学位論文

Potential function of Scx+/Sox9+ cells as progenitor cells in rotator cuff tear repair in rats
（ラットにおける肩腱板断裂修復術での前駆細胞としての Scx/Sox9 共陽性細胞の機能）

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Potential function of Scx+/Sox9+ cells as progenitor cells in rotator cuff tear repair in rats

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ABSTRACT

Tendons and their attachment sites to bone, fibrocartilaginous tissues, have poor self-repair capacity when they rupture, and have risks of retear even after surgical repair. Thus, defining mechanisms underlying their repair is required in order to stimulate tendon repairing capacity. Here we used a rat surgical rotator cuff tear repair model and identified cells expressing the transcription factors Scleraxis (Scx) and SRY-box 9 (Sox9) as playing a crucial role in rotator cuff tendon-to-bone repair. Given the challenges of establishing stably reproducible models of surgical rotator cuff tear repair in mice, we newly established Scx-GFP transgenic rats in which Scx expression can be monitored by GFP. We observed tissue-specific GFP expression along tendons in developing ScxGFP transgenic rats and were able to successfully monitor tissue-specific Scx expression based on GFP signals. Among 3-, 6-, and 12-week-old ScxGFP rats, Scx+/Sox9+ cells were most abundant in 3-week-old rats near the site of humerus bone attachment to the rotator cuff tendon, while we observed significantly fewer cells in the same area in 6- or 12-week-old rats. We then applied a rotator cuff repair model using ScxGFP rats and observed the largest number of Scx+/Sox9+ cells at postoperative repair sites of 3-week-old relative to 6- or 12-week-old rats. Tendons attach to bone via fibrocartilaginous tissue, and cartilage-like tissue was seen at repair sites of 3-week-old but not 6- or 12-week-old rats during postoperative evaluation. Our findings suggest that Scx+/Sox9+ cells may function in rotator cuff repair, and that ScxGFP rats could serve as useful tools to develop therapies to promote rotator cuff repair by enabling analysis of these activities.

1. Introduction

Rotator cuff (RC) tears frequently lead to shoulder pain and dysfunction and adversely affect daily activities. Surgical arthroscopic RC repair (ARCR) is often performed in patients who do not respond positively to nonoperative therapy. Currently, ~460,000 patients undergo RC repair surgery yearly in the US [1]. However, retear of a repaired tendon occurs with a frequency of ~20% based on

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meta-analysis and is an important postoperative complication [2]. Thus, understanding the RC repair process is mandatory to develop therapies to prevent retear after ARCR.

The RC, which consists of four tendons, attaches to the humerus by the fibrocartilaginous enthesis, a tissue found between tendon and bone [3]. Thus, stimulation of regeneration of a functional fibrocartilaginous enthesis after surgery likely promotes strength of the repaired RC tendon-to-bone attachment site. However, regeneration of a functional fibrocartilaginous enthesis after injury or surgical tendon-bone repair is challenging, even in animal models, because of poor healing capacity, and weak fibrovascular scar tissues formed between tendon and bone [4].

Scleraxis (Scx) is a basic helix-loop-helix transcription factor expressed in tendons from the early progenitor stage to the formation of mature tendons [5–8]. Scx−/− mice reportedly exhibit defective limb tendon development, resulting severely reduced mobility after birth [9]. Sox9 functions in chondrocyte development, and mutations or translocations of the Sox9 gene or enhancer reportedly cause a type of congenital osteochondral dysplasia known as campomelic dysplasia in humans [10]. During the skeletal development, genetic ablation of the Sox9 gene reportedly results in complete absence of endochondral skeletal elements [11]. Scx/Sox9 doubly positive (Scx+/Sox9+) cells reportedly contribute to establishment of the junction between cartilage and tendon in mice [12,13]. Conditional Sox9 inactivation in Scx+/Sox9+ cells causes defective enthesis formation [13,14]. Thus, Scx+/Sox9+ cells are considered to be potential multipotent progenitors that give rise to tenocytes, ligamentocytes and chondrocytes to establish the chondro-tendinous junction.

Recently, we reported use of ScxGFP transgenic (Tg) mice to assess participation of Scx+/Sox9+ cells in postnatal formation of fibrocartilaginous entheses of supraspinatus tendon [15]. In that study we observed a greater number of Scx+, Sox9+ and Scx+/Sox9+ cells during the healing process after RC dissection performed in 3-week-old versus 20-week-old mice, and detected fibrocartilage formation 4 weeks after surgery in mice that had undergone surgery at 3-weeks of age, suggesting that Scx+/Sox9+ cells function as entheseal progenitor-like cells during healing after RC injury. However, it remained difficult to investigate Scx+/Sox9+ cell function after RC tendon-to-bone "surgical repair" in mice, as their small size made it difficult to create reproducible models.

