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Tendon Progenitor Cells in Injured Tendons Have Strong Chondrogenic Potential: The CD105-Negative Subpopulation Induces Chondrogenic Degeneration

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Abstract

To study the cellular mechanism of the tendon repair process, we used a mouse Achilles tendon injury model to focus on the cells recruited to the injured site. The cells isolated from injured tendon 1 week after the surgery and uninjured tendons contained the connective tissue progenitor populations as determined by colony-forming capacity, cell surface markers and multipotency. When the injured tendon-derived progenitor cells (inTPCs) were transplanted into injured Achilles tendons, they were not only integrated in the regenerating area expressing tenogenic phenotype but also trans-differentiated into chondrogenic cells in the degenerative lesion that underwent ectopic endochondral ossification. Surprisingly, the micromass culture of the inTPCs rapidly underwent chondrogenic differentiation even in the absence of exogenous BMPs or TGFβs. The cells isolated from human ruptured tendon tissues also showed connective tissue progenitor properties and exhibited stronger chondrogenic ability than bone marrow stromal cells. The mouse inTPCs contained two subpopulations one positive and one negative for CD105, a co-receptor of the TGFβ superfamily. The CD105-negative cells showed superior chondrogenic potential *in vitro*

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and induced larger chondroid degenerative lesions in mice as compared to the CD105-positive cells. These findings indicate that tendon progenitor cells are recruited to the injured site of tendons and have a strong chondrogenic potential and that the CD105-negative population of these cells would be the cause for chondroid degeneration in injured tendons. The newly identified cells recruited to the injured tendon may provide novel targets to develop therapeutic strategies to facilitate tendon repair.

Keywords

Tendon; progenitor; injury; chondrogenesis; TGF β

INTRODUCTION

Tendons have limited repair capacity. Damaged tendons do not usually regain their original levels of biological and biomechanical characteristics and can undergo chronic structural and functional degeneration [1–4]. The injured site is initially filled with blood and inflammatory cells and becomes occupied over time by the intrinsic and extrinsic cells that likely migrate from the paratenon, endotenon, sheaths and/or surrounding tissues such as the synovium [1, 5, 6]. These various types of cells proliferate and can synthesize collagen and other extracellular matrix proteins and organize dense collagen fibers, thus resulting in some restoration of damaged tendon structure [1, 5, 6]. However, they often fail to reconstruct normal tendon tissue organization and the neo-tendons undergo degenerative changes that cause decreases in normal mechanical strength, flexibility and elasticity. Hence, it is necessary to understand the exact nature of the cells responsible for the complex repair processes and to determine which cellular and molecular mechanisms impede the complete recovery of tendon structure and function.

Recent studies have reported that tendon stem/progenitor cells (TSPCs) reside and can be isolated from embryo and adult tendons from human and vertebrate animals including mice, rats and rabbits [7–12]. The TSPCs not only express tendon cell markers, but also increase expression of tendon cell traits in response to platelet-rich plasma or an instructive microenvironment [7, 12]. Although involvement of TSPCs in tendon repair and tendinopathy is suggested [11, 13], it is still unclear how TSPC populations respond to tendon injury and whether TSPCs contribute to tendon repair as reparative cells or result in structural and functional degeneration. Connective tissue progenitor cells expand and/or migrate in the injured sites or pathological lesions of the connective tissues such as muscle, bones and cartilages [14–19]. These progenitor cells can support or hamper tissue regeneration depending on their origins [20–22]. In this study, we hypothesize that connective tissue progenitors also reside in or are recruited into injured tendons and that these progenitor cells may participate in repair processes, but also cause degenerative changes. The results from experiments using the mouse Achilles injury model indicate that two populations of tendon progenitor cells appeared and were expanded in injured tendons, and had distinct potentials to participate in tendon healing and degeneration.

MATERIALS AND METHODS

Mice

All mouse studies were conducted with approval by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia. The CD-1 mice and CD-1 Nude mice (Crl:CD-1Foxn1^{nu}) were purchased from Charles River Laboratories International, Inc. (Wilmington, MA). The RFP transgenic mice that ubiquitously express red fluorescence protein (RFP) (B6.Cg-Tg(CAG-DsRed*MST)1Nagy/J) were purchased from the Jackson Laboratory (Bar Harbor, ME). The GFP transgenic mice that ubiquitously express enhanced green fluorescence protein (EGFP) (H2K-eGFP) have been established previously [23]. Scleraxis-GFP reporter (Scx-GFP) mice were kindly provided by Dr. R. Schweitzer (Oregon Health & Science University) [24]. The Scleraxis-GFP reporter mouse were mated with the RFP-mouse to generate the compound transgenic mouse (Scx-GFP;RFP).

Tendon surgery and cell transplantation

A complete transverse incision, without attempt at repair, was made at the midpoint of the right Achilles tendon in 6 week-old male CD-1, CD-1 Nude, H2K-eGFP, Scx-GFP reporter or Scx-GFP;RFP mice (Supplement Figure 1A). Animals were euthanized 1, 2, 4, 8 and 12 weeks post-operatively and tendon tissues were harvested to isolate progenitor cells or perform histological, immunohistochemical or gene expression analysis. The CD-1 Nude mice (n=5/group) received transplantation of injured tendon-derived progenitor cells (inTPCs) (200,000/mouse) in the Achilles tendon gap just after the incision. The injured Achilles tendons were harvested 10 or 12 weeks after the cell transplantation. The inTPCs isolated from RFP-expressing mice were subcutaneously transplanted in Matrigel (BD Bioscience, Inc., Franklin Lakes, NJ) containing recombinant human bone morphogenetic protein 2 (Gene Script Corp., Piscataway, NJ) (1.5×10^6 cells/200 μ l/transplant) as previously reported [25] and harvested 10 days after transplantation.

