distinct receptors (FGFR1–4) with differential binding properties, and the main downstream signaling pathways are the mitogen-activated protein kinase (RAS/MAPK) pathway, the phosphoinositide 3 (PI3) kinase/AKT pathway, and phospholipase C gamma (PLCγ) pathway (Yun et al., 2010). Our result revealed FGF-8b promoted the expression of FGFR1 and 2 (Fig. 2A), which suggested the FGF-8b supplementation can alter downstream FGF signaling. While FGF-8b has been poorly explored in fate decision of MSCs, FGF-2 has been extensively analyzed. The effects of FGF-2 in fate decision of MSCs are to suppress osteogenesis (Huang et al., 2010; Lai et al., 2011; Wang et al., 2018) and promote chondrogenesis (Correa et al., 2015; Solchaga et al., 2005), adipogenesis (Kakudo et al., 2007; Neubauer et al., 2004) and tenogenesis (Cai et al., 2013). FGF-2 also has been reported to promote proliferation, but suppress myogenesis in MPCs (Olwin and Rapraeger, 1992; Yablonka-Reuveni et al., 2015). Interestingly, FGF-8b showed the opposite effect from FGF-2 in some lineages (adipogenesis, tenogenesis, and myogenesis) although FGF-2 expression was enhanced by FGF-8b supplementation (Figs. 2A, C, 5–7). Of note, the dose-dependent biphasic effects of FGF-2 on osteogenesis, chondrogenesis, and adipogenesis has been reported with or without the combination of other growth factors (Chiou et al., 2006; Hanada et al., 1997; Kim et al., 2015). One of our limitations was that the trial of FGF-8b supplementation in differentiation assays were at a single dose (100 ng/mL), which was chosen based on the proliferative effect. Nonetheless this report describes the foundational effects of FGF-8b supplementation on MSCs differentiation. Future studies will evaluate the precise mechanistic control of FGF signaling in combination or comparison with FGF-2 signaling pathway including exploration of optimal dose condition.

4.3. FGF signaling regulates adipogenesis

Adipose tissue acts as an endocrine organ that can regulate inflammation and healing processes, and FGFs have shown the ability to regulate adipogenesis. The effect of FGFs on adipogenesis is isoform dependent. Exogenous supplementation of FGF-1 and FGF-2 to medium have been shown to promote adipogenic differentiation of human preadipocytes and ADSCs *in vitro*, respectively (Kakudo et al., 2007; Widberg et al., 2009). Furthermore, these studies demonstrate that FGF-1 and -2 supplementation act to prime human ADSCs for adipogenic differentiation by showing that FGF supplementation is needed in the proliferative stages but not during the differentiation stage of the *in vitro* assay. Interestingly, Hutely et al. demonstrated that FGF-1 supplementation to human preadipocytes leads to

early adipogenic commitment while reducing endogenous FGF-2 levels, suggesting that paracrine interplay of FGFs in adipose tissue is complex (Hutley et al., 2011).

In the present study, we demonstrate that FGF-8b supplementation to adipogenic medium suppress lipid production and the expression of adipogenic mRNA in rat ADSCs (Fig. 5). To our knowledge, of the 22 FGFs, only FGF-8b has shown the ability to downregulate the adipogenic differentiation of rat ADSCs. However, it has been shown that FGF-8b downregulates the adipogenic differentiation of mice preadipocytes. This provides more evidence for the adipogenic inhibitory effect of FGF-8b in mesenchymal derived cells (Westphal et al., 2019). Additionally, the expression of PPARg was found to be downregulated when mouse brown preadipocytes were supplemented with FGF-6 and -9 *in vitro* (Shamsi et al., 2020). The results of our study and current literature on FGF signaling in adipogenesis demonstrate the significant role of FGFs in the regulation of adipose.

4.4. FGF-8 signaling in somite myogenesis is recapitulated in vitro with muscle progenitor cells

The role of FGF-8 in muscle development has been well documented in zebrafish and chick models (Edom-Vovard et al., 2001; Groves et al., 2005). In particular, the role of FGF-8 has been shown to be necessary for the expression of the myogenic markers, myoD and myogenin in the zebrafish somite (Groves et al., 2005). Within the somite there are three major compartments, the sclerotome, myotome, and dermomyotome. It has been found that FGF-8 is expressed in the myotome giving rise to the syndetome, a structure that forms between the myotome and sclerotome (Groves et al., 2005). Additionally, in chick limb development FGF-8 expression is localized to mature tendon but restricted to the myotendinous junction (Edom-Vovard et al., 2001). Therefore, the role of FGF-8 in muscle and tendon differentiation is of interest.

In the present study FGF-8 had opposing effects on tendon and muscle gene expression (Figs. 6F and 7C). Tendon markers of ADSCs were found to be downregulated in the presence of FGF-8 supplementation suggesting that FGF-8 might be involved in the maintenance of mature tendon rather than the induction of tenogenesis. Likewise, FGF-8b treatment of ADSCs in myogenic induction medium did not affect the presence of muscle fiber proteins (Supplementary Fig. 3). These results may be due to the naïve state of ADSCs, thus the effect of FGF-8b on muscle progenitor cells was evaluated. Interestingly, FGF-8b was found to enhance the expression of myoD and myogenin, which is consistent with the FGF-8 driven myogenic progression in the developing somite (Groves et al., 2005). This suggests that FGF-8 may enhance muscle maturation and could be of therapeutic interest for muscle pathologies.