Here, to circumvent that issue, we established ScxGFP Tg rats to visualize Scx-positive cells based on GFP activity, as anti-Scx antibodies are as yet unavailable. Using that rat model, we show that Scx+/Sox9+ cells are present in fibrocartilaginous entheses forming postnatally and that those cells appear to participate in healing process after RC surgical repair. We also observed a greater number of Scx+/Sox9+ cells in rats subjected to surgery at 3-weeks rather than 6- or 12-weeks of age.

2. Materials and methods

2.1. Generation of ScxGFP transgenic rats

To construct ScxGFP transgenes, we used an 11 kb genomic clone from the mouse Scx locus, which extends 4 kb upstream and 5 kb downstream of the *Scx* gene, as reported [16]. Transgene constructs were injected into pronuclei of fertilized eggs, which were transferred into oviducts of pseudopregnant recipient rats. Founder rats were identified by genotyping of tail DNA. Sprague-Dawley rats were purchased from Japan SLC (Sizuoka, Japan). Animals were housed under a 12-h dark–light cycle (light from 07:00 to 19:00) at 22 ± 1 °C with ad libitum access to food and water. The Animal Care and Use Committee of Kumamoto University School of Medicine approved protocols used for animal experiments (C29-174).

2.2. Surgical procedures

RC surgical repair models in rats were created as previously described [17,18]. In brief, after administering general anesthesia via intraperitoneal injection of 0.375 mg/kg medetomidine hydrochloride, 2 mg/kg midazolam, and 2.5 mg/kg butorphanol, we made 1.5-cm longitudinal anterolateral skin and deltoid muscle incisions on the left shoulder. Then, the supraspinatus tendon was detached sharply at its insertion and the fibrocartilaginous layer at the bone surface was removed. Next, we made a 0.8-mm anterior-posterior transverse bone tunnel at the humeral head and refixed the detached tendon to its proper anatomic position using a 5–0 prolene suture passed through the bone tunnel. We then sutured the torn tendon end to bone using No. 5–0 prolene. The incision was closed in layers using No. 4–0 nylon sutures. For the sham procedure, skin and deltoid incisions were made on the right shoulder to expose the supraspinatus tendon, and they were closed in the same way. All rats were permitted cage activities without immobilization.

2.3. Histological evaluation

As described [15], after perfusion fixation, supraspinatus tendon-to-bone specimens were dissected and fixed in 4% paraformaldehyde, treated with 20% sucrose in 4% paraformaldehyde, and frozen in SCEM-L1 compound (Section-lab, Hiroshima, Japan). Samples were then cryosectioned to 5 μm thickness on a Leica CM3050 S cryostat (Leica, Wetzlar, Germany). To evaluate histomorphology of entheses, sections were stained with either a 0.05% toluidine blue (TB) solution (pH 4, Wako, Osaka, Japan) or for ALP/alizarin red S, the latter using an alkaline phosphatase (ALP) staining kit (Cosmobio, Tokyo, Japan) and alizarin red S (Sigma-Aldrich, Tokyo, Japan). Sections were observed under a BZ-X700 microscope (Keyence, Osaka, Japan) and digital images were captured.

2.4. Immunohistochemistry

To assess Scx and Sox9 expression at healing sites at 2, 4, 6 weeks after surgery, we washed tissue sections with PBS, soaked them 10 min at 37 °C in a 0.05% Proteinase K solution (Kanto Kagaku, Japan), incubated them 30 min in 0.1% Triton-X/3% BSA in PBS and incubated them overnight at 4 °C with primary antibodies diluted with 1% BSA in PBS. After washing, sections were incubated with secondary antibodies and washed again in PBS. Primary antibodies used were goat anti-GFP antibody (abcam, ab6673) and rabbit anti-Sox9 antibody (MILLIPORE, AB5535), and secondary were Alexa Fluor 488 Donkey anti-goat IgG and Alexa Fluor Plus 594 Donkey anti-rabbit IgG (both from Thermo Fisher Scientific). Nuclei were counterstained with 4',6-Diamidino-2-phenylindole (DAPI). Images were captured under a BZ-X700 microscope. The percentage of cells positive for each antibody was determined by dividing the number of immunostained cells by the total number of cells in a 200 × 200 μm area selected randomly at the insertion site under 200-fold magnification. The average percentage of three areas from each slide was recorded.