Isolation and culture of the mouse injured tendon-derived progenitor cells (inTPCs)

Tendon progenitor cells were isolated from injured and uninjured tendons following the method previously reported with some modification [8, 10, 11]. Briefly, the fibrous tissues that had formed in the incised Achilles tendons (Supplement figure 1B, squared) were dissected 1 week post surgery and incubated with 2.5 unit/ml Dispase (MP Biomedicals, LLC., Solon OH) and 600U/ml (3mg/ml) type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ) in HBSS for 1h at 37°C with gentle shaking. The dissociated cells were plated on the culture dish at a density of 70–140 cells/cm² in 60-mm or 100-mm dishes and cultured in DMEM containing 20% FBS (Gemini Bio-Products, West Sacramento, CA). The cells were also isolated from the uninjured Achilles tendon of the 7-weeks old CD-1 mice in a similar manner. The cultures were passaged twice and used for the experiments.

To perform a colony-forming unit fibroblast (CFU-f) assay, the freshly isolated cells were plated at 2,000 cells/60-mm dish, grown for 10 days, and then stained with crystal violet (n=4). The number of colonies, clusters more than 2 mm in diameter that contain more than

50 cells, was counted, and the total number of colonies per tendon was calculated based on the total number of dissociated cells per tendon.

The inTPCs and uninjured tendon-derived cells were plated on the collagen 1 substrate (Cellmatrix, Nitta Gelatin Inc., Osaka, Japan) at a density of 100,000/well in the 24-well plate (n=4/group) and cultured in DMEM containing 10% FBS and either mouse StemXVivo Adipogenic or Osteogenic Supplement (R&D Systems, Inc., Minneapolis, MN). The cultures were stained with Oil red O or Alizarin red 2 or 3 weeks after the induction, respectively. The cells were also spotted on the collagen 1 substrate at a density of 200,000/20 μ l, cultured in 10%FBS- containing DMEM for 7 days with or without recombinant human bone morphogenetic protein 2 (BMP2, R&D Systems, Inc) at the concentration of 100 ng/ml and then stained with alcian blue at pH 1.0 or toluidine blue. The cultures were treated with BMP type 1 receptor (ALK-2, -3) inhibitor (LDN-193189) or TGF β receptor (ALK-4, -5, -7) inhibitor (SB431542 Stemgent Inc., Cambridge, MA). The integrated density of alcian blue stained cultures was semi-quantified using Image J software.

Isolation and culture of mouse bone marrow stromal cells (BMSCs)

Bone marrow cells were isolated from C57BL/6 mice and cultured at a density of 1×10^6 cells/cm² in 10% FBS-containing DMEM. Non-adherent cells were removed by washing with PBS 3 days after the initial plating. Bone marrow stromal cells (BMSCs) were obtained by expanding the adherent cells [26]. To reduce contamination of hematopoietic cells, cultures were passaged repeatedly and the absence of hematopoietic cells confirmed by flow cytometry using CD45 and hematopoietic lineage markers such as CD3, B220, CD11b, Gr-1 and Ter119 [26].

Isolation and culture of human tendon cells and bone marrow stromal cells (BMSCs)

The human tendon tissues we used in this study had been made for clinical diagnosis, but were no longer needed and completely de-identified before they were provided. As a result, the study was determined not to be human subject research by CHOP's IRB committee. Tendon tissues (5–10 mm cubic) were collected from the damaged edge prior to suturing the ruptured tendon during the reparative surgery: One was from the posterior tibial tendon of a 55-year old female (F55) and the other was from the Achilles tendon of a 56-year old male (M56). The tissues were digested by incubating with 2.5 unit/ml Dispase and 600U/ml in HBSS for 1h at 37°C with gentle shaking. The dissociated cells were plated on the culture dish at a density of 70–140 cells/cm² in 60-mm or 100-mm dishes and cultured in DMEM containing 20% FBS.

Human bone marrow stromal cells from two healthy donors (a 42-year old male (M42) and a 54-year old female (F54)) were obtained from the Stem Cell and Xenograft Core Facility at the Perelman School of Medicine of the University of Pennsylvania and isolated as previously reported [27]. The bone marrow was diluted with an equal volume of saline and layered over LSM (MP Biomedicals, LLC, Solon, OH). The mononuclear cells were collected after centrifugation at 400g for 30 min at room temperature. After washing with saline, the mononuclear cells were plated in DMEM supplemented with 10% FBS at a

density of 1.65×10^5 cells/cm². The non-adherent cells were removed by changing the medium at day 3. Human BMSCs were isolated from the expanded adherent cells. The human tendon cells were plated on the collagen 1 substrate (Cellmatrix, Nitta Gelatin Inc., Osaka, Japan) at a density of 100,000/well in the 24-well plate (n=4/group) and cultured in DMEM containing 10% FBS and either human StemXVivo Adipogenic or Osteogenic Supplement (R&D Systems, Inc., Minneapolis, MN). The cultures were stained with Oil red O or Alizarin red 2 or 3 weeks after the induction, respectively. The tendon cells or human BMSCs were also spotted on the collagen 1 substrate at a density of 200,000/20 μ l, cultured in 10%FBS- containing DMEM for 7 days with or without recombinant mouse TGF β 1 (R&D Systems, Inc) at the concentration of 10 ng/ml and then stained with alcian blue at pH 1.0.

Statistical methods

Results were analyzed using InStat 3 version 3.1a (GraphPad Software, Inc., La Jolla, CA). One-way Analysis of Variance (ANOVA) with a Tukey-Kramer Multiple Comparison Test was used to identify the differences. The threshold for significance for all tests was set as $p < 0.05$.

