Transplantation of Fetal Instead of Adult Fibroblasts Reduces the Probability of Ectopic Ossification During Tendon Repair

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Although cell transplantation therapy can effectively promote functional tendon repair, occasional ectopic ossification during tendon regeneration undermines its efficacy. The effect of transplanted cell types on ectopic ossification has not yet been systematically evaluated. This study compared the rate of ectopic ossification during tendon repair upon transplantation with mouse fetal fibroblasts (FFs) and their adult counterparts (adult fibroblasts [AFs]). Alkaline phosphatase (ALP) staining, immunofluorescence, and gene expression analysis were used to compare the spontaneous osteogenic differentiation of FFs and AFs *in vitro*. X-ray, histology, and gene expression analysis were used to investigate the ectopic ossification in a mouse Achilles tendon repair model *in vivo*. ALP staining and immunofluorescence data *in vitro* showed that FFs had less spontaneous osteogenic differentiation capacity, and lower expression of *runx-related transcription factor 2 (runx2)*. For the *in vivo* study, the FFs transplant group displayed reduced ectopic ossification (2/7 vs. 7/7, Mann–Whitney test $p < 0.01$) at 14 weeks post-transplantation and enhanced tendon repair (general histological score at week 6, 7.53 vs. 10.56, $p < 0.05$). More chondrocytes formed at 6 weeks, and all mice developed bone marrow at 14 weeks post-transplantation in the AFs transplant group. Gene expression analysis of the regenerated tissue showed significantly higher expression levels of transforming growth factor beta1 (TGF- β 1) and transforming growth factor beta3 (TGF- β 3) in the AFs group during the early stages of tendon repair. Our study demonstrates that transplantation of fetal instead of AFs is more promising for tendon repair, underscoring the importance of the origin of seed cells for tendon repair.

Introduction

TENDONS ARE UNIQUE dense connective tissues that are responsible for mechanical transmission of muscle force to bone.¹ Upon injury, tendons possess poor self-regeneration capacity. A variety of therapeutic strategies such as xenografts,^{2,3} allografts,^{4–6} autografts,^{7,8} suture^{9,10} and fixation^{11,12} have been utilized for the repair of tendon injuries, with cell transplantation showing the greatest potential for tendon regeneration.^{13,14} Currently, commonly used seed cells in tendon tissue engineering include mesenchymal stem cells (MSCs), embryonic stem cells, tenocytes and fibroblasts. However, lack of optimal tenolineage differentiation induction or ethical problems limit the use of different types of stem cells. Tenocytes have to be directly isolated from tendon tissues, which may cause tendon defect and donor site morbidity. It is not practical to utilize these cells for tendon repair.

Since both tenocytes and skin fibroblasts are derived from mesoderm and have similar characteristics in cell morphology and extracellular matrix components, fibroblasts showed great potential to form neo-tendon tissue under specific condition.^{15,16} Skin fibroblasts are an easily accessible cell source from patients and do not cause donor site defect. Neo-tendon tissues engineered from human skin fibroblasts were reportedly similar to human tenocytes *in vitro*.¹⁵ Additionally, fibroblasts-engineered tendon are similar to tenocyte-engineered tendon in terms of histology and tensile strength in a porcine model, which suggest fibroblasts may have the potential as seed cells for tendon regeneration.¹⁶ Fibroblasts derived from foreskin have previously been utilized in Apligraf—the first tissue-engineered skin that is now widely utilized in nonhealing wound.¹⁷ However, compared to adult cells, fetal cells are essential for soft tissue regeneration, both in tendon and skin.^{18–20}

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Fetal fibroblasts (FFs) showed enhanced growth and collagen production in two- and three-dimensional culture in comparison with adult fibroblasts (AFs).¹⁸ FFs also retain hyperactive migratory and contractile phenotype, which is crucial for avoiding fibrosis or scarring.²¹ Moreover, spontaneous ectopic ossification of transplanted cells impedes repair of tendon injuries through cell transplantation. Hence, preventing the osteogenic differentiation of transplanted cells is crucial for successful tendon repair through cell therapy. Further studies on the mechanism of ectopic bone formation during cell transplantation therapy are therefore required.

During tendon repair, ectopic bone may form.²² There are two types of ectopic bone formation in tendon wounds: (i) endogenous cells and growth factors, including bone-related growth factors such as *transforming growth factor beta* (TGF- β) and *bone morphogenetic protein* (BMP), *hypoxia-inducible factor* (HIF), and *vascular endothelial growth factor* (VEGF) cause ectopic bone formation at the repair site.²³ (ii) For stem cell transplantation and tissue engineering therapy, exogenous materials or seed cells can induce ectopic bone formation.²² The presence of seed cells is thought to be the main causative factor that induces ectopic bone formation.^{22,24,25} However, there have so far been very few studies that have focused on the effect of seed cell origin on ectopic bone formation, which is a risk for tendon repair.^{22,26} In our preliminary work, we found that there is a difference of ossification ratio between FFs and AFs transplantation, which indicate the cell source influence ectopic bone formation.