2.5. Biomechanical testing

Supraspinatus tendon-to-bone specimens were dissected at 6 and 12 after surgery and stored at −80 °C until analysis. After thawing, all samples were subjected to biomechanical testing as described [17,18]. In brief, we removed surrounding tissues, including supraspinatus muscle belly and scar tissue, down to the layer where sutures used for tendon repair could be identified and then measured width and thickness of the repaired insertion site with a digital micro-caliper to estimate the cross-sectional area. We secured each sample to a conventional tensile tester (EZ test, SHIMADZU, Japan) using two vise grips preloaded to 0.1 N and loaded grips to failure at a rate of 10 mm/min; the ultimate

load-to-failure and failure sites were recorded. Stiffness and ultimate stress-to-failure were calculated from respective load-displacement curves and assessment of load-to-failure divided by the cross-sectional area of the repair site.

2.6. Statistical analysis

All numerical data are shown as means ± SD. Statistical significance of differences between groups was evaluated using Student's t-test or the Mann-Whitney *U* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS, not significant, throughout the paper).

3. Results

3.1. Generation of ScxGFP transgenic (Tg) rats

First, we established ScxGFP Tg rats in which the GFP sequence was inserted between the promoter and enhancer locus of mouse Scx (Fig. 1A) We previously detected GFP protein expression in tendon tissues in developing ScxGFP Tg mice established using that construct [16]. We assessed GFP expression in newborn (day 0) ScxGFP Tg rats and detected it in tendons of upper limbs (Fig. 1B), as previously reported in ScxGFP Tg mice [13,15,16], confirming successful establishment of this ScxGFP model.

We then evaluated enthesis regions of RCs at the humerus in 3-, 6- or 12-week-old ScxGFP Tg rats by staining with either toluidine blue (Fig. 2A–C) or alkaline phosphatase and alizarin red (Fig. 2D–F). Toluidine blue-positive fibrocartilaginous tissue was detected between tendon and bone. We also stained specimens with anti-GFP and anti-Sox9 antibodies and observed Scx+/Sox9+ cells in 3- and 6- but not 12-week-old ScxGFP Tg rats (Fig. 2G–I). Moreover, the number of Scx+/Sox9+ cells was higher in 3- relative to 6-week-old ScxGFP Tg rats.

3.2. Induction of Scx+/Sox9+ cells at repair sites after RC repair surgery in ScxGFP Tg rats

We next created an RC surgical repair model by cutting the supraspinatus tendon at the fibrocartilaginous layer between tendon and humerus bone and then re-suturing dissected tendon to the humerus in 3-, 6- and 12-week-old ScxGFP Tg rats. By two weeks after surgery, some Scx+/Sox9+ cells were induced at repair sites in ScxGFP Tg rats subjected to surgery at 3- and 6-weeks of age (Fig. 3A–F), but those cells were less frequent in rats undergoing surgery at 12-weeks (Fig. 3G–I). Quantitative analysis revealed a significantly greater number of Scx+/Sox9+ cells in 3-week-old ScxGFP Tg rats compared to either 6- or 12-week-old rats, and the number of Scx+/Sox9+ cells at the repair sites decreased over the healing period in both 3- and 6-week-old ScxGFP Tg rats (Fig. 3J).

Finally, to test the roles of Scx+/Sox9+ cells induced at the repair sites, we performed histological analysis of undecalcified frozen sections of specimens acquired from ScxGFP Tg rats subjected to surgery at 3-, 6- or 12-weeks of age and analyzed at day0 or 2, 4 or 6 weeks post-surgery by toluidine blue, alizarin red or alkaline phosphatase staining (Fig. 4). In these analyses, humerus bone was stained by alizarin red, and repair sites were located above the humerus. Alkaline phosphatase activities indicative of osteoblastic cells located at the humerus bone surface were equivalent in all three ScxGFP Tg rats at 2, 4 or 6 weeks after surgery, while toluidine blue-positive chondrogenic cells were more robustly induced at repair sites of ScxGFP Tg rats undergoing surgery at 3-weeks of age as compared to rats operated on at 6 or 12 weeks, at any period post-surgery. Thus, Scx+/Sox9+ cells likely contribute to promote toluidine blue-positive fibrocartilaginous tissue repair after surgery.