RESULTS

Histology of injured tendons in a mouse Achilles tendon injury model

One week after the mouse Achilles tendon injury was created, many fibroblastic cells appeared in the injured site (Fig. 1C, D). The center of the injured site had reduced cell density and was restructured by aligned cells over time (Fig. 1F, J, R). In contrast, round or polygonal cells were found in the proximal and distal edges of the injury site 2 weeks after injury (Fig. 1E) and became more apparent at 4 weeks (Fig. 1G). The lesions containing these round cells (Fig. 1I) displayed alcian-blue and toluidine blue positive proteoglycan matrix (Fig. 1K and 1M) and collagen 2 accumulation (Fig. 1O) by 8 weeks, and organized mineralized and bony tissues (Fig. 1Q and 1T, arrows) by 12 weeks at 100% reproducibility. Thus this tendon injury model represents tendon regeneration in the center and chondroid degeneration/ossification at the edges of injured tendons in a site-specific manner. This observation is consistent with other reports in rats [28, 29].

Progenitor-like cells in the injured tendons

To investigate whether the cells appearing in the injured tendon contained connective tissue progenitor cell population, we dissected the injured tendons 1 week after the surgery, and dissociated and plated the cells following the method for stem/progenitor cell isolation from tendons as previously reported [8, 10, 11]. In a similar manner we also isolated the connective tissue progenitor cell population from the uninjured tendons as a control. The number of cells isolated from the injured tendons was much higher than from the uninjured tendon (Fig. 2A), and both cultures gradually formed colonies. The injured tendons contain an approximately 60-fold higher number of colony forming cells as compared to the control, uninjured tendon (Fig. 2B). The FACS analysis for cell surface markers revealed that the injured tendon-derived cells as well as the control tendon-derived cells are positive for

Sca-1, CD29, CD44 and CD49e and negative for CD45, CD11b and Gr-1 (Fig. 2C and Supplement Fig. 2).

Both cultures showed an ability to differentiate into osteogenic (Fig. 2D, H), adipogenic (Fig. 2E, I) and chondrogenic (Fig. 2F, J) cells under each appropriate inductive condition *in vitro*. To test the ability to differentiate into tenogenic cells, we isolated injured and uninjured tendon-derived cells from the transgenic mice (Scx-GFP) that harbor the GFP reporter gene under the control of scleraxis promoter [24]. Both injured and uninjured tendon-derived cell cultures contained a small number of GFP-positive cells in monolayer culture. When the cultures were treated with TGF β 1 for 5 days, a majority of cells expressed GFP in both cultures (Fig. 2G,K), indicating that most of the cells in these cultures expressed a tenogenic phenotype in response to TGF β 1. We prepared total RNA from the P2 passage monolayer cultures of injured and uninjured tendon-derived cells and from bone marrow stromal cell (BMSC) cultures and analyzed their gene expression by the qPCR assay. We found that both tendon-derived cell cultures expressed scleraxis at much higher levels compared to BMSCs (Fig. 2L, Scleraxis) and that the aggrecan expression was undetectable among the three types of cell cultures (Fig. 2L, Aggrecan). We prepared injured tendon-derived cells from the transgenic mice that ubiquitously express red fluorescence protein (RFP) and subcutaneously transplanted them with BMP2 into athymic mice. We observed formation of cartilaginous tissue (Fig. 2M) that contained RFP-positive cells (Fig. 2O) and was positive for collagen 2 (Fig. 2P) and collagen 10 (Fig. 2Q). We also observed bony-like tissues inside the transplant (Fig. 2N, arrows).

Contribution of injured tendon-derived progenitor cells (inTPCs) to tendon repair

We next asked whether the population of the injured tendon-derived progenitor cells (inTPCs) could contribute to tendon repair. To do so, we isolated inTPCs from the injured Achilles tendons of the Scx-GFP;RFP mice that express GFP under the control of scleraxis promoter and ubiquitously express red fluorescence protein (RFP), and transplanted them in the Achilles tendon gap in the Nude CD-1 mice immediately following the incision. The injured tendons were harvested 4 weeks after the cell transplantation. RFP-positive cells were found in the entire site of the injured tendon (Fig. 3A) while the Scx-GFP-positive cells were predominantly detected in the center of the injured tendon (Fig. 3B). The difference in distribution between RFP- and GFP-positive cells was clear when the two fluorescence images were merged (Fig. 3C). The Achilles tendon that had not received cell transplantation did not show any significant fluorescence (Fig. 3D,E). We also transplanted the inTPCs isolated from the H2K-eGFP mice that ubiquitously express EGFP (enhanced green fluorescence protein) or the Scx-GFP mice into the injured Achilles tendons. The injured tendons were harvested 12 weeks after the cell transplantation. Macroscopic views of EGFP- positive (Fig. 3F, G) or Scx-GFP- positive (Fig. 3J, K) cells were similar to the results obtained using the Scx-GFP;RFP cells (Fig. 3A–C). The immunohistochemical analysis for the GFP-positive cells revealed that the EGFP-inTPCs were distributed in both the center and edges of the injured tendons (Fig. 3H, I). Scx-GFP-expressing cells were detected in the center region (Fig. 3L, arrows) where the elongated cells were aligned, but marginally in the chondroid lesion at the edges (Fig. 3M). The injured Achilles tendons without GFP-cell transplantation showed marginal staining (Fig. 3N and 3O). These findings

indicate that the inTPCs were integrated into both the regenerating region and chondroid lesion in injured tendons and that inTPCs can express both tenogenic and chondrogenic phenotype.

Strong chondrogenic potential of inTPCs

Interestingly, when the inTPCs were grown in a high-density mass culture in the absence of pro-chondrogenic factors such as BMPs and TGF β s, the cells accumulated a large amount of cartilage-specific proteoglycan (Fig. 4C, Injured). In contrast, the uninjured tendon-derived cells showed much less chondrogenic differentiation (Fig. 4B, Uninjured), and the BMSCs did not show such a phenotype without BMP2 treatment (Fig. 4A, BMSC). The integrated density measurement of the staining (Fig. 4D) and analysis of aggrecan gene expression (Fig. 4E) confirmed this observation. Chondrogenic differentiation of the inTPCs was partially inhibited by treatment with the inhibitor of BMP receptor kinase and strongly inhibited by the inhibitor of TGF β receptor kinase (Fig. 4F), indicating that TGF β /BMP signaling is required for chondrogenic differentiation of the inTPCs.

