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■ **MUSCLE & TENDON**

Biological analysis of flexor tendon repair-failure stump tissue

A POTENTIAL RECYCLING OF TISSUE FOR TENDON REGENERATION

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Objectives

Re-rupture is common after primary flexor tendon repair. Characterization of the biological changes in the ruptured tendon stumps would be helpful, not only to understand the biological responses to the failed tendon repair, but also to investigate if the tendon stumps could be used as a recycling biomaterial for tendon regeneration in the secondary grafting surgery.

Methods

A canine flexor tendon repair and failure model was used. Following six weeks of repair failure, the tendon stumps were analyzed and characterized as isolated tendon-derived stem cells (TDSCs).

Results

Failed-repair stump tissue showed cellular accumulation of crumpled and disoriented collagen fibres. Compared with normal tendon, stump tissue had significantly higher gene expression of collagens I and III, matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF). The stump TDSCs presented both mesenchymal stem and haematopoietic cell markers with significantly increased expression of CD34, CD44, and CD90 markers. Stump TDSCs exhibited similar migration but a lower proliferation rate, as well as similar osteogenic differentiation but a lower chondrogenic/adipogenic differentiation capability, compared with normal TDSCs. Stump TDSCs also showed increasing levels of SRY-box 2 (Sox2), octamer-binding transcription factor 4 (Oct4), tenomodulin (TNMD), and scleraxis (Scx) protein and gene expression.

Conclusion

We found that a failed repair stump had increased cellularity that preserved both mesenchymal and haematopoietic stem cell characteristics, with higher collagen synthesis, MMP, and growth factor gene expression. This study provides evidence that tendon stump tissue has regenerative potential.

Cite this article: *Bone Joint Res* 2019;8:232–245.

Article focus

■ To characterize the biological changes in the ruptured tendon stumps after repair failure.

■ To evaluate the stemness and regenerative capability of the stump tendon tissue and cells.

Key messages

■ The failed-repair tendon stump had increased cellularity that preserved both mesenchymal and haematopoietic stem cell characteristics.

■ The stump tendon tissue presented high gene expression of collagens I and III, matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF).

■ The stump tendon-derived stem cells (TDSCs) showed increasing levels of SRY-box 2 (Sox2), octamer-binding

transcription factor 4 (Oct4), tenomodulin (TNMD), and scleraxis (Scx) protein and gene expression but a lower proliferation rate compared with normal TDSCs.

Strengths and limitations
- This is, to the authors' knowledge, the first study to investigate both the stump tissue and cellular changes in the flexor tendon after repair failure.
- The study tissue and cells were harvested six weeks after the initial repair; the associated changes in cell characteristics at other timepoints need to be evaluated.

Introduction
Tendon injuries are the most common and severe hand injuries. They usually result in prolonged rehabilitation, impaired work function, and a huge socioeconomic burden.[1,2] Re-rupture is a common complication after primary flexor tendon repair (5% to 20%) and increases the need for secondary flexor tendon reconstruction.[3-6] In general, the healing of repaired tendons follows the typical wound healing course, including an early inflammatory phase, followed by proliferative and remodelling phases.[7-9] After failure repair, the ruptured tendon stumps are exposed to prepare for autograft or allograft reconstruction during revision. However, the cellular and molecular properties of ruptured tendon stumps are not clear.

Zhao et al[10] introduced a unique primary repair failure model in dogs that was a reproducible, reliable, and clinically relevant animal model with which to study flexor tendon reconstruction. This animal model also provided an opportunity to study the biological changes in the repaired failed tendon stump. Previous studies showed that the stump tissue and cells affect the implanted graft incorporation, revascularization, and vitalization after tendon or ligament reconstruction.[11-15] In recent years, stem cells have been isolated from rupture remnants and injured tissue, such as ruptured rotator cuff, shoulder synovium, and anterior cruciate ligament (ACL) ruptured remnants.[11,12,16,17] The tendon-derived stem cells (TDSCs) have been proven to show clonogenicity and high proliferation potential, multi-differentiation, self-renewal capacity, and enhancement of soft-tissue regeneration.[18-22] However, the stemness and regeneration capability of cells from the stump tendon have not been well studied. Understanding the variation in phenotypic expression of stump tissue and cells in the failed repair site will help us to manipulate the stump tendon tissue and cells in order to enhance tendon healing after repair or grafting.