Hence, we compared the induction of ectopic ossification in a mouse Achilles tendon injury model transplanted with fetal and adult skin fibroblasts. We hypothesize that transplantation of FFs can reduce ectopic bone formation with better tendon reparation, compared with AFs. This study can be subdivided into two phases: (i) isolation and identification of AFs and FFs *in vitro*; (ii) comparison of ectopic ossification and regeneration efficacy upon transplanting scaffold-free engineered tendons comprised of AFs and FFs, within a mouse Achilles tendon injury model.

Materials and Methods

Cell isolation and culture

AFs were isolated from the dorsal dermis of female ICR mice (2 months). The excised skin pieces were digested with 2 mL of 0.2% collagenase solution (Gibco, Grand Island, NY), followed by culture in 2 mL of Dulbecco's modified Eagle's medium (DMEM) (high glucose; Gibco, www.invitrogen.com) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Inc., Carlsbad, CA, www.invitrogen.com-Gibco) and 1% penicillin-streptomycin (Gibco). After 24 h following digestion, fresh medium was replaced every 3 days. FFs were isolated from mouse embryos explanted from pregnant ICR mice between 10 and 12 days of gestation (E10–E12). The epidermis and dermis were separated by floating the skin on 0.5% (w/v) dispase in phosphate-buffered saline (PBS) at 4°C overnight. Fibroblasts were isolated by mincing the dermis, and then they were grown in DMEM (high-glucose) containing 10% (v/v) FBS. Fresh culture medium was replaced every 3 days.

For the *in vitro* study, AFs and FFs were utilized for cell differentiation, proliferation, migration, and gene expression analysis at different time points. For the *in vivo* study, AFs and

FFs were cultured in 10 cm dishes until they reached 90% confluency, where upon 50 μ g/mL of ascorbic acid was supplemented to the culture medium for 2 weeks to encourage formation of cell sheet as engineered tendon.²⁷ The cell sheets that formed could be detached from the substratum by applying a small roll-up force to form scaffold-free tissue-engineered tendon that was utilized for subsequent *in vivo* experiments. Each cell sheet formed in one 10 cm dish can be divided into six parts, each part can be applied into one leg of mouse.

Osteogenic differentiation

The osteogenic differentiation capacity of AFs and FFs were investigated *in vitro* as described previously.²⁷ Spontaneous osteogenesis was confirmed by alkaline phosphatase (ALP) staining²⁸ after 3 days in DMEM (high-glucose) condition. The rate of osteogenesis was considered to be the ratio of the number of ALP-positive cells to the total cell number determined by 4,6-diamidino-2-phenylindole (DAPI) staining (Beyotime Institute of Biotechnology, Inc., Jiangsu, China).

Proliferation and migration capacity

Cell proliferation was measured with CCK-8. AFs and FFs cultured in DMEM (high-glucose) at desired time points (1, 3, 5, 7, and 10 days), was incubated in CCK-8 solution in a 5% CO₂ incubator at 37°C for 3 h. The intense orange-colored formazan derivative formed by cell metabolism is soluble in the culture medium. The absorbance was measured at 450 nm. Cell number was correlated to optical density (OD). We detected cell proliferation of implanted cells by KI67 staining.

For cell migration study, cells were grown in DMEM (high-glucose) containing 10% FBS to form confluent monolayers in six-well plates and were serum-starved overnight. An artificial wound was made in the cell monolayer with a 100- μ L micropipette tip. Then, the culture medium was removed and the cells washed twice with serum-free medium. At desired time points (0, 8, 24, and 48 h), wound closure was photographed to show migration capacity of AFs and FFs. Further, we used Image-pro plus software to quantify the migratory activity of AFs and FFs.

Immunofluorescence

Immunofluorescence was utilized to determine the expression of *runx-related transcription factor 2* (*runx2*) in AFs and FFs after culture in DMEM (high-glucose) with 50 μ g/mL of ascorbic acid for 3 days *in vitro*. Mouse anti-mouse monoclonal antibody against *runx2* (1:100 dilution; Abcam, Inc., Cambridge, MA) was used to detect the expression of transcriptional factor-*runx2*. The primary antibody was omitted in the control experiment. Donkey anti-mouse IgG was used to detect the primary antibody (1:15,000 dilution; LI-COR, Inc., Lincoln, NE). We observed and imaged the results under confocal laser scanning microscope (Zeiss LSM 510 Meta). Further, we used Image-pro plus software to quantify the density of immunofluorescence data corresponding to the mean level of expression in nuclei.

Animal model

Sixteen female ICR mice weighing 25–30 g were utilized. The Achilles tendon was exposed through a lateral incision under general anesthesia. In the left leg, part of the Achilles

tendon was removed to create a defect about 2 mm in length. Scaffold-free AFs-engineered tendons were joined to the remaining Achilles tendon using a nonresorbable suture (6-0 nylon; AFs-treated group, $n = 16$). In the right leg, the FFs-engineered tendons were implanted (FFs-treated group, $n = 16$). The wound was then irrigated and skin was closed with suture. Post-operatively, mice were allowed free cage activity at constant temperature with a 12-h dark–light cycle, together with unrestricted access to a standard diet and water.