4.5. FGF-8 effects on chondrogenesis are likely dependent on the source of mesenchyme

The effect of FGF-8 signaling on the development of cartilage is dependent on the source of mesenchymal tissue and can have both positive and negative effects (Bobick et al., 2007; Schmidt et al., 2018; Zhou et al., 2007). For example, Schmidt et al. demonstrated that FGF-8 overexpression promotes chondrogenesis across the skull in mice (Schmidt et al., 2018). In a study of mesenchyme explants, it was found that FGF-8 treatment causes

upregulation of chondrogenic markers and sulfated GAGs in mandibular mass explants, but not in frontonasal or limb bud mesenchyme (Bobick et al., 2007). In our present study, we demonstrate that micromass cultures of ADSCs are enhanced in size and have increased proteoglycan content. Furthermore, on the molecular level chondrocyte markers were enhanced when ADSC chondrogenic differentiation medium was supplemented with FGF-8b. This suggests that inguinal ADSCs chondrogenic potential is enhanced by FGF-8b.

4.6. Potential use of FGF-8b for regenerative medicine

The findings on the fundamental roles of exogenous FGF-8b on MSCs, demonstrates its diverse biological function for tissue regeneration and provides a basis to engineer therapies for musculoskeletal applications. Based on the data, there are two potential orthopedic applications for FGF-8b: 1) Downregulation of adipose induced inflammation in the knee joint for the treatment of osteoarthritis, and 2) regeneration of rotator cuff tissue. These therapeutic areas are of great interest as the prevalence of osteoarthritis and rotator cuff tears continues to increase with an aging population worldwide (Minetto et al., 2020).

Conservative treatment of osteoarthritis aims to alleviate patient pain through the administration of anti-inflammatory agents (i.e. corticosteroids) or viscosupplementation to increase lubrication during joint loading (Abramoff and Caldera, 2020). Yet, in many cases a total knee replacement is necessary for the patient to relieve pain and increase joint function. The knee joint is a complex organ comprised of several tissue types (e.g. synovium, adipose, cartilage, ligament, and synovial fluid), that experience physical forces and diverse paracrine signaling events. The main actor in the progression of osteoarthritis is controversial, however a greater appreciation of the role adipose tissue (infrapatellar fat pad) plays in promoting osteoarthritis has recently garnered more interest (Collins et al., 2021; Greif et al., 2020). Moreover, the combinatorial application of FGF-2 and FGF-8 in the knee joint was recently found to suppress adipokine secretion and demonstrated a decreased inflammatory profile (Mengsteab et al., 2020). Evidence has begun to reveal that adipose tissue promotes inflammation within the knee, which leads to cartilage degeneration.

Our findings that FGF-8b inhibits adipogenic differentiation and promotes chondrogenesis makes it an interesting candidate to treat osteoarthritis. However, the literature on the effect of FGF-8b on cartilage is controversial. One study injected 50 μg of FGF-8b in the knee joint of rats and found that FGF-8b promoted cartilage degeneration, which was evidenced by increased sulfated glycosaminoglycan content in the synovial fluid and decreased patella bone weight (Uchii et al., 2008). This result is in contrast to our observations in this *in vitro* study. FGF-8 may have a role in controlling the balance of catabolic and anabolic activity of cartilage formation. Therefore, the dosing effect of FGF-8b on the knee joint remains to be explored. Future therapies may need to consider directly targeting the fat pads of the knee joint to promote an anti-inflammatory effect.

FGF-8b is also a promising candidate to administer during the repair of rotator cuff tears due to its pro-myogenic effect on MPCs and anti-adipogenesis effect on ADSCs. The muscles of a rotator cuff tear atrophy after injury and there is fatty infiltration into the muscle belly (Gladstone et al., 2007; Osti et al., 2013). Studies have demonstrated that there is a correlation between baseline fatty infiltration and a failed rotator cuff repair and that fatty

infiltration progresses after repair (Gladstone et al., 2007; Lansdown et al., 2017). The adverse outcomes associated with fatty infiltration and its progression, and the need to promote myogenesis to gain patient function makes FGF-8b delivery a compelling therapy. Future studies will need to assess the effect of FGF-8b on rotator cuff derived cells *in vitro* and rotator cuff repair *in vivo*.