The current study had two aims. First, we aimed to characterize the healing potential and behavioural changes of stump tissue after failure of flexor digitorum profundus (FDP) tendon repair by comparing tissue histology and gene expression with normal healthy tendon. Second, we investigated the regeneration capability of stem cells from the failed-repair FDP tendons by identifying stem cell surface markers, colony formation, cell migration, cell proliferation, multi-differentiation capability, immunofluorescence, and gene expression.

Materials and Methods
Study design. A total of eight mixed-breed dogs (mean age, 10 months (SD 1); mean weight, 21.3 kg (SD 3)) were used under Mayo Clinic Institutional Animal Care and Use Committee (number A51613-14) approval. A repaired tendon failure model was created using previous protocols.[10] Briefly, FDP tendons from the second and fifth digits were lacerated and repaired immediately in one forepaw; the dogs were then kept protected and non-weight-bearing with a custom-made jacket. Five days later, the jacket was removed and the dogs were allowed free cage activity with weight-bearing on the surgical paw. Six weeks after tendon repair, the second and fifth digits were opened to assess the tendon repair status, and the second and fifth repaired ruptured FDP tendon stumps were then harvested for analysis. The second and fifth FDP tendons from the non-surgical forepaw served as the control group. The collected failed repair stumps and normal FDP tendons were assigned randomly to the histological examination or cell culture group.

Histological analysis and gene expression changes in tendon stump after repair failure. Stump and normal FDP tendon tissues were stained with haematoxylin and eosin (H&E) and Masson's trichrome staining to assess collagen fibre alignment. These samples were stained with Picrosirius red to determine collagen orientation and collagen fibre type.[23]

Six FDP stumps and six normal tendons were collected for gene expression comparison. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to quantitatively measure levels of collagen I, collagen III, chondrogenic genes (collagen II, aggrecan), matrix metalloproteinases (MMPs: MMP2, MMP3, MMP9, and MMP13), tenogenic markers (tenomodulin (TNMD), tenascin C (TNC), scleraxis (Scx), and fibronectin (FBN)), and growth factors (vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), epidermal growth factor (EGF), and transforming growth factor beta (TGF-β)). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the reference gene. All PCR primers in this study are listed in Table I.

Isolation and characterization of TDSCs from stumps and normal tendons. After six weeks of primary suture repair, 2 cm of the failed-repair proximal stumps of the second and fifth FDP tendons were harvested. For the normal healthy tendons, the same part of zone II tendons was harvested as for the control group. These tendon tissues were minced into pieces 1 mm to 2 mm thick, digested with 5 mg/ml collagenase type I (Sigma) in phosphate-buffered saline (PBS) at 37°C with 5% CO_2 for six hours,