We next tested whether similar progenitor cells appeared in human injured tendons. The tendons were obtained from human patients and digested in the same way as the mouse tendons for cell isolation. The cells isolated from both samples showed the ability to make colonies, maintain active proliferation over 6 passages and differentiate into osteoblastic, adipogenic and chondrogenic cells (Supplement figure 3). Consistent with the results of the mouse inTPCs, the human injured tendon-derived cells showed much stronger chondrogenic potential without TGF β 1 in mass culture (Fig. 4H, I, Tendon) compared to the human bone marrow stromal cells (Fig. 4G, I, BMSC).

CD105-positive and -negative inTPCs

As described above, the flow cytometric analysis revealed that inTPCs showed very similar profiles of cell surface markers as compared to those of uninjured tendon-derived cells (Supplement figure 2). Interestingly, we found that the inTPCs contained two populations one CD105-positive and the other CD105-negative (Fig. 5A). The uninjured tendon-derived cells also contained a small peak of the CD105-negative subpopulation, but its percentage was lower (Supplement figure 4). We examined the nature of these two subpopulations. Both were able to undergo osteogenic (Fig. 5B, E) and adipogenic (Fig. 5C, F) differentiation under appropriate culture conditions. However, the CD105-negative cells showed superior chondrogenic potential compared to the CD105-positive cells at high-density mass culture (Fig. 5D, G) as confirmed by integrated density of alcian blue staining (Fig. 5H) and gene expression of aggrecan (Fig. 5I). Both cells also expressed scleraxis in response to TGF β 1 and the CD105-positive cells expressed scleraxis at a significantly higher level than the CD105-negative cells (Fig. 5J).

To test the contribution of each population to tendon regeneration or chondroid degeneration, the sorted CD105-positive or negative inTPCs isolated from the Scx-GFP;RFP mice were transplanted into injured Achilles tendons in Nude CD-1 mice. The RFP-expressing cells were broadly found in the injured site (Fig. 6A, D) while the Scx-GFP-expressing cells were limited to the center part (Fig. 6B, E) in both groups. The tendons

receiving CD105-positive and negative cells showed similar intensity of RFP (Fig. 6G), but the tendons receiving CD105-positive cells had stronger fluorescence of GFP compared to those receiving the CD105-negative cells (Fig. 6H)

When CD105-positive or negative iTPCs isolated from the H2K-eGFP transgenic mice were transplanted into injured Achilles tendon the injured tendons that received the CD105-negative cells contained larger chondroid degenerative lesions as compared to the injured tendons receiving the CD105-positive cells (Fig. 6I, M, Q). Image analysis of immunohistochemical staining for EGFP revealed that both CD105-positive and negative cells were integrated into the center region of the injured tendons to a similar extent (Fig. 6S), but more CD105-negative cells were found in chondroid degenerative lesions (Fig. 6R). Thus, it is indicated that the CD105-negative cells have a stronger chondrogenic potential and may participate in chondroid degeneration in injured tendons at a higher efficiency compared to the CD105-positive cells.

Lastly, we examined the mechanism by which CD105-negative cells have superior chondrogenic ability. CD105 has been reported to be a co-receptor of TGF β s and to regulate function of vascular endothelial cells by modifying smad-mediated TGF β signaling [30, 31]. Because we found that chondrogenesis of iTPCs was dependent on TGF β receptor and BMP receptor kinases (Fig. 4E), we first investigated how TGF β s and BMPs activate smad signaling pathways. TGF β 1 stimulated phosphorylation of smad 2/3 and smad1/5 within 30 min after the treatment (Supplement Fig. 6A, TGF β 1). In a similar manner, BMP2 treatment increased phosphorylation of smad 2/3 and smad 1/5 (Supplement Fig. 6A, BMP2). These findings suggest that TGF β s and BMPs do not have a strict selectivity to smad2/3 and smad1/5/8 pathways in the iTPCs, which has been observed in other types of cells [32–35]. Because CD105 has been shown to potentiate a smad1/5 pathway in response to TGF β s in endothelial cells [32, 33], we examined whether CD105-positive and -negative cells showed different responsiveness to TGF β 1. In contrast to the endothelial cells, CD105-negative iTPCs showed stronger response of phosphorylation of smad1/5 compared to the response in CD105-positive iTPCs: the content of phospho-smad1/5 in the CD105-negative iTPC culture was higher 30 and 60 min after TGF β 1 treatment and still significantly high 120 min after the treatment (Fig. 7A). Response of smad2 phosphorylation in the CD105-negative group was comparable to that in the CD105-positive group (Fig. 7A).

To further examine the significance of CD105 in chondrogenic differentiation, we used bone marrow stromal cell clones that we have established. We picked 2 clones that presented very similar expression patterns of cell surface markers except that one is CD105 positive while the other is negative (Supplement figure 5). The CD105-negative clone did not show spontaneous chondrogenic ability when cultured at high density, but exhibited much stronger chondrogenic ability in the presence of TGF β 1 than the CD105-positive clone (Supplement figure 6B). Consistent with the results of iTPCs, the CD105-negative clone showed a much higher content of phospho-smad 1/5 in response to TGF β 1 compared to that of the CD105-positive clone while the amount of phospho-smad 2 was similar between both clones (Supplement figure 6C). Lastly, we forcedly expressed CD105 in CD105-negative iTPCs and treated with TGF β as described above. The CD105-expressing group (adeno-CD105) had greatly reduced phosphorylation of Smad1/5 (P-smad1/5) while

phosphorylation of Smad2 was not much affected (Fig. 7B). Furthermore, forced-expression of CD105 in CD105-negative iTPCs strongly reduced aggrecan gene expression in mass culture (Fig. 7C). These findings indicate that CD105 negatively regulates Smad1/5 activity and chondrogenic potential in connective tissue progenitor cells.