5. Conclusion

In summary, the results provide fundamental insights into the role of exogenous FGF-8b on the proliferation and differentiation of MSCs. FGF-8b supplementation enhances cell proliferation under all differentiation medium assessed. We were able to recapitulate the enhanced expression of genes related to limb development with exogenous FGF-8b. Also, we show that FGF-8b enhances myogenic and chondrogenic lineage commitment, whereas it suppresses adipogenic and tenogenic differentiation. These results propose a potential application of FGF-8b in expansion and fate determination of MSCs towards complex tissue regeneration. Future studies will analyze the effect of FGF-8b under the pathological conditions to enhance complex tissue regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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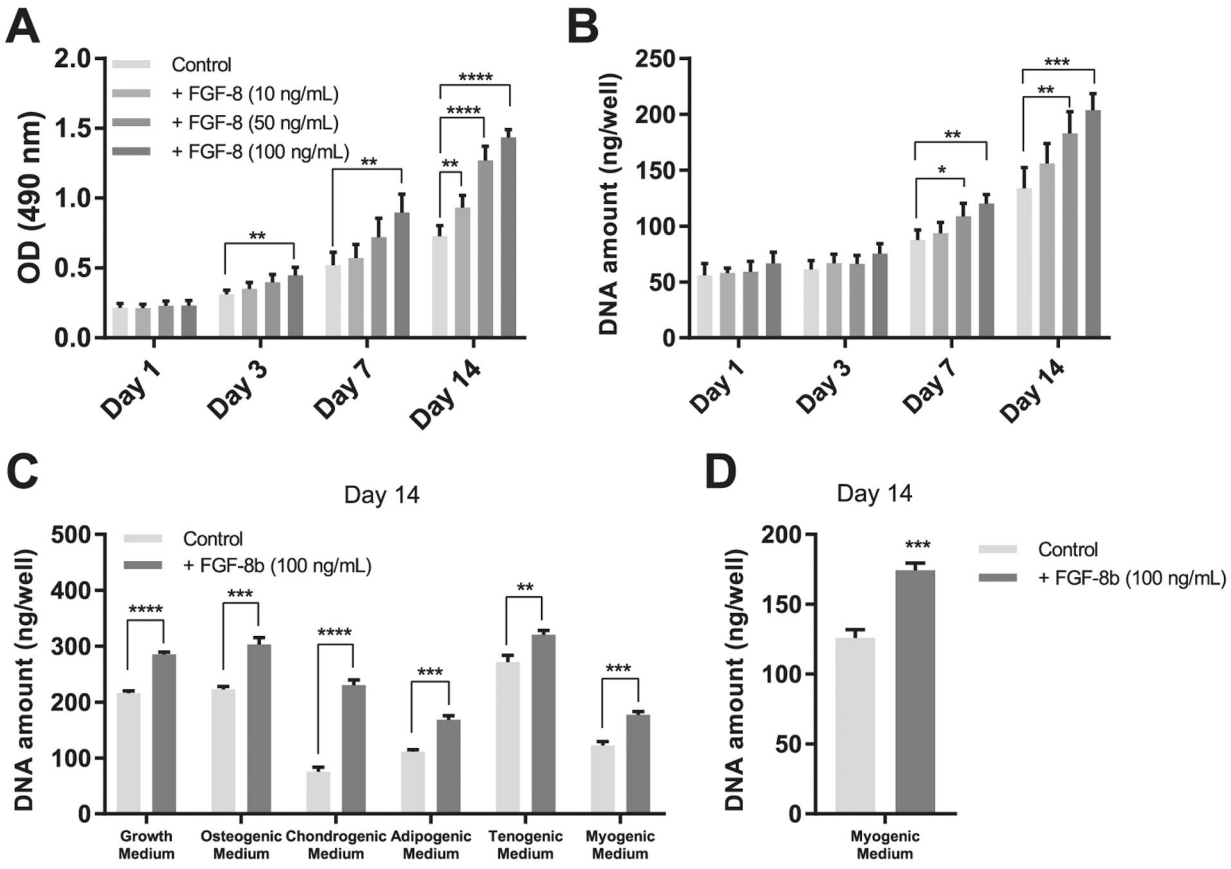

Fig. 1.

FGF8-b supplementation enhances cell proliferation in rat ADSCs and MPCs. (A) MTS assay and (B) DNA quantification of rat ADSCs cultured in growth medium (GM) supplemented with or without FGF-8b at Day 1, 3, 7, and 14. (C) DNA quantification of rat ADSCs cultured in growth and differentiation medium. (D) DNA quantification of rat MPCs in myogenic medium at Day 14. Statistical analyses: one-way ANOVA (A, B), unpaired Student's t-test (C, D). * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