4. Discussion

Full-thickness RC tears do not heal spontaneously; therefore, surgical treatment is the preferred approach for patients whose injuries interfere with activities of daily living. However, the risk of re-tear after surgical repair and the need for prolonged post-surgery rest periods remain roadblocks to successful surgical treatment. Thus, understanding mechanisms underlying RC tendon-to-bone repair could enhance post-surgical repair. This study reveals that Scx+/Sox9+ cells are present in the repaired area, and that younger rats exhibited a greater number of these cells. We also observed strong induction of toluidine blue-positive chondrogenic cells during the healing process at repair sites of rats that had undergone surgery at 3-weeks of age.

It is commonly acknowledged that tissue repair is more efficient in younger individuals. Here, we demonstrate that during the post-surgery repair period, Scx+/Sox9+ cells are more abundant in growing 3-week-old rat tendons than in more mature 6- or 12-week-old rat tendons. Also, as tissues repair, embryonic or developmental processes are likely activated. For example, endochondral ossification, which normally occurs

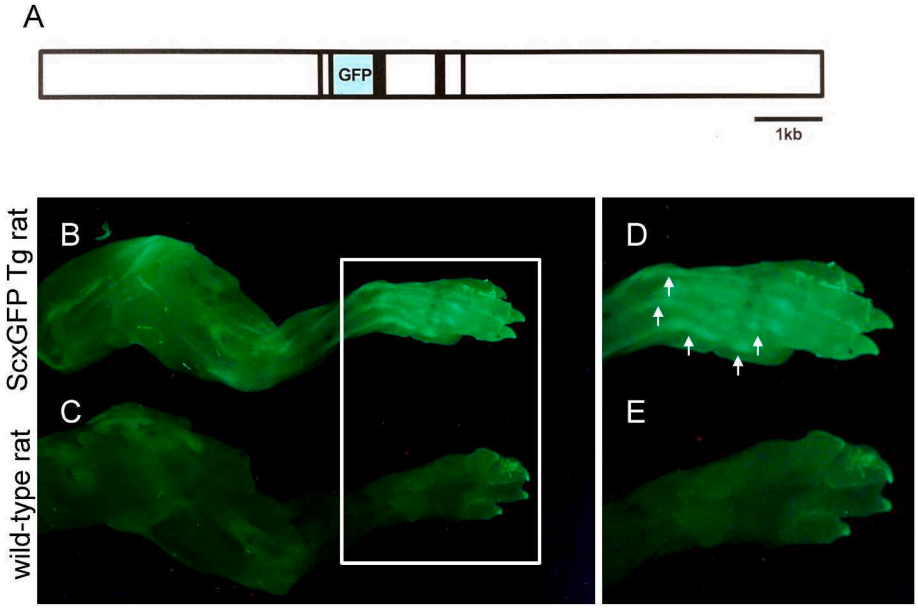


Fig. 1. Generation of ScxGFP Tg rats.
(A) Schematic of the ScxGFP transgene. A GFP sequence was inserted at the exon 1 start codon of the mouse Scleraxis (Scx) gene to yield a ScxGFP transgene. Bar = 1 kb. (B–D) GFP fluorescence was detected in forelimb tendons and ligaments in 0-day-old female ScxGFP Tg (B and D) but not wild-type (C and E) rats. Low (B and C) and high (D and E) magnification views are shown. Arrows indicate ScxGFP-positive tendons and ligaments in ScxGFP Tg rats.