DISCUSSION

Tendon progenitor cells present in injured tendons

Our findings indicate that the progenitor cells appear in injured tendons in both mice and human and that these cells have stronger chondrogenic potential than the progenitor cells present in uninjured normal tendons. The data also indicate that the progenitor cells from injured tendons were integrated into neo-tendon tissues as well as into chondrogenic lesions when transplanted into the injured tendon, indicating that these progenitor cells have the potential to contribute to both regeneration and degeneration of tendons. It is likely that a large number of progenitor cells appear after acute tendon injury and the injured tendons might be relatively rich in tendon progenitor cells. However, the data also indicate that the iTPCs can trans-differentiate into chondrogenic cells and induce chondrogenic degenerative lesions. It is suggested that the progenitor cells appearing in the injured tendons are not directed to acquire tendon cell phenotype efficiently and that they require additional step-wise guidance towards full maturation. The origin of iTPCs needs to be clarified. They might originate from normal tendon stem/progenitor cells [11], but change their phenotype during propagation in response to injury and/or reside in the paratenon or epitenon [1]. They could migrate from the surrounding tissue or from the circulation [36].

Cell therapies for tendon repair using mesenchymal stem cells have been widely and actively studied and these studies have represented various degrees of success [37–40]. It is important to clarify whether and how local tendon stem/progenitor cells are different from other connective tissue progenitor cells [41]. Interestingly Tan et al. [42] have reported that tendon-derived stem cells showed higher levels of gene expression for not only the tenogenic phenotype but also other cell lineage phenotypes compared to BMSCs *in vitro*. However, further studies including *in vivo* experiments are required for a definitive conclusion. Results from our study suggest that we could obtain enough tendon progenitor cells from the injured tendons, or from the trimmed tendon tissue in the restoration surgery of ruptured tendons to be used in treatment. Since these cells rapidly proliferate, autologous cell application into the restored tendon could be used to attempt improvement of healing. We would select and use CD105 negative or positive iTPCs in accordance with the demand; CD105-negative cells can be used for reconstruction of fibrocartilage in the enthesis; CD105-positive cells can be used for stimulation of tendon regeneration.

The iTPCs should be considered as target cells to develop drugs to stimulate tendon cell differentiation. Our results demonstrate that the iTPCs have different characteristics from BMSCs, suggesting that the iTPCs could show distinct responses to the drugs and growth factors which have been studied using mesenchymal stem cells isolated from other origins [37, 40] and that we may need to re-evaluate the pharmacological potency of these reagents in this context. Third, further comparison of the CD105-positive iTPCs with other

connective tissue progenitor cells would lead us to develop a method to select specific populations of progenitor cells for tendon repair.

The mechanism of strong chondrogenic potential of the inTPCs

Chondrogenic differentiation of the inTPCs was inhibited by treatment with TGF β or BMP receptor inhibitors, indicating that spontaneous chondrogenic potential is closely related with TGF β /BMP signaling. Indeed, we found strong and long-term increases in gene expression of the TGF β /BMP signaling related molecules in injured tendons (manuscript in preparation). This signaling pathway also should be considered in understanding our findings that the CD105-negative cell population showed superior chondrogenic potential *in vitro* and *in vivo*. CD105, also called Endoglin is a co-receptor of the TGF β family proteins and is involved in ALK1 (activin-like kinase-1) and ALK5 (type I TGF β receptor) signaling [30, 31]. It has been shown that this molecule plays particularly important and essential roles in the vasculature, physiologically and pathologically [30, 31]. Although the regulatory mechanism of TGF β signaling pathway by CD105 has not been fully elucidated, recent studies have indicated that CD105 takes a balance between smad2/3 and smad1/5 pathways and enhances the smad1/5 signaling in endothelial cells [32, 33], myoblastic cell line cells [34] and human immortalized chondrocytes [35]. Furthermore, CD105 physically interacts with the scaffolding protein β -arrestin and inhibits ERK signaling, one of the non-canonical TGF β pathways [43]. Our results indicate that CD105-negative and -positive inTPCs have different modes of smad1/5 and smad2/3 signaling activation in response to TGF β s, which could lead to distinct potential for chondrogenic differentiation although the response to TGF β 1 in the inTPCs is different from that in the previous reports [32, 33, 35].

Interestingly, chondroid degeneration was dominantly induced at the edges of injured tendons, and tenogenic differentiation of transplanted inTPCs was detected in the center of injured tendons as determined by SCX-GFP reporter. When CD105-negative cells were transplanted in injured tendons, they were also distributed to the regenerating region of the center where chondroid degeneration does not occur. This indicates that additional microenvironmental factors are required for induction of site-specific chondrogenesis of CD105-negative inTPCs. It is likely that the cells in the regenerating region are exposed to unidirectional tensile force in a longitudinal direction while the cells at the edges receive both tensile and compressive forces in a multidirectional manner. We assume that differences in type and direction of the mechanical force are critical in regulation of chondroid degeneration of the inTPCs in addition to absence of CD105.