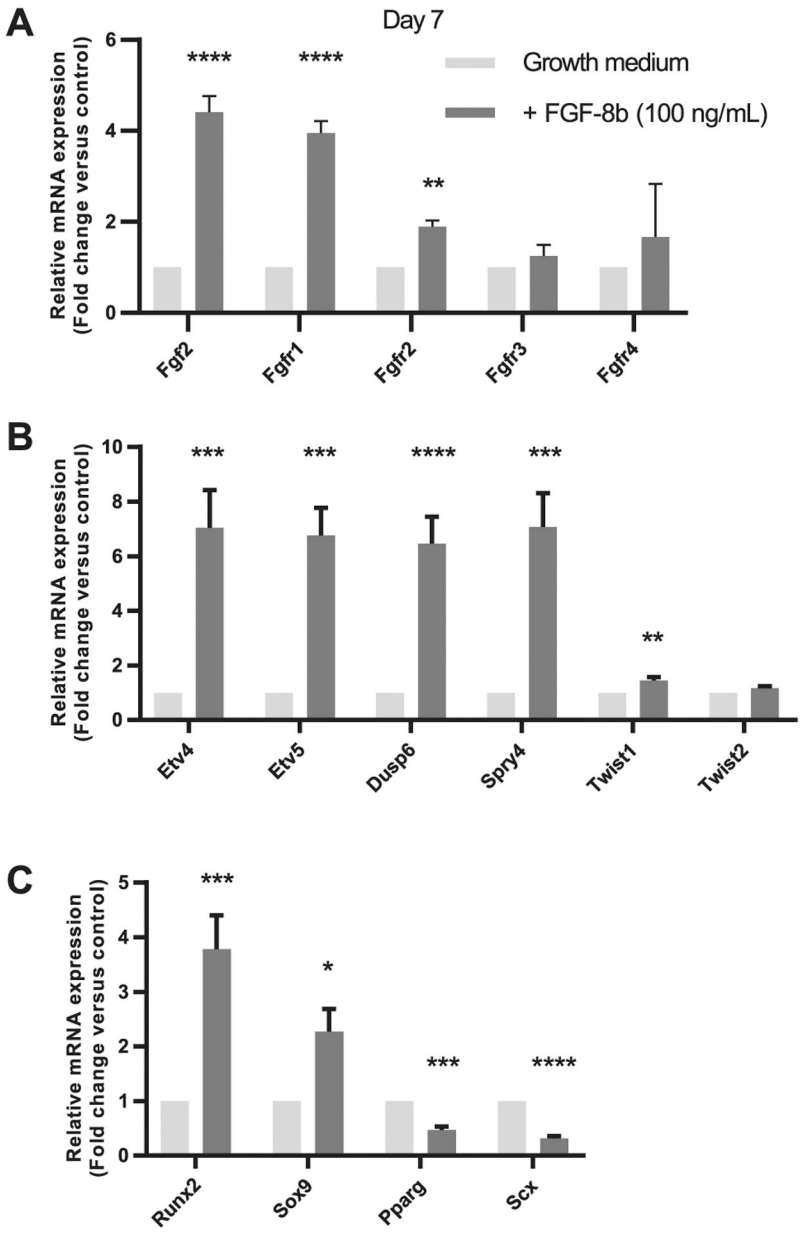

Fig. 2.

FGF-8b supplementation alters FGF downstream signaling gene expression and differentiation potentials. (A) mRNA expression of Fgf2 and FGF receptors. (B) mRNA expression of FGF signaling downstream genes. (C) mRNA expression of lineage specific transcription factors. All data are expressed as fold change relative to both the housekeeping gene and growth medium control (without FGF-8b supplementation) condition. Statistical analyses: unpaired Student's t-test (A, B, C). * P < .05, ** P < .01, *** P < .001, and **** P < .0001.