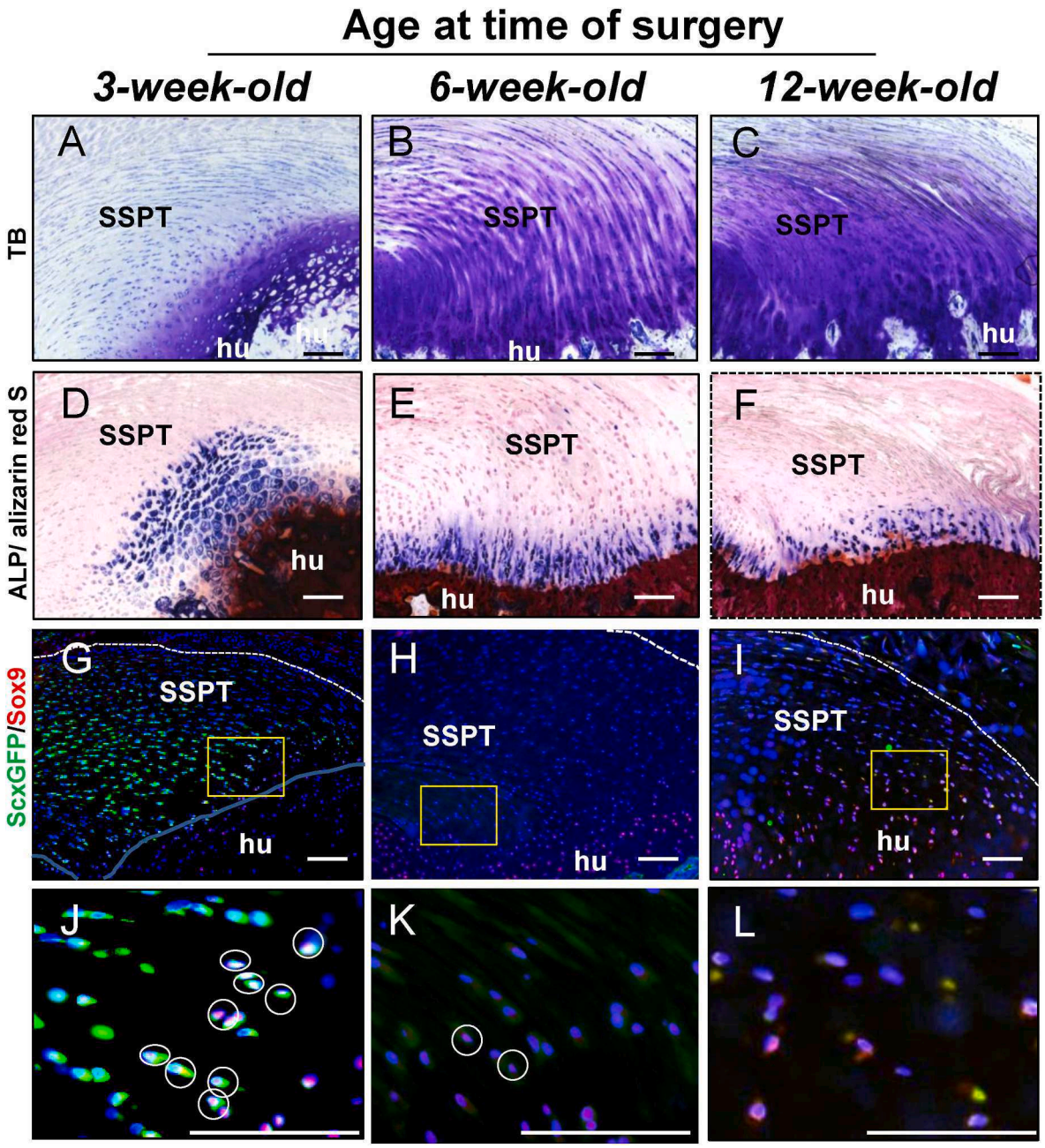
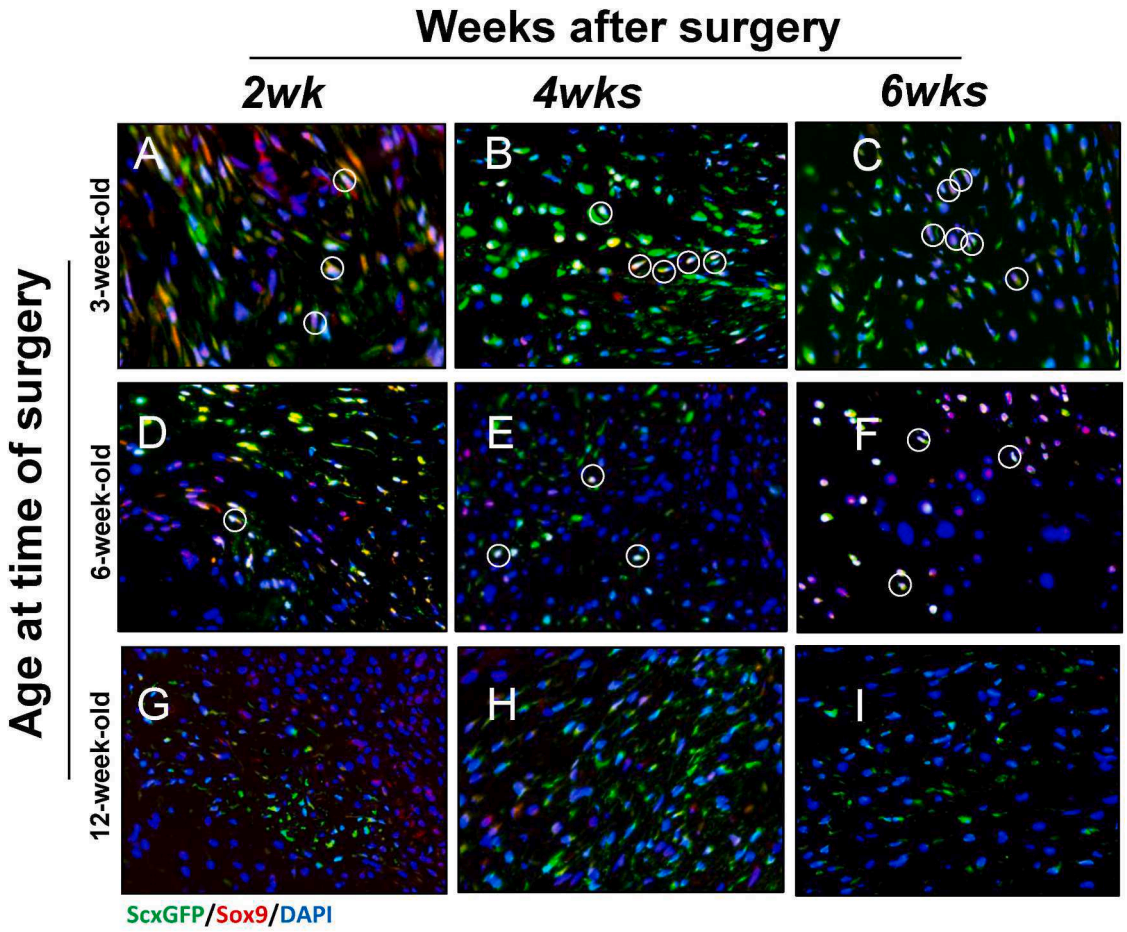