CONCLUSION

Our data indicate that injured tendons contain progenitor cells and likely contribute to tendon regeneration and chondroid degeneration. These progenitor cells can be distinguished as CD105-positive and -negative cell populations and that the CD105-negative cells appear to be the culprits for chondroid degeneration. Furthermore, we suggest that human injured tendons also contain a large number of tendon progenitor cells supporting the findings in the mouse model. Characterization of the newly identified cells recruited to the tendon injury

site may provide novel targets to develop therapeutic strategies to facilitate tendon repair and improve clinical outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Sharma P, Maffulli N. Tendon injury and tendinopathy: healing and repair. *J Bone Joint Surg Am*. 2005; 87(1):187–202. [PubMed: 15634833]
2. Rees JD, Wilson AM, Wolman RL. Current concepts in the management of tendon disorders. *Rheumatology (Oxford)*. 2006; 45(5):508–521. [PubMed: 16490749]
3. Aslan H, Kimelman-Bleich N, Pelled G, et al. Molecular targets for tendon neoformation. *J Clin Invest*. 2008; 118(2):439–444. [PubMed: 18246194]
4. Tozer S, Duprez D. Tendon and ligament: development, repair and disease. *Birth Defects Res C Embryo Today*. 2005; 75(3):226–236. [PubMed: 16187327]
5. Manske PR, Gelberman RH, Vande Berg JS, et al. Intrinsic flexor-tendon repair. A morphological study in vitro. *J Bone Joint Surg Am*. 1984; 66(3):385–396. [PubMed: 6699055]
6. Gelberman RH, Manske PR, Vande Berg JS, et al. Flexor tendon repair in vitro: a comparative histologic study of the rabbit, chicken, dog, and monkey. *J Orthop Res*. 1984; 2(1):39–48. [PubMed: 6491797]
7. Yin Z, Chen X, Chen JL, et al. The regulation of tendon stem cell differentiation by the alignment of nanofibers. *Biomaterials*. 2010; 31(8):2163–2175. [PubMed: 19995669]
8. Zhang J, Wang JH. Characterization of differential properties of rabbit tendon stem cells and tenocytes. *BMC Musculoskelet Disord*. 2010; 11:10. [PubMed: 20082706]
9. Zhang J, Wang JH. BMP-2 mediates PGE(2) -induced reduction of proliferation and osteogenic differentiation of human tendon stem cells. *J Orthop Res*. 2011
10. Rui YF, Lui PP, Li G, et al. Isolation and characterization of multipotent rat tendon-derived stem cells. *Tissue Eng Part A*. 2010; 16(5):1549–1558. [PubMed: 20001227]
11. Bi Y, Ehrichtou D, Kilts TM, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med*. 2007; 13(10):1219–1227. [PubMed: 17828274]
12. Zhang J, Wang JH. Platelet-rich plasma releasate promotes differentiation of tendon stem cells into active tenocytes. *Am J Sports Med*. 2010; 38(12):2477–2486. [PubMed: 20802092]
13. Zhang J, Pan T, Liu Y, et al. Mouse treadmill running enhances tendons by expanding the pool of tendon stem cells (TSCs) and TSC-related cellular production of collagen. *J Orthop Res*. 2010; 28(9):1178–1183. [PubMed: 20225313]
14. Medici D, Shore EM, Lounev VY, et al. Conversion of vascular endothelial cells into multipotent stem-like cells. *Nat Med*. 2010; 16(12):1400–1406. [PubMed: 21102460]

15. Lounev VY, Ramachandran R, Wosczyzna MN, et al. Identification of progenitor cells that contribute to heterotopic skeletogenesis. *J Bone Joint Surg Am.* 2009; 91(3):652–663. [PubMed: 19255227]
16. Lee SY, Miwa M, Sakai Y, et al. Isolation and characterization of connective tissue progenitor cells derived from human fracture-induced hemarthrosis in vitro. *J Orthop Res.* 2008; 26(2):190–199. [PubMed: 17763429]
17. Matsumoto T, Mifune Y, Kawamoto A, et al. Fracture induced mobilization and incorporation of bone marrow-derived endothelial progenitor cells for bone healing. *J Cell Physiol.* 2008; 215(1): 234–242. [PubMed: 18205179]
18. Kurth TB, Dell'accio F, Crouch V, et al. Functional mesenchymal stem cell niches in adult mouse knee joint synovium in vivo. *Arthritis Rheum.* 2011; 63(5):1289–1300. [PubMed: 21538315]
19. Koelling S, Kruegel J, Irmer M, et al. Migratory chondrogenic progenitor cells from repair tissue during the later stages of human osteoarthritis. *Cell Stem Cell.* 2009; 4(4):324–335. [PubMed: 19341622]
20. Liu R, Birke O, Morse A, et al. Myogenic progenitors contribute to open but not closed fracture repair. *BMC Musculoskelet Disord.* 2011; 12:288. [PubMed: 22192089]
21. Park D, Spencer JA, Koh BI, et al. Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. *Cell Stem Cell.* 2012; 10(3):259–272. [PubMed: 22385654]
22. Schindeler A, Liu R, Little DG. The contribution of different cell lineages to bone repair: exploring a role for muscle stem cells. *Differentiation.* 2009; 77(1):12–18. [PubMed: 19281760]
23. Dominici M, Tadjali M, Kepes S, et al. Transgenic mice with pancellular enhanced green fluorescent protein expression in primitive hematopoietic cells and all blood cell progeny. *Genesis.* 2005; 42(1):17–22. [PubMed: 15828004]
24. Schweitzer R, Chyung JH, Murtaugh LC, et al. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development.* 2001; 128(19):3855–3866. [PubMed: 11585810]
25. Shimono K, Morrison TN, Tung WE, et al. Inhibition of ectopic bone formation by a selective retinoic acid receptor alpha-agonist: a new therapy for heterotopic ossification? *J Orthop Res.* 2010; 28(2):271–277. [PubMed: 19725108]
26. Otsuru S, Gordon PL, Shimono K, et al. Transplanted bone marrow mononuclear cells and MSCs impart clinical benefit to children with osteogenesis imperfecta through different mechanisms. *Blood.* 2012; 120(9):1933–1941. [PubMed: 22829629]
27. Otsuru S, Hofmann TJ, Olson TS, et al. Improved isolation and expansion of bone marrow mesenchymal stromal cells using a novel marrow filter device. *Cytotherapy.* 2013; 15(2):146–153. [PubMed: 23321326]
28. Lin L, Shen Q, Xue T, et al. Heterotopic ossification induced by Achilles tenotomy via endochondral bone formation: expression of bone and cartilage related genes. *Bone.* 2010; 46(2): 425–431. [PubMed: 19735753]
29. Rooney P, Grant ME, McClure J. Endochondral ossification and de novo collagen synthesis during repair of the rat Achilles tendon. *Matrix.* 1992; 12(4):274–281. [PubMed: 1435511]
30. Nassiri F, Cusimano MD, Scheithauer BW, et al. Endoglin (CD105): a review of its role in angiogenesis and tumor diagnosis, progression and therapy. *Anticancer Res.* 2011; 31(6):2283–2290. [PubMed: 21737653]
31. Valluru M, Staton CA, Reed MW, et al. Transforming Growth Factor-beta and Endoglin Signaling Orchestrate Wound Healing. *Front Physiol.* 2011; 2:89. [PubMed: 22164144]
32. Lee NY, Ray B, How T, et al. Endoglin promotes transforming growth factor beta-mediated Smad 1/5/8 signaling and inhibits endothelial cell migration through its association with GIPC. *J Biol Chem.* 2008; 283(47):32527–32533. [PubMed: 18775991]
33. Park S, Dimaio TA, Liu W, et al. Endoglin regulates the activation and quiescence of endothelium by participating in canonical and non-canonical TGF-beta signaling pathways. *J Cell Sci.* 2013; 126(Pt 6):1392–1405. [PubMed: 23418351]