At 2, 4, and 6 weeks post-transplantation, three mice were sacrificed for histological evaluation and gene expression analysis at each time point. To quantify and compare ectopic bone formation between the AFs and FFs groups, the remaining seven mice were exposed under X-ray using a noninvasive Kodak *in vivo* FX small animal imaging system every week. The seven mice were eventually sacrificed for further histological evaluation and gene expression analysis at 14 weeks post-transplantation. All animals were from Zhejiang University Laboratory Animal Center and treated according to the standard guidelines approved by the Zhejiang University Ethics Committee (ZJU2011101005).

Cell labeling and detection

The ADFs and FDFs utilized in the mouse Achilles tendon repair model were prestained with 1,1'-diiododecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI; Sigma-Aldrich, Inc., St. Louis, MO). Briefly, the fibroblasts were incubated with 5 μ L/mL DiI at 37°C for 20 min, and then washed with PBS. The DiI-dyed cells were observed under fluorescence microscopy (IX71; Olympus, Tokyo, Japan) at 543 nm. The culture medium was replaced by serum-free medium on the day before the tissue-engineered tendon was to be implanted into animals.

Histological assessment

Specimens were fixed, dehydrated, and embedded within paraffin blocks. Histological sections (8 μ m) were prepared

using a microtome, and they were subsequently deparaffinized with xylene, hydrated with decreasing concentrations of ethanol and then stained with hematoxylin and eosin (H&E). To detect proteoglycan synthesis as an indicator of cartilage production, sections were stained with 0.1% Safranin O (Sangon, Shanghai, China) for 8 min, followed by counter staining with 0.02% Fast Green, FCF (Cellchipbj, Beijing, China) for 4 min.

To quantify the degree of ectopic bone formation, we scored animal sample according to cell formation, bone structure, and bone tissue as describe in Table 1.^{29,30}

To quantify the degree of chondrogenesis based on Safranin O staining, we scored the Safranin O staining sample according to several parameters, including uniformity and darkness, distance between cells, and cell morphology.³¹ Further, we used Image-pro plus software to quantify the density of Safranin O staining.

General histological scoring was performed using a blinded semi-quantitative scoring system based on six parameters (fiber structure, fiber arrangement, rounding of the nuclei, inflammation, vascularity, cell population) of H&E staining.³²

X-ray imaging and quantization using Image-pro plus software

After surgery, seven mice were exposed under X-ray (1 min duration) using a noninvasive Kodak *in vivo* FX small animal imaging system every week to evaluate ectopic bone formation. At 14 weeks post-transplantation, we calculated the ossification ratio of each group (number of ectopic bone formed mice/seven).

Captured images were analyzed with the Image-pro plus software to quantify the area, mean density, max density, and sum density of ectopic bone formation.

RNA isolation and real-time polymerase chain reaction

Total cellular RNA was isolated by lysis in TRIzol (Invitrogen) followed by a one-step phenol chloroform-isoamyl

TABLE 1. BONE HISTOLOGICAL SCORING SYSTEM

Cell level	Bone structure	Tissue level
Osteoblasts	Bone trabecules	Bone tissue
0. Absent	0. Absent	0. Absent
1. Present at the periphery	1. Present at the periphery	1. Present at the periphery
2. Centrally present	2. Centrally present	2. Centrally present
3. Present at the periphery and centrally	3. Present at the periphery and centrally	3. Present at the periphery and centrally
Osteocytes	Neoformation vessels	Percentage area of bone tissue
0. Absent	0. Absent	0. Absent
1. Present at the periphery	1. Present at the periphery	1. Up to 25%
2. Centrally present	2. Centrally present	2. Between 25% and 50%
3. Present at the periphery and centrally	3. Present at the periphery and centrally	3. Between 50% and 75%
		4. Between 75% and 100%
Osteoclasts		
0. Absent		
1. Present at the periphery		
2. Centrally present		
3. Present at the periphery and centrally		

alcohol extraction procedure, following the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) analysis of seven genes including transforming growth factor beta1 (TGF- β 1), transforming growth factor beta2 (TGF- β 2), transforming growth factor beta3 (TGF- β 3), BMP4, acidic sulfated integral membrane glycoproteins (*CD44*), interleukin-6 (*IL-6*), osteocalcin (*OCN*), osteopontin (*OPN*), *msh-like 2* (*MSX2*), *alpha-smooth muscle actin* (α -*SMA*), *myogenin* (*Myog*), *runx2*, and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) were quantitatively analyzed by utilizing Brilliant SYBR Green QPCR Master Mix (TakaRa, DaLian, China) with a Light Cycler apparatus (ABI 7900HT), as described previously.²⁸ The primer sequences were designed using primer 5.0 software and are listed in Table 2. Each RT-PCR was performed on at least three different experimental samples, and representative results are displayed as target gene expression normalized to *GAPDH*.²⁸ Error bars represent standard deviation from the mean of technical replicates as previously described.³³

Statistical analysis

All quantitative data sets are expressed as mean $\pm$ SD. Student's *t*-test was performed to assess statistical significance of differences in results between datasets. We analyzed X-ray data with the Mann-Whitney test (short for "M-W test") in the SPSS system. Values of $p < 0.05$ were considered to be significantly different.