Fig. 2. A greater number of Scx/Sox9 double-positive cells is seen at the supraspinatus tendon enthesis in younger relative to older rats.
The supraspinatus tendon (SSPT) enthesis region was dissected from 3-, 6- or 12-week-old female ScxGFP rats, and undecalcified frozen sections were prepared and stained with toluidine blue (TB) (A, B and C), alkaline phosphatase (ALP)/alizarin red S (D, E and F) or goat anti-GFP (green) and rabbit anti-Sox9 (red) antibodies followed by Alexa488-conjugated donkey-anti goat and Alexa594-conjugated donkey-anti rabbit antibodies (G–L). Nuclei were DAPI-stained (blue), and samples were observed under a fluorescence microscope (G–L). Lower (G–I) and higher (J–L) magnification images are shown. Solid and dotted lines highlight surface of the humerus (hu) and the cFC/ucFC interface, respectively. White circles indicate Scx+/Sox9+ double-positive cells. Scale bar = 100 μm.

during mammalian development, also occurs during fracture repair. Scx+, and Scx+/Sox9+ cells are also seen in tendons and ligaments of growing mice and chick [5,8,12,13], and growing Scx-deficient mice exhibit defective tendon and ligament phenotypes [9]. Additionally, muscle bundle shapes and attachment sites were markedly altered in mice embryos with Scx-lineage cell ablation [19]. These observations suggest that Scx+/Sox9+ cells play a crucial role in tendon and enthesis repair.

Expression of the transcription factor Sox9 is associated with growth in several tissues, and mice heterozygous for Sox9 loss exhibit embryonic lethality [11]. Sox9 also functions in chondrocyte development, and mutations or translocations of the Sox9 gene or enhancer reportedly cause a type of congenital osteochondral dysplasia known as campomelic dysplasia in humans [10]. Several cell-lineage tracing studies indicated that at least part of the cells of the ligaments and tendons originate from Sox9-expressing condensed mesenchymal cells of the cartilage primordia and possibly contribute to the formation of the enthesis [11,12,20,21]. Here, we observed robust cartilage formation in the RC repair area in rats subjected to surgery at 3-weeks of age, a location also showing abundant Scx+/Sox9+ cells. Tendons are connected to bone through Sharpey's fibers at the fibrocartilaginous enthesis, and tears often occur in this area. Repair of RC tears may require Sharpey's fiber repair, and cells expressing transcription factors essential for tendon and ligament formation (Scx) and cartilage