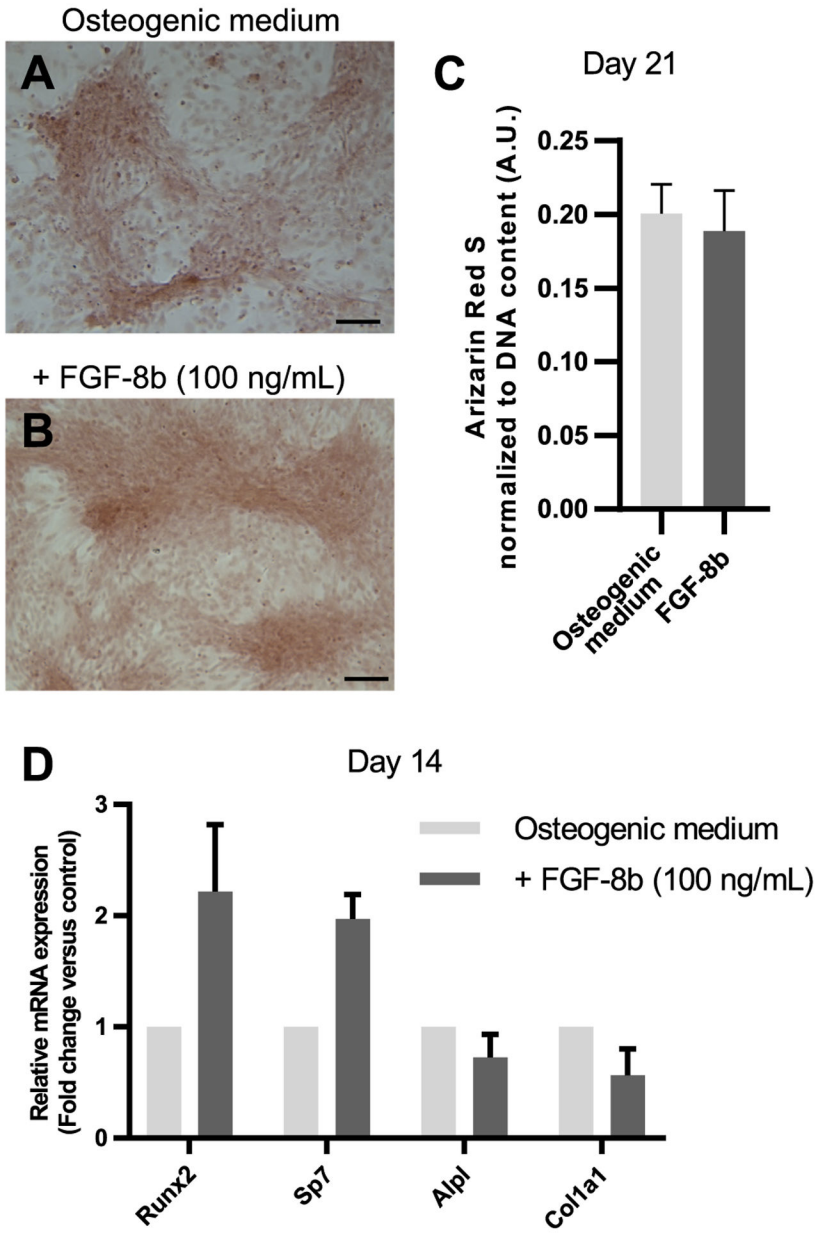

Fig. 3.

FGF-8b does not affect osteogenesis in rat ADSCs. (A, B) Representative images of the stained calcium deposit after 21 days of culture. Treatment conditions were (A) osteogenic medium and (B) osteogenic medium supplemented with 100 ng/mL FGF-8b. Scale bar = 100 μm. (C) Quantification of the stained calcium deposit were performed using the eluted Alizarin red S stain via measuring absorbance at 450 nm. The readings were normalized to background values of non-induced control ADSCs. (D) mRNA expression of osteogenic markers. Data are expressed as fold change relative to both the housekeeping gene and osteogenic medium condition.