Results

FFs displayed less spontaneous osteogenic differentiation capacity in vitro compared with AFs

AFs and FFs were isolated from adult and fetal dermis, and both cell types displayed spindle-shaped morphology (Fig. 1A, B). Generally, AFs (Fig. 1C insert) displayed significantly higher ALP activity compared with FFs (Fig. 1D insert) cultured in normal medium without induction. The percentage of ALP positively stained cells in the AFs group is also significantly higher compared with the FFs group (Fig. 1C–G, $23.72\% \pm 5.71\%$ vs. $4.12\% \pm 1.11\%$, $p < 0.01$). Additionally, the immunofluorescence data showed significantly higher density of *runx2* in the nuclei of AFs compared with FFs, when cultured in DMEM (high-glucose with ascorbic acid) for 3 days (Fig. 1H–N). Higher gene expression of *runx2* (Fig. 1O) coincided with immunofluorescence data. These results thus indicated less spontaneous osteogenic differentiation capacity of FFs compared with AFs.

RT-PCR was employed to evaluate the gene expression levels of osteogenic genes and myogenic genes by FFs and AFs on day 0, 3, 7, and 14 in scaffold-free tendon induction medium. The results showed that AFs expressed significantly higher levels in the early stage of α -*SMA* (Fig. 2A, day 0, 1.23-fold, $p < 0.05$), *Myog* (Fig. 2A, day 0, 3.65-fold, $p < 0.01$), *OCN* (Fig. 2B, day 0, 16.6-fold, $p < 0.01$), *MSX2* (Fig. 2B, day 0, 1.53-fold, $p < 0.05$), and BMP4 (Fig. 2B)

TABLE 2. LIST OF PRIMER SEQUENCES UTILIZED FOR REAL-TIME POLYMERASE CHAIN REACTION

Genes	5'-3'	Primers	Production size (bp)
TGF- β 1	Forward	CACCATCCATGACATGAACCG	377
	Reverse	GCTTGCGACCCACGTAGTAGA	
TGF- β 2	Forward	ATCTCCTGCTAATGTTGTTGCC	288
	Reverse	CGGAAGCTTCGGGATTTATGGT	
TGF- β 3	Forward	ATACCTCCGACGCGCAGACA	185
	Reverse	ACTTACACGACTTCACCACCAT	
BMP4	Forward	GGAGGAGGAGGAGGAAGAGCA	162
	Reverse	CTGAGGTTGAAGAGGAAACGAAA	
<i>CD44</i>	Forward	TCATCCCAACGCTATCTGTGC	216
	Reverse	CTATACTCGCCCTTCTTGCTGT	
<i>IL-6</i>	Forward	TAACAAGAAAGACAAAGCCAGAGT	117
	Reverse	GCATTGGAAATTGGGGTAGGAAG	
<i>OCN</i>	Forward	GACCATCTTTCTGCTCACTCTG	124
	Reverse	TACCTTATTGCCCTCCTGCTTG	
<i>OPN</i>	Forward	CGGCACTCCAACCTGCCCAAGA	192
	Reverse	TGCCATTCCCGCCATCCACC	
<i>MSX2</i>	Forward	TTCACCACATCCCAGCTTCTA	159
	Reverse	TTGCAGTCTTTTCGCCTTAGC	
α - <i>SMA</i>	Forward	GGACGTACAACCTGGTATTGTGC	179
	Reverse	TCGGCAGTAGTCACGAAGGA	
<i>Myog</i>	Forward	CGATCTCCGCTACAGAGGC	115
	Reverse	GTTGGGACCGAAGCTCCAGT	
<i>Runx2</i>	Forward	CCAACTTCCTGTGCTCCGTG	238
	Reverse	GTGAAACTCTTGCTCGTCCG	
<i>GAPDH</i>	Forward	GCAAATTCAACGGCACAG	141
	Reverse	CACCAGTAGACTCCACGAC	

TGF- β 1, transforming growth factor beta1; TGF- β 2, transforming growth factor beta2; TGF- β 3, transforming growth factor beta3; BMP4, bone morphogenetic protein 4; *CD44*, acidic sulfated integral membrane glycoproteins; *IL-6*, interleukin-6; *OCN*, osteocalcin; *OPN*, osteopontin; *MSX2*, *msh-like 2*; α -*SMA*, *alpha-smooth muscle actin*; *Myog*, *myogenin*; *runx2*, *runt-related transcription factor 2*; *GAPDH*, *glyceraldehyde 3-phosphate dehydrogenase*.