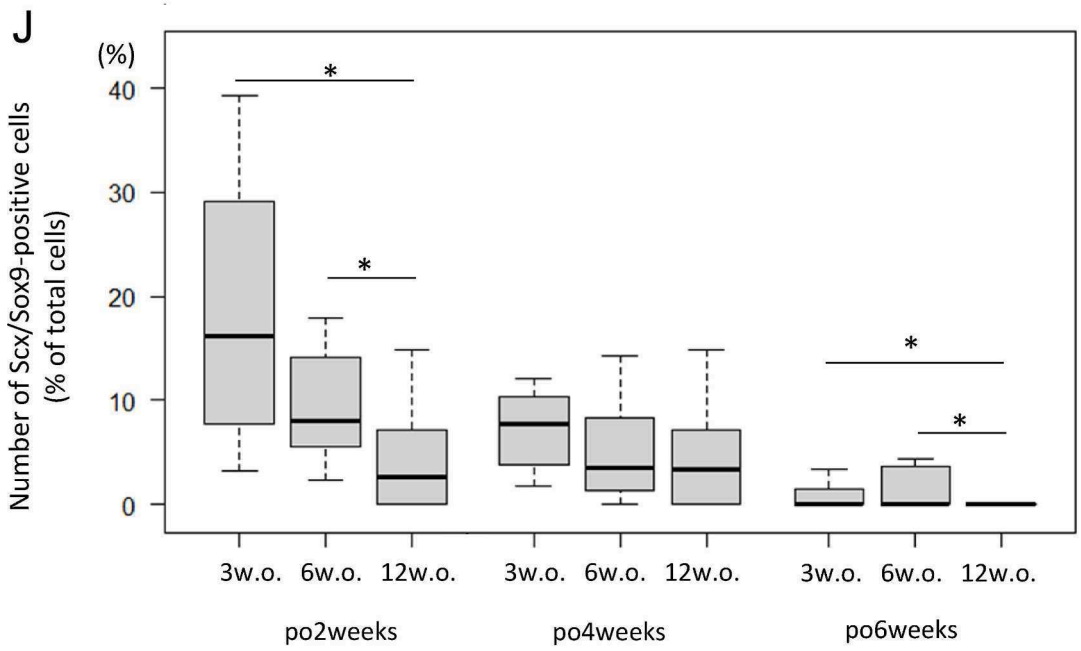


Fig. 3. Scx/Sox9 double-positive cells are more robustly induced in younger than in older rats during RC tear repair.
(A–I) RC tear repair models were created in 3-, 6- or 12-week-old female ScxGFP rats. After 2, 4, or 6 weeks (wks), repair sites were dissected from rats in each group, and undecalcified frozen sections were prepared and stained with goat anti-GFP and rabbit anti-Sox9 antibodies followed by Alexa488-conjugated donkey-anti goat and Alexa594-conjugated donkey-anti rabbit antibodies. Nuclei were stained with DAPI, and sections were observed under a fluorescence microscope. White circles indicate Scx+/Sox9+ double-positive cells. po, post-operative. Scale bar = 100 μm. (J) The number of Scx+/Sox9+ double-positive cells was counted and normalized to the total number of DAPI-stained nuclei in repaired tissues in each section. All values represent the mean number of Scx+/Sox9+ double-positive cells per total cells (%) ± SD. N = 3 for each time point. *$p < 0.05$.