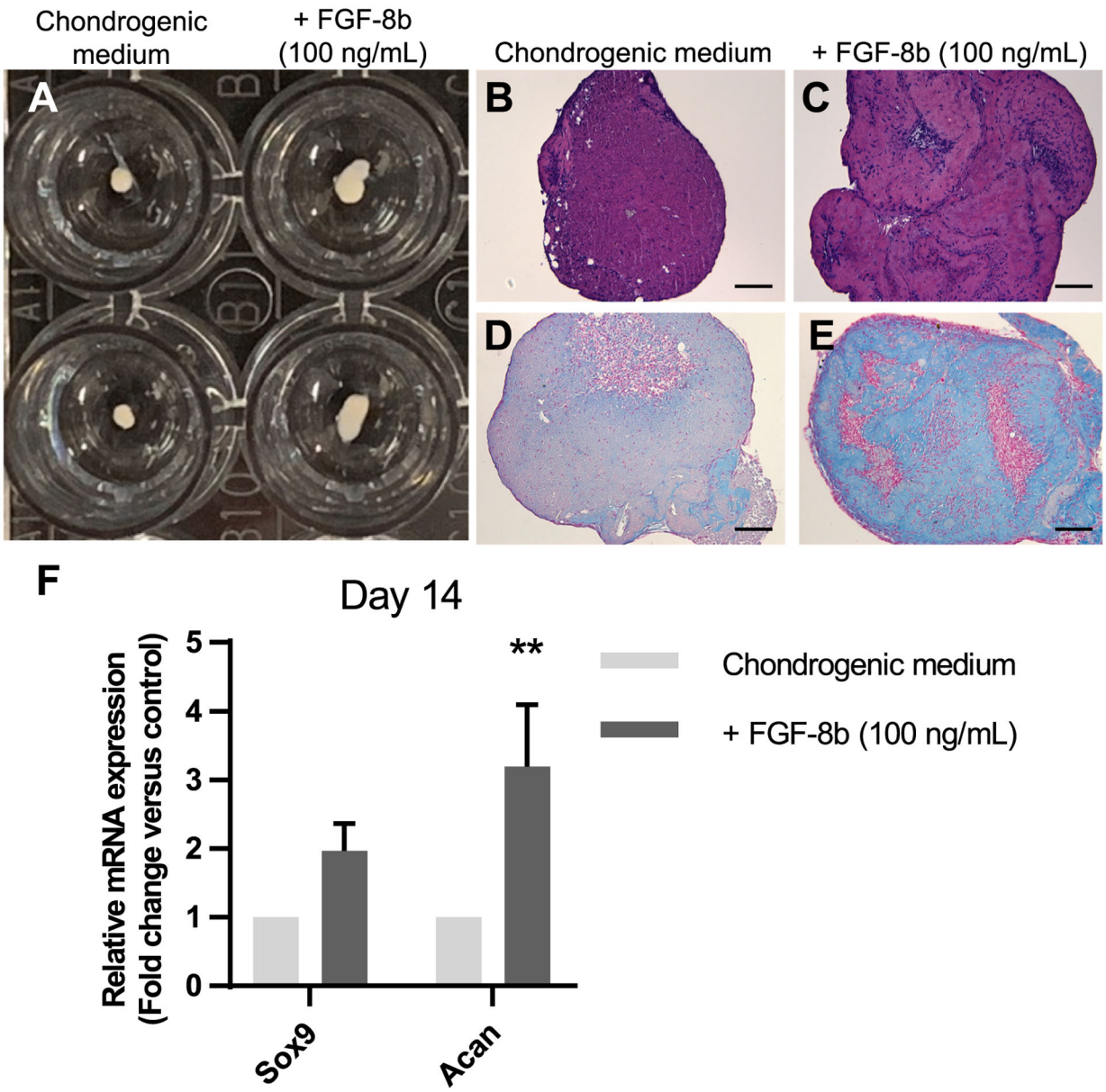

Fig. 4.

FGF-8b enhances chondrogenesis in rat ADSCs. (A) Macroscopic aspect of micromasses after 21 days of culture in chondrogenic medium with and without FGF-8b supplementation. (B-D) Histological staining of micromass section after 21 days of culture; (B, C) hematoxylin/eosin staining; (D, E) alcian blue/nuclear fast red staining. Scale bar = 100 μm. (F) mRNA expression of chondrogenic markers. Data are expressed as fold change relative to both the housekeeping gene and chondrogenic medium condition. Statistical analyses: unpaired Student's t-test (F). ** $P < .01$.

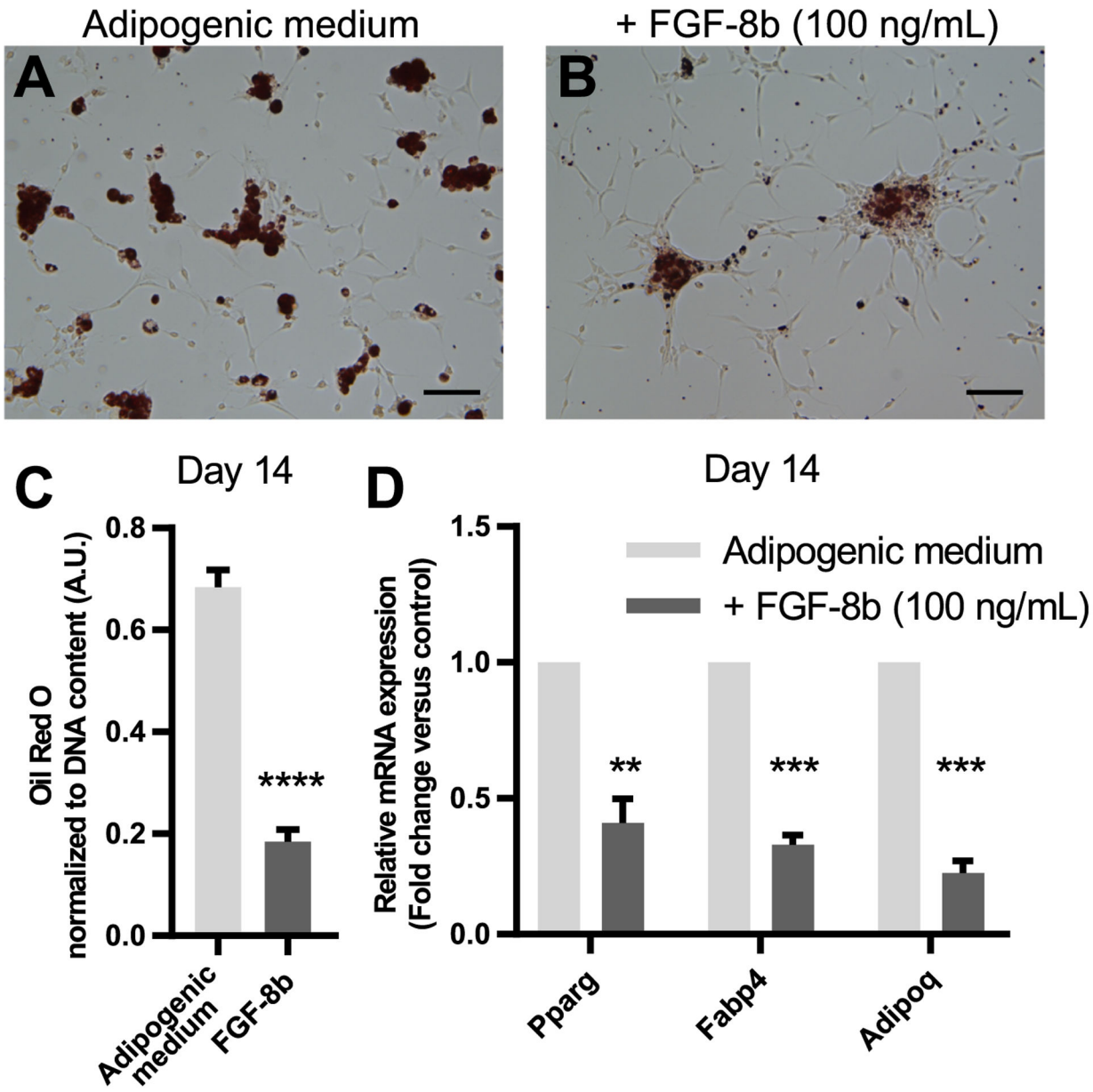

Fig. 5.