FIG. 1. Isolation, culture and osteogenic differentiation potential of AFs and FFs. (A, B) Morphology of AFs (A) and FFs (B). (C–G) ALP staining of AFs and FFs cultured in DMEM (high-glucose) for 14 days showed spontaneous osteogenic differentiation ($n=3$). Gross view and microscopic view of ALP staining showed ALP-positive cells of AFs (C, E) compared to FFs (D, F). Quantification of ALP-positive cells showed that FFs had significantly lower percentage of spontaneously osteogenic differentiation cells (G, 4.1% vs. 27.3% $p<0.01$, $n=3$). (H–O) Immunofluorescence and RT-PCR results of *runx2* *in vitro* of AFs and FFs cultured in medium with ascorbic acid ($n=3$). Immunofluorescence of *runx2* showed higher expression in AFs group (H, J, L) compared with FFs group (I, K, M). (N) Density of *runx2* was significantly higher in AFs group. (O) Gene expression of *runx2* showed higher expression in AFs group ($n=3$). Scale bar (A–F): 200 μ m, scale bar (H–M): 100 μ m. ALP, alkaline phosphatase; AFs, adult fibroblasts; FFs, fetal fibroblasts; RT-PCR, real-time polymerase chain reaction; *runx2*, runt-related transcription factor 2; DMEM, Dulbecco's modified Eagle's medium. Color images available online at www.liebertpub.com/tea

and *OPN* (Fig. 2B). In the later stage, AFs expressed significantly lower levels of α -SMA (Fig. 2A, day 3, 1.48-fold, $p<0.05$; day 7, 1.70-fold, $p<0.01$; day 14, 3.67-fold, $p<0.01$), *Myog* (Fig. 2A, day 3, 4.35-fold, $p<0.01$; day 14, 1.57-fold, $p<0.05$), and higher levels of *MSX2* (Fig. 2B, day 3, 2.96-fold, $p<0.05$; day 7, 4.80-fold, $p<0.05$; day 14, 8.12-fold, $p<0.01$).

Proliferation and migration capacity of FFs compared to AFs *in vitro*

Proliferation capacity were determined on day 1, 3, 5, 7, and 10 cultured in normal medium, and the result showed higher proliferation capacity of FFs (Supplementary Fig. S2A; Supplementary Data are available online at www.liebertpub.com/tea). Wound closure was photographed on 0, 8, 24, and 48 h to show migration capacity; the migration distance showed similar migration capacity between FFs and AFs (Supplementary Fig. S3A–B).

Lower expression levels of inflammatory factors and TGF- β family of cytokines in FFs compared with AFs *in vitro*

RT-PCR was employed to evaluate the gene expression levels of inflammatory factors and the TGF- β family of cytokines by FFs and AFs on day 0, 3, 7, and 14 in scaffold-free tendon induction medium. The results showed that AFs expressed significantly higher levels of TGF- β 1 (Fig. 2D, day 3, 9.44-fold, $p<0.05$), TGF- β 3 (Fig. 2D, day 0, 2.16-fold, $p<0.05$; day 3, 3.50-fold, $p<0.01$), and relatively higher expression of TGF- β 2 (Fig. 2D), compared with FFs. AFs also expressed significantly higher levels of inflammatory factors, such as *CD44* (Fig. 2C, day 0, 4.68-fold, $p<0.05$; day 3, 17.41-fold, $p<0.05$) and *IL-6* (Fig. 2C, day 3, 3.80-fold, $p<0.01$) compared with FFs. Lower expression levels of the TGF- β family of cytokines and inflammatory factors by FFs may result in less spontaneous osteogenic differentiation capacity of FFs, compared with AFs.

FIG. 2. RT-PCR results of AFs and FFs *in vitro* cultured in medium with ascorbic acid ($n=3$). RT-PCR analysis of α -SMA, *Myog* (A), *OCN*, *MSX2*, *BMP4*, *OPN* (B), *CD44* and *IL-6* (C), *TGF- β 1*, *TGF- β 2*, and *TGF- β 3* (D) were conducted. The results of the target gene expression were normalized to the housekeeping gene, *GAPDH*. *Significant differences between two groups (day 0 for α -SMA, *MSX2*, *CD44*, *TGF- β 1*, and *TGF- β 3*; day 3 for α -SMA, *MSX2*, and *CD44*; day 7 for *MSX2*; and day 14 for *Myog*) ($p<0.05$). **Highly significant differences between two groups (day 0 for *Myog* and *OCN*; day 3 for *Myog*, *IL-6*, and *TGF- β 3*; day 7 for α -SMA; and day 14 for α -SMA and *MSX2*) ($p<0.01$). Bar (A): 100 μ m. α -SMA, *alpha-smooth muscle actin*; *Myog*, *myogenin*; *OCN*, *osteocalcin*; *MSX2*, *msh-like 2*; *BMP4*, *bone morphogenetic protein 4*; *OPN*, *osteopontin*; *CD44*, *acidic sulfated integral membrane glycoproteins*; *IL-6*, *interleukin-6*; *TGF- β 1*, *transforming growth factor beta1*; *TGF- β 2*, *transforming growth factor beta2*; *TGF- β 3*, *transforming growth factor beta3*; *GAPDH*, *glyceraldehyde 3-phosphate dehydrogenase*. Color images available online at www.liebertpub.com/tea