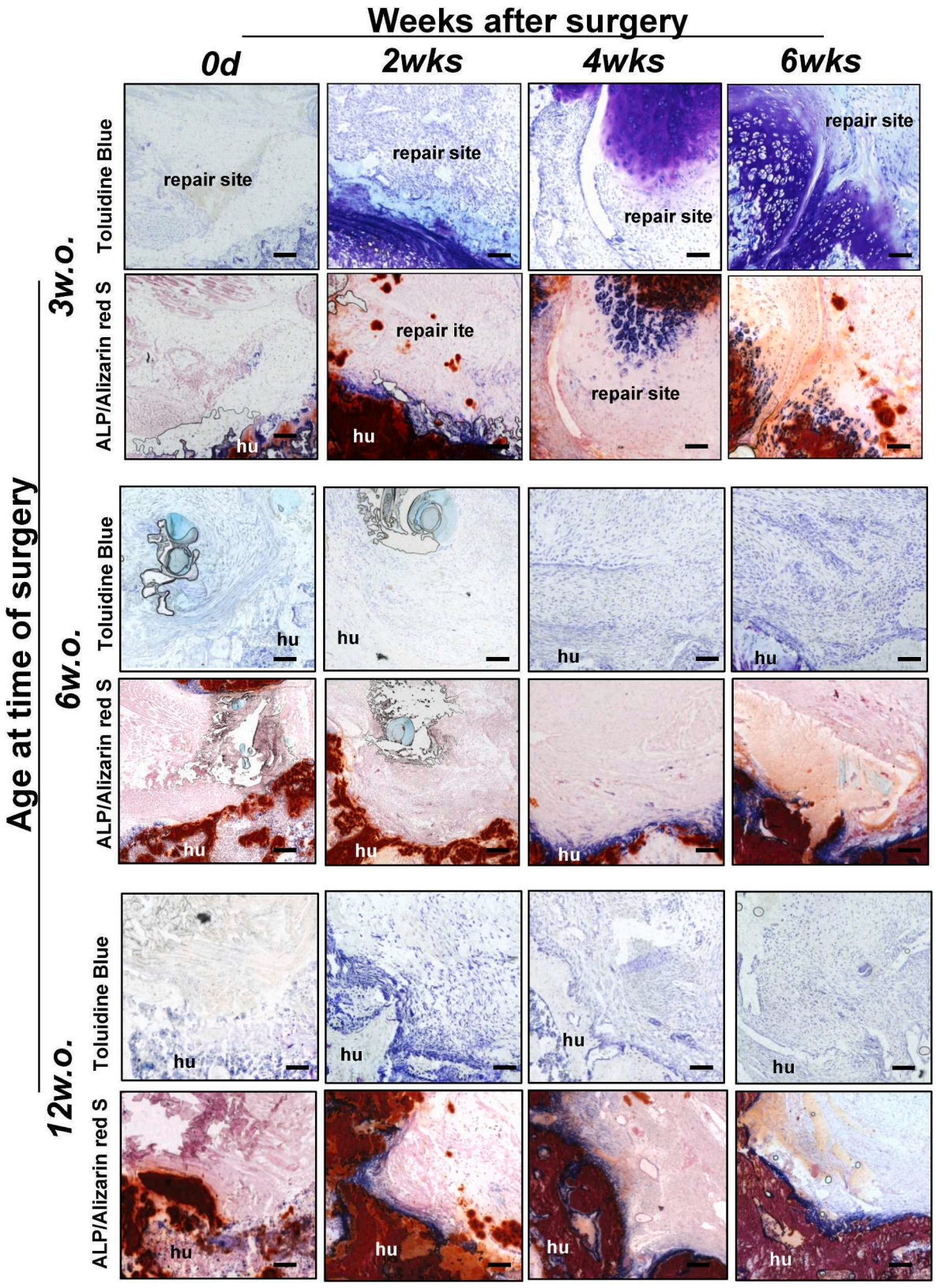


Fig. 4. Emergence of toluidine blue-positive chondrogenic cells in young rats during RC tear repair.
RC repair models were created in 3-, 6- or 12-week-old female ScxGFP rats, and either immediately (0d) or after 2, 4, or 6 weeks (wks), repair sites were dissected from rats in each group, undecalcified frozen sections were prepared and stained with toluidine blue or alkaline phosphatase (ALP)/alizarin red S and sections were observed under a microscope. hu, humerus bone. Black lines highlight surface of the humerus (hu). Scale bar = 100 μm.

formation (Sox9) are likely required for that process.

Animal models are essential to analyze physiological processes, such as RC tear repair, that cannot be assessed *in vitro*. Although numerous genetic mouse models and antibodies suitable for analysis are now available, the small size of mice hinders establishment of a stable model of RC surgical repair. Indeed, most mouse models relevant to this condition are RC injury models without repair [15,22]. In contrast, rats provide a more tractable model for analysis of RC repair due to their larger size, although genetically modified rats and necessary antibodies (such as anti-Scx) are limited in this species. Thus, we newly established ScxGFP Tg rats to assess Scx expression based on GFP activity. We anticipate that this model will provide a useful tool for observing tendon and ligament repair. We had wanted to assess function of Scx+/Sox9+ cells at repair sites in each group by performing post-operative biomechanical tests. However, we observed induction of epiphysiolysis in 54% of ScxGFP Tg rats operated on at 3-weeks of age (data not shown), preventing us from performing that analysis. Other analyses of wild-type rats have shown that FGF-2 administration enhances biomechanical strength after RC repair [17,18]. In the future, further monitoring of the emergence and function of Scx+/Sox9+ cells may lead to identification of agents that enhance RC tendon-to-bone repair.

Author contributions

Investigation: YF, ST, YY, TM (Mashimo) and TK (Kaneko); conceptualization: TT, TK (Karasugi) and TM (Miyamoto); resources: YY, TM (Mashimo) and TK (Kaneko); data curation: XT, KI, RY, KM, KS, MY, SH, TN, YU and TM (Masuda); funding acquisition: TT, TK (Karasugi) and TM (Miyamoto); supervision: TT, YY, CS, TK (Karasugi) and TM (Miyamoto); writing: TT and TM (Miyamoto).

Declaration of competing interest

All authors state that they have no conflicts of interest with the contents of this article.

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