FGF-8b suppresses adipogenesis in rat ADSCs. (A, B) Representative images of the stained lipid droplets after 14 days of culture. Treatment conditions were (A) adipogenic medium and (B) adipogenic medium supplemented with 100 ng/mL FGF-8b. Scale bar = 100 μm. (C) Quantification of the stained lipid droplets were performed using the eluted Oil red O stain via measuring absorbance at 510 nm. The readings were normalized to background values of non-induced control ADSCs. (D) mRNA expression of adipogenic markers. Data are expressed as fold change relative to both the housekeeping gene and adipogenic control condition. Statistical analyses: unpaired Student's t-test (C, D). ** $P < .01$, *** $P < .005$, and **** $P < .0001$.

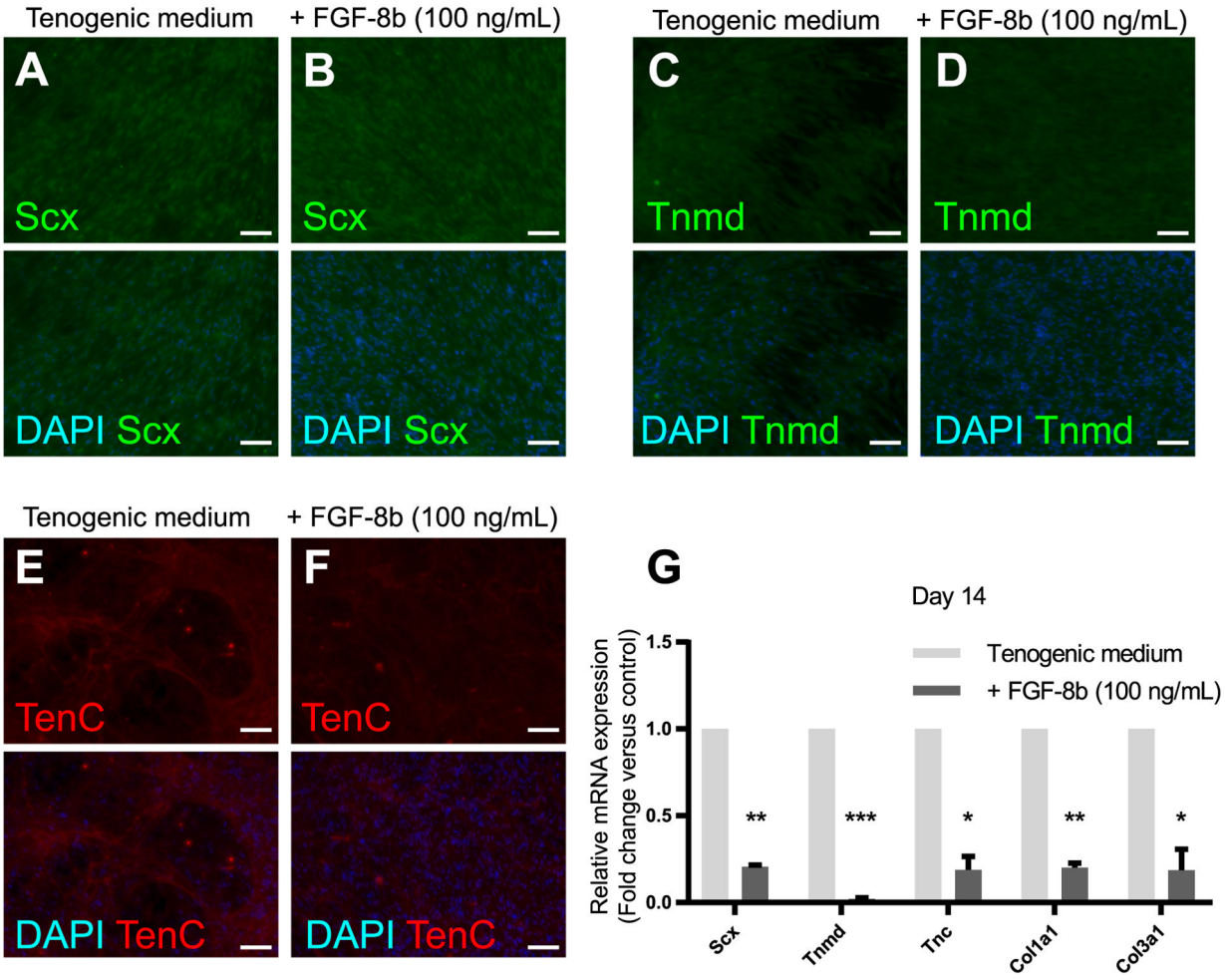

Fig. 6.