FFs reduced ectopic ossification with better Achilles tendon regeneration *in vivo*

X-ray imaging was used to determine the ossification level in each group. The results showed that the AFs transplant group displayed significantly higher levels of spontaneous ossification (Fig. 3A first row, arrows showed the ectopic bone formed during regeneration) compared with the FFs group (Fig. 3A second row, arrows showed the ectopic bone formed during regeneration) (7/7 vs. 2/7, M–W test $p<0.01$). We also calculated the area and density of

ectopic bone formed, and found that the AFs group displayed significantly larger area (Fig. 3B, M–W test: $p<0.05$), higher mean density, and max density (Fig. 3B, M–W test, $p<0.05$) of ectopic bone compared with the FFs group. The results thus indicated that utilizing FFs as seed cells had significantly lower potential to induce ectopic bone formation compared with AFs.

Bone histological scoring showed significantly lower score in the FFs group at 14 weeks post-transplantation (Fig. 4F, 1.00 vs. 8.25, $p<0.05$), both groups scored 0 at 6 weeks (not shown). Safranin O stain scoring showed significantly

FIG. 3. X-ray images of repaired tendons of mice 14 weeks post-transplantation and evaluation of ossification extent ($n=7$). **(A)** X-ray images showed ossification ratio of 7/7 in AFs group (first row), 2/7 in FFs group (second row) (arrow showed the ectopic bone inside repaired tendon). **(B)** The distribution and medians of area, mean density, max density, and sum density of ectopic bone in AFs group (rhombus) and FFs group (square). The differences between two groups were determined by Mann-Whitney test. The lines indicate the medians.*Significant differences between two groups [area, density (mean), density (max), $p<0.05$]. **Highly significant differences between two groups (Ossification ratio, $p<0.01$). Color images available online at www.liebertpub.com/tea

lower score of the FFs group at 6 weeks post-transplantation (Fig. 4C, 4.56 vs. 7.06, $p<0.05$), with less chondrocytes being present in the repaired tendon of the FFs group. The density of Safranin O staining showed significantly lower density of FFs group (Fig. 4D, 0.77 vs. 1.00, $p<0.05$), which correspond to scoring results. General histological scoring showed significantly lower score of the FFs group at 6 weeks post-transplantation (Fig. 4E, 7.53 vs. 10.56, $p<0.05$), with better histological structure of the repaired tendon being observed in the FFs group.

Both H&E staining and Safranin O staining showed that there were consistently more chondrocyte-like (Fig. 4A, yellow arrow) and Safranin O-stained cells (Fig. 4B, yellow arrow) at 6 weeks post-transplantation in the AFs group compared with the FFs group. At 14 weeks post-transplantation, all mice in the AFs group developed ectopic bone, whereas only two out of seven mice in the FFs group had ectopic bone formation (Fig. 3A). Bone marrow was found in the AFs group (Fig. 4A), while only a few chondrocytes were present in the FFs group (Fig. 4A).

These results thus suggested that utilizing FFs as seed cells could reduce ectopic bone formation leading to enhanced tendon regeneration.

Few transplanted cells contributed to ossification in vivo

Six weeks after transplantation, we can track the DiI-dyed transplanted cells by using noninvasive Kodak *in vivo* FX small animal imaging system (Supplementary Fig. S1) and confocal image. A few implanted cells were KI67 positive; while the majority of implanted cells were KI67 negative; these results indicated that a few of implanted cells are proliferative (Supplementary Fig. S2B). Under confocal laser scanning microscope, we observed that the majority of implanted cells contribute in neo-tendon (Fig. 4G, second row). We also found that a few of implanted cells differentiated into osteo/chondrocytic-like cells in ossification region (Fig. 4G, first row, arrows showed DiI-positive osteo/chondrocytic-like cells). These were found in some samples

FIG. 4. Morphology and production of cartilage matrix of cells inside repaired tendon of AFs and FFs group at 6 weeks ($n=3$) and 14 weeks ($n=3$) post-transplantation. (A, B) Typical H&E staining, Safranin O (SO) staining of regenerated tendon at 6 and 14 weeks post-transplantation. (C, D) Safranin O staining score and density of regenerated tendon at 6 and 14 weeks post-transplantation were shown. (E) Histological score of regenerated tendon at 6 and 14 weeks post-transplantation. (F) Bone histological score of regenerated tendon at 14 weeks post-transplantation. (G) DiI-dyed cells (arrow) were seen in both repaired tissue and ossification region. Scale bar (A, B): 200 μ m of first and third columns, 50 μ m of second and fourth columns; scale bar (G): 100 μ m. H&E, hematoxylin and eosin; DiI, 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate. Color images available online at www.liebertpub.com/tea

(three of four specimens in AFs group and two of four specimens in FFs group). These results indicated that the majority of transplanted cells contribute to tendon repair and cause ossification by paracrine, while few of them may differentiate into osteo/chondrocytes and contribute ossification.