34. Scherner O, Meurer SK, Tihaa L, et al. Endoglin differentially modulates antagonistic transforming growth factor-beta1 and BMP-7 signaling. *J Biol Chem.* 2007; 282(19):13934–13943. [PubMed: 17376778]
35. Finnsen KW, Parker WL, Chi Y, et al. Endoglin differentially regulates TGF-beta-induced Smad2/3 and Smad1/5 signalling and its expression correlates with extracellular matrix production and cellular differentiation state in human chondrocytes. *Osteoarthritis Cartilage.* 2010; 18(11): 1518–1527. [PubMed: 20833252]
36. Daher RJ, Chahine NO, Razzano P, et al. Tendon repair augmented with a novel circulating stem cell population. *Int J Clin Exp Med.* 2011; 4(3):214–219. [PubMed: 21977235]
37. Lui PP, Wong OT. Tendon stem cells: experimental and clinical perspectives in tendon and tendon-bone junction repair. *Muscles Ligaments Tendons J.* 2012; 2(3):163–168. [PubMed: 23738293]
38. Sakabe T, Sakai T. Musculoskeletal diseases--tendon. *Br Med Bull.* 2011; 99:211–225. [PubMed: 21729872]
39. Chaudhury S. Mesenchymal stem cell applications to tendon healing. *Muscles Ligaments Tendons J.* 2012; 2(3):222–229. [PubMed: 23738300]
40. Ahmad Z, Wardale J, Brooks R, et al. Exploring the application of stem cells in tendon repair and regeneration. *Arthroscopy.* 2012; 28(7):1018–1029. [PubMed: 22381688]
41. Lui PP, Chan KM. Tendon-derived stem cells (TDSCs): from basic science to potential roles in tendon pathology and tissue engineering applications. *Stem Cell Rev.* 2011; 7(4):883–897. [PubMed: 21611803]
42. Tan Q, Lui PP, Rui YF, et al. Comparison of potentials of stem cells isolated from tendon and bone marrow for musculoskeletal tissue engineering. *Tissue Eng Part A.* 2012; 18(7–8):840–851. [PubMed: 22011320]
43. Lee NY, Blobe GC. The interaction of endoglin with beta-arrestin2 regulates transforming growth factor-beta-mediated ERK activation and migration in endothelial cells. *J Biol Chem.* 2007; 282(29):21507–21517. [PubMed: 17540773]

Figure 1.

Histological changes in the Achilles tendons after injury surgery. Longitudinal sections were prepared from uninjured control Achilles tendons (Control) (A, B, S) or injured Achilles tendons (Injury) 1 (C, D), 2 (E, F), 4 (G, H), 8 (I–P) or 12 (Q–T) weeks after injury, and stained with hematoxylin-eosin (A–J, Q, R) or alcian blue (K, L), toluidine blue (M, N) or anti-collagen 2 antibody (O, P). The images represent the edges (A, C, E, G, I, K, M, O, Q) and the center parts (B, D, F, H, J, L, N, P, R) of the injured tendons. The measurement bars are 200 μm for A–J, Q and R, 50 μm for K–P. S and T, Radiology images of the hind limb.

The radiopaque materials were detected at both edges of the injured Achilles tendon (arrows).

Figure 2.

Clonogenicity, cell surface marker expression and multipotency of uninjured and injured tendon derived cells. The cells were isolated from uninjured control (Uninjured) and injured (Injured) Achilles tendons 1 week after the injury surgery. A, The total cell number was counted. B, The number of colonies was counted 10 days after the cells were plated at a low density. C, The cells expressed Sca-1, but not CD45, a hematopoietic stem cell marker. D–J, The cells were cultured under the osteogenic (D, H), adipogenic (E, I) or chondrogenic (F, J) condition, and stained with Alizarin red (D, H), Oil red O (E, I) and alcian blue (F, J),

respectively. G and K, The cells were isolated from uninjured and injured tendons of Scx-GFP mice and cultured in the presence of TGF β 1 (10 ng/ml) for 5 days. GFP fluorescence images were taken. L and M, Total RNAs were prepared from Achilles tendons (7 weeks old mice), epiphyseal cartilage (4 days old mice) and monolayer cultures of mouse bone marrow stromal cells (BMSC), uninjured (Uninjured) or injured (Injured) tendon derived cells, and subjected to qPCR analysis for *scleraxis* and *aggrecan* (L). The injured tendon-derived cells from RFP-expressing mice were mixed with Matrigel and subcutaneously transplanted into athymic mice, and histologically inspected 10 days after transplantation (M–Q). The frozen sections of the transplant were observed under the fluorescence microscope (O), and the paraffin sections were stained with hematoxylin and eosin (M and N), anti-collagen 2 (P) and collagen 10 (Q) antibodies.