FGF-8b suppresses tenogenesis in rat ADSCs. (A-F) Representative fluorescent images stained with Scx, Tnmd, or TenC after 14 days of culture. Treatment conditions were (A, C, E) tenogenic medium and (B, D, F) tenogenic medium supplemented with 100 ng/mL FGF-8b. Scale bar: 100 μm. (G) mRNA expression of tenogenic markers. Data are expressed as fold change relative to both the housekeeping gene and tenogenic medium condition. Statistical analyses: unpaired Student's t-test (G). * P < .05, ** P < .01, and *** P < .001.

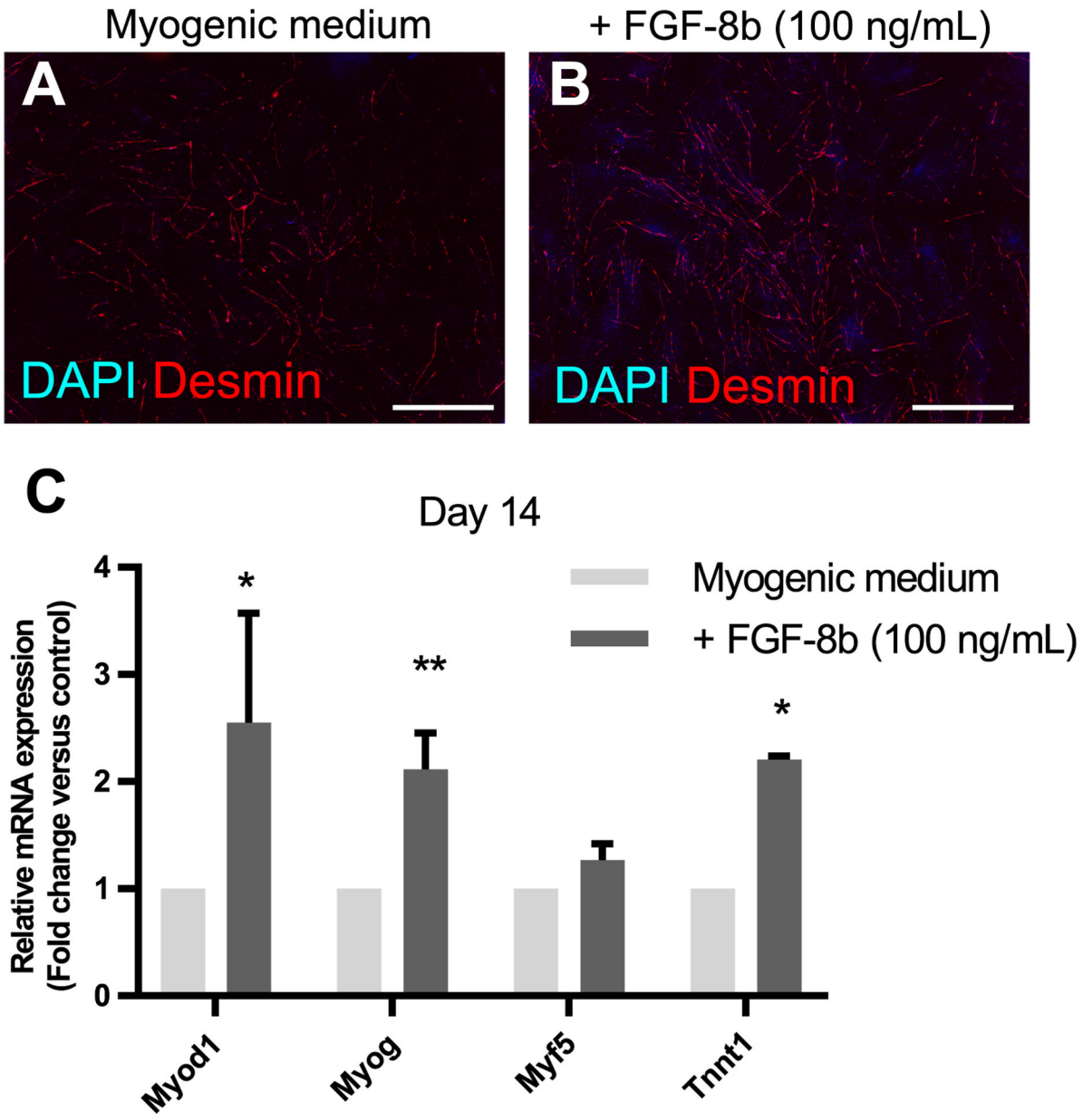

Fig. 7.

FGF-8b enhances myogenesis in rat MPCs. (A, B) Representative fluorescent images stained with Desmin after 14 days of culture. Treatment conditions were (A) myogenic medium and (B) myogenic medium supplemented with 100 ng/mL FGF-8b. Scale bar = 2 mm. (C) mRNA expression of myogenic markers. Data are expressed as fold change relative to both the housekeeping gene and myogenic control condition. Statistical analyses: unpaired Student's t-test (C). * $P < .05$ and ** $P < .01$.