FFs had lower expression levels of inflammatory factors and TGF- β family of cytokines in vivo

RT-PCR was utilized to analyze the expression of inflammatory factors and the TGF- β family of cytokines within the repaired tendon at 2, 4, 6, and 14 weeks post-transplantation. The results showed that AFs expressed significantly higher levels of TGF- β 1 (Fig. 5A, week 2, 6.38-fold, $p < 0.05$; week 4, 30.90-fold, $p < 0.05$), TGF- β 2 (Fig. 5A, week 4, 10.78-fold, $p < 0.05$), and TGF- β 3 (Fig. 5A, week 2, 3.23-fold, $p < 0.05$; week 4, 10.81-fold, $p < 0.05$) compared with FFs. AFs also displayed significantly higher expression levels of inflammatory factors, such as *CD44* (Fig. 5B, week 2, 1.63-fold, $p < 0.05$) and IL-6 (Fig. 5B, week 2, 6.14-fold) compared with FFs.

Discussion

To our knowledge, this is the first study to investigate the age of donor cells on spontaneous ectopic ossification in tendon repair and its underlying mechanism. It was found that (i) there was significantly lower probability of ectopic bone formation in FFs treated tendon, and less chondrocytes and bone marrow formation compared with AFs-treated tendon; (ii) FFs had less osteogenic differentiation potential both *in vitro* and *in vivo*; (iii) FFs had significantly lower expression levels of TGF- β 1, TGF- β 2, and TGF- β 3 both

in vitro and *in vivo*, and significantly lower levels of inflammatory factors *in vitro*, such as *CD44* and IL-6.

Osteogenic potential of AFs and FFs may be due to presence of dermal stem cell subpopulation. Dermal stem cells derived from mammalian skin demonstrated osteogenic potential *in vitro* with expression of *OCN* and apparent formation of osteogenic nodules.³⁴ An *in vivo* animal study showed type I collagen expression 4 weeks after dermal stem cells were injected into the backs of immunodeficient NOD-SCID mice,³⁴ which indicated that dermal stem cells possessed the potential for bone formation *in vivo*. In another study, stem cells isolated from adult dermis were utilized as seed cells for cartilage tissue engineering.³⁵ These studies suggested that there exists a cellular subpopulation within mammalian dermis that can be considered to be *bona fide* stem cells, which has the potential to differentiate into mesenchymal lineages. In our study, we also found that the heterogeneous cells derived from skin may contain subpopulations of stem cells, which may differentiate into tenocyte and osteogenic lineages. Moreover, lower expression of osteogenic genes of FFs cell sheet may be the reason of lower ossification during tendon repair. While higher expression of α -SMA and *Myog* of FFs cell sheet indicated higher capacity of FFs to differentiate into myofibroblasts, which may promote the regeneration of connective tissues³⁶ including tendon. These endogenous differences between AFs and FFs may contribute to tendon regeneration potential and ectopic ossification risk.

Osteogenic differentiation *in vitro* and ossification *in vivo* are closely associated with the expression of the TGF- β family and *BMP* family of cytokines. Growth factors play an important role in mediating cell fate decisions. TGF- β could induce differentiation of fibroblasts during tissue repair.^{37,38} However, as TGF- β is crucial during fibrosis,^{39,40} scarring,⁴¹

FIG. 5. FFs expressed lower TGF- β and inflammatory factors *in vivo* 2, 4, 6, and 14 weeks after transplantation ($n = 3$). (A, B) RT-PCR analysis of TGF- β 1, TGF- β 2, TGF- β 3, *CD44*, and IL-6 were shown. The results of the target gene expression were normalized to the housekeeping gene, *GAPDH*. The results showed that AFs expressed significantly higher levels of TGF- β 1 (A, week 2, 6.38-fold, $p < 0.05$; week 4, 30.90-fold, $p < 0.05$), TGF- β 2 (A, week 4, 10.78-fold, $p < 0.05$), TGF- β 3 (A, week 2, 3.23-fold, $p < 0.05$; week 4, 10.81-fold, $p < 0.05$), *CD44* (B, week 2, 1.63-fold, $p < 0.05$), and IL-6 (B, week 2, 6.14-fold) compared to FFs. *Significant differences between two groups ($p < 0.05$). *CD44*, acidic sulfated integral membrane glycoproteins; *IL-6*, interleukin-6. Color images available online at www.liebertpub.com/tea