Figure 3.

Transplantation of injured tendon derived cells in injured Achilles tendons. The cells were isolated from injured Achilles tendons in the Scx-GFP;RFP (A–C), H2K–GFP (F–I, N, O) or Scx-GFP (J–M) mice 1 week after the injury surgery and transplanted in the injured Achilles tendons in CD1 Nude mice (n=5/group) just after the injury surgery. After 4 weeks (A–E) or 12 weeks (F–O), the injured Achilles tendons that had received cell transplantation (A–C, F, G, J, K) or no transplantation (D, E) were observed under the fluorescence stereomicroscope. The red (A, D) and green (B, E–G, J, K) fluorescence images were

superimposed to the brightfield images (BF+RFP or BF+GFP) or each other (RFP+GFP). The sections of the injured tendons that had received HK2-EGFP or Scx-GFP cell transplantation were subjected to the immunohistochemical staining for GFP (H, I, L–O). N and O were the injured Achilles tendons that had no transplantation. The bar is 50 μ m for H, I, L–O.

Figure 4.

Chondrogenic potential of tendon-derived progenitor cells and bone marrow stromal cells. A–E, Mouse bone marrow stromal cells (A, BMSC), or tendon-derived progenitor cells isolated from mouse uninjured (B, Uninjured) or injured (C, Injured) Achilles tendons were cultured in micromass in 10% FBS-DMEM for 7 days and stained with alcian blue (A–C). Integrated density of the alcian blue-stained cultures (n=4) was measured (D). Aggrecan gene expression was examined by qPCR (E). F, The micromass cultures of the inTPCs were treated with LDN-193189 (BMP inhibitor) at the concentration of 200 nM or SB431542 (TGFβ inhibitor) at the concentration of 2 μM for 7 days, and integrated density of alcian blue-stained cultures (n=4) was measured. G–I, The cells isolated from human bone marrow stromal cells (F, BMSC) or human tendons (H, Tendon) were cultured in micromass in 10% FBS-DMEM for 7 days and stained with alcian blue. Integrated density of the alcian blue-stained cultures (n=3–8) was measured (I). The values are average and SD. *, p<0.05.

Figure 5. Multipotency of CD105-positive and negative injured tendon-derived progenitor cells. A, The inTPCs were sorted to two subpopulations, Sca1+/CD105+ and Sca1-/CD105- populations. B–H, The CD105+ and CD105- cells were cultured under the osteogenic, adipogenic or chondrogenic condition and stained with Alizarin red (B, E), Oil red O (C, F) or alcian blue (D, G), respectively. Integrated density of the alcian blue-stained cultures (n=4) was measured (H). I and J, The micromass cultures (I) and TGFβ1-treated monolayer cultures (J) of CD105+ and CD105- cells were subjected to qPCR analysis for aggrecan (I) or scleraxis (J) gene expression, respectively. The values are the average and SD. *, p<0.05.

Figure 6.

Distribution of transplanted CD105-positive and negative tendon progenitor cells in injured Achilles tendons. The sorted CD105+ and CD105- inTPCs isolated from Scx-GFP;RFP mice (A–H) or H2K-GFP mice (I–S) were transplanted in the injured Achilles tendon of CD1 Nude mice just after the injury surgery. A–H, The injured Achilles tendons that had received cell transplantation were examined under the fluorescence microscope (A–F) 4 weeks after the transplantation. The red (RFP) and green (Scx-GFP) fluorescence images were superimposed to the brightfield images (A/D and B/E, respectively) or each other (C,

F). Integrated intensity of RFP (G) and Scx-GFP (H) fluorescence of the samples (n=4) was measured. I–S, The injured Achilles tendons that had received transplantation of H2K-GFP mouse-derived iTPCs were harvested 10 weeks after the transplantation (n=5). Longitudinal sections of the injured tendons were subjected to alcian blue staining (I, K, M, O) and immunohistochemical staining for GFP (J, L, N, P). The chondroid lesion area positive to alcian blue staining at the edges of injured tendons (I, M) was measured (Q). The number of GFP-positive cells was counted at the edges (J, N) and the center parts (L, P) of injured tendons (R and S, respectively). The values are the average and SD. *, $p < 0.05$.

A Fractionated inTPC, Immunoblot

B CD105-negative inTPC, Immunoblot

C. CD105-negative inTPC, Gene expression

Figure 7.

Phosphorylation of smad proteins and chondrogenesis in injured tendon derived progenitor cells. A and B, The sorted CD105-positive and negative inTPCs (A) or CD105-negative inTPCs transduced with adenovirus encoding CD105 or GFP (B) cultures were treated with TGFβ1 (10 ng/ml) for 30–120 min, and subjected to immunoblot with anti-phospho-smad 2, phospho-smad1/5, total smad 2, total smad 1. The immunoblot band intensity was evaluated by Image J and the ratio of P-Smad2 to Smad 2 or P-Smad1/5 to Smad 1 was calculated. The value represents ratio to the value of the non-treated control group. C, CD105-negative

inTPCs transduced with adenovirus encoding CD105 or GFP were cultured in 10% FBS-DMEM for 7 days, and gene expression levels of aggrecan and CD105 were examined. The values are the average and SD. *, $p < 0.05$