and inducing chondrogenesis of fibroblasts,⁴² which also decrease tendon repair, the proper expression level of *TGF-β* may be essential for tendon regeneration. *TGF-β* signaling is specifically required for cell proliferation in Meckel's cartilage and the mandibular anlagen, and for the formation of the coronoid condyle and angular processes.⁴³ After chondrogenic differentiation, some cells will undergo terminal differentiation into osteoblasts, in the absence of inhibitors, such as basic fibroblast growth factors or parathyroid hormone-like peptide.⁴⁴ A previous study showed that *TGF-β3* could enhance chondrogenesis,⁴⁵ and calcification occurred upon subcutaneous implantation into nude mice for 8 weeks.⁴⁶ Chondrogenic differentiation of human mesenchymal stem cells (hMSCs) was demonstrated to occur in the presence of *TGF-β3* with expression of Collagen type I, Collagen type II, and Collagen type X.⁴⁴ Further, *TGF-β3* could also directly induce hypertrophy and osteogenesis of MSCs.⁴⁷ *TGF-β1* also had similar potential to induce chondrogenic differentiation and hypertrophy of hMSCs.^{47,48} *TGF-β2* is known to be necessary for mandibular development because without *TGF-β2*, severe defects in mandibular development occurred.⁴³ *BMP* family also showed great osteoinductive potential.^{49–51} These results thus suggested that the presence of *TGF-β1*, *TGF-β2*, *TGF-β3*, or *BMP* might cause spontaneous calcification.

In this study, we cultured AFs and FFs in high cell density with multilayer and in high glucose DMEM with 10% FBS and 50 μg/mL of ascorbic acid as described in previous work.^{27,52} We analyzed the mRNA during the formation of scaffold-free engineered tendon and found that mRNA expression of *TGF-β1*, *TGF-β2*, *TGF-β3*, *BMP4*, and some inflammatory cytokines were significantly increased up to day 14 in AFs compared with FFs. The mechanisms responsible for the upregulation of these genes may be due to intrinsic trait of AFs in response to environment. The *in vitro* study showed that transcriptional factor—*runx2*, an essential transcription factor that governs osteoblastic differentiation,⁵³ was significantly more highly expressed in the protein level in the AFs group compared with the FFs group. As reported, *runx2* respond to *TGF-β* and *BMP* family.^{54,55} Hence, it is possible that the obvious chondrocytes morphology observed at 6 weeks post-transplantation and formation of bone marrow-like cavity at 14 weeks post-transplantation in the AFs group may be due to high expression levels of *TGF-β* and *BMP* during the early stages of tendon repair.

FFs are superior to AFs for tendon repair due to lower osteogenic potential and greater capacity for tissue regeneration. Our study demonstrated lower osteogenic potential *in vitro* and significantly lesser ossification (2/7 in the FFs group compared to 7/7 in the AFs group) upon *in vivo* transplantation. Further, we noticed that the expression levels of inflammatory factors in FFs cultured *in vitro* was significantly lower compared with AFs. During fetal tendon regeneration, lower expression levels of *CD44* indicated superior material properties and reduced cross-sectional area, resulting in better healing parameters compared with adult tendon injury.⁵⁶ During scarless human fetal wound repair, diminished IL-6 expression play an important role in regeneration.⁵⁷ Other studies also demonstrated the great potential of fetal dermal cells to regenerate extracellular matrix similar to original tissue.^{58,59} In our study, we found

that FFs expressed lower levels of IL-6, which made them preferred seed cells in tendon tissue engineering. Collectively, our results indicated that fetal cells were more suitable than adult cells for tendon repair, due to lower risk of ossification and greater potential for scarless wound healing.

The limitation of this study is that the detailed signaling pathway by which ossification is mediated by the *TGF-β* superfamily of cytokines was not determined. As previously reported, the induction of the signaling pathway by *TGF-β* leads to phosphorylation of *smad2* and *smad3*.⁶⁰ The Mad homology 1 and Mad homology 2 (MH1 and MH2) domains of *smad3* are known to interact with *runx* transcription factors,⁶⁰ which are key targets of the *TGF-β* superfamily.⁵⁵ *Runx2* is an essential transcription factor that governs osteoblastic differentiation.⁵³ Hence, we hypothesize that *TGF-β* signaling was transduced by *smad2/3* to activate *runx2* to initiate ossification. The mechanism of ectopic bone formation by cell-treated tendon will be explored in our future studies.

In conclusion, our study suggested that fetal-derived cells are more appropriate seed cells for tendon tissue engineering due to lower risk of ossification and greater potential for scarless regeneration. This may be due to decreased expression of inflammatory factors by fetal cells compared with adult cells. Significantly higher expression of *TGF-β1* and *TGF-β3* by AFs suggested a relationship between ectopic bone formation and the *TGF-β* family of cytokines in cell transplantation therapy for tendon repair.

Acknowledgments

This work was supported by NSFC grants (81330041, 81125014, 31271041, 81071461, 81201396, 31000440, J1103603, and 1073032), The National Key Scientific Program (2012CB966604), the National High Technology Research and Development Program of China (863 Program) (No. 2012AA020503), Regenerative Medicine in Innovative Medical Subjects of Zhejiang Province and Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents, Medical and Health Science and Technology Plan of the Department of Health of Zhejiang Province (2013RCA010). The authors thank Ping Lu for assistance in article preparation and Linzhao Xiao Nan for the confocal imaging.

Disclosure Statement

No competing financial interests exist.

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Received: May 19, 2013

Accepted: January 2, 2014

Online Publication Date: May 14, 2014