Table I. Polymerase chain reaction (PCR) primers used in this study

Gene	Amplicon size, bp	Primer sequence	Accession number
Collagen I	232	Forward: 5'-TGGTTCTCCTGGCAAAGAT-3' Reverse: 5'-ATCACCGGGTTCACCTTTA-3'	AF153062
Collagen III	156	Forward: 5'-ACAGCAGCAAGCTAT TGAT-3' Reverse: 5'-GGACAGTCTAATTCTTGTTCG-3'	XM_535997
Collagen II	177	Forward: 5'-GCCGAATCTGTGTCTGTGAC-3' Reverse: 5'-CTCCGGGTTCTCCTTTCTGT-3'	NM_001006951.1
Aggrecan	116	Forward: 5'-AGGGAAGGCTGCTATGGAGA-3' Reverse: 5'-ACCTCACCCTCCATCTCCTC-3'	NM_001113455.2
Tenomodulin	154	Forward: 5'-GATCCCATGCTGGATGAG-3' Reverse: 5'-TACAAGGCATGATGACACG-3'	AF234259
Tenascin C	82	Forward: 5'-CCACAGTCAGGGCGTTAACT-3' Reverse: 5'-GCTGGGCCTCAACTTCATCT-3'	NM_001195149
Scleraxis	120	Forward: 5'-AAGCTCTCCAAGATCCGAGACACTG-3' Reverse: 5'-AAGAAGGGCCCAGAGTGGCA-3'	XM_005628297.3
Fibronectin	139	Forward: 5'-GAGCTGCACATGTCTTGGAA-3' Reverse: 5'-TGTGCAGGAGCAAATAGAC-3'	XM_005640741
VEGF	146	Forward: 5'-CTATGGCAGGAGGAGAGCAC-3' Reverse: 5'-GCAGGATGGCTTGAAGATGT-3'	NM_001003175
PDGF	150	Forward: 5'-CCGAGGAGCTCTACGAGATG-3' Reverse: 5'-AACTCTCCAGCTCGTCTCCA-3'	NM_001003383.1
EGF	92	Forward: 5'-CTATGGCCGTGTTTGAGGAT-3' Reverse: 5'-TACCCTGTTTTTGCCAGTCC-3'	NM_001003094.1
IGF	121	Forward: 5'-TGTACTGTGCACCCCTCAAG-3' Reverse: 5'-CCTGCACTCCCTCTACTTGC-3'	XM_861842.3
TGF-β	85	Forward: 5'-TGGTGGAAAACACCAACAAA-3' Reverse: 5'-CGGAGCTCTGATGTGTTGAA-3'	NM_001003309.1
MMP2	112	Forward: 5'-CACGGCCAACTATGATGATG-3' Reverse: 5'-AGAATGCTCCAGTCCCATTG-3'	XM_535300.4
MMP3	150	Forward: 5'-CATTCCCTGGGTCTCTTTCA-3' Reverse: 5'-GGAGGAATCAGAGGGAGGTC-3'	AY183143.1
MMP9	102	Forward: 5'-GACAAGAAGTGGGGCTTCTG-3' Reverse: 5'-TGGCACCGATGAATGATCTA-3'	XM_005634678.1
MMP13	138	Forward: 5'-CTTCTGGCTCATGCTTTTCC-3' Reverse: 5'-ACCTAAGGAGTGGCCGAACT-3'	XM_005619857.1
Sox2	397	Forward: 5'-TTTGTCGGAGACGGAGAAGC-3' Reverse: 5'-CGAGCTGGTCATGGAGTTGT-3'	XM_005639752.1
Oct4	88	Forward: 5'-GCTCCTGAAGCAGAAGAGGA-3' Reverse: 5'-GCTGAACACCTTCCCAAAGA-3'	XM_538830.1
Nanog	166	Forward: 5'-CCCAACTCTAGGGACCCTTC-3' Reverse: 5'-GACTGGTGGAAGAATCCGGG-3'	XM_005642425.1
GAPDH	154	Forward: 5'-TATGATTCTACCCACGGCAA-3' Reverse: 5'- CAGTGGACTCCACAACATAC-3'	NM_002046.3

VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; IGF, insulin-like growth factor; TGF-β, transforming growth factor beta; MMP, matrix metalloproteinase; Sox2, SRY-box 2; Oct4, octamer-binding transcription factor 4; Nanog, homeobox protein Nanog; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

and then filtered through a 70 μm nylon sieve to remove the undigested tissue.[21] After centrifugation at 1500 rpm for five minutes, the supernatant was discarded and the cells resuspended in culture medium.[24] The third passage TDSCs were used for the following studies.

Flow cytometry identification of TDSCs: We investigated the third passage TDSCs with CD31, CD34 (endothelial or haematopoietic cell markers), CD44, and CD90 (mesenchymal stem cell markers) with flow cytometry.[18,21,25] These cells were analyzed using flow cytometry (BD FACSCalibur; BD Biosciences, San Jose, California). Triplicates of cells from three dogs in both groups were examined in this study. The CD marker antibodies used in this study are listed in Supplementary Table i.

Colony-forming assays: The colony-forming assays were performed with P1 and P3 cells to investigate the self-renewal capabilities of TDSCs harvested from stumps and normal tendons in different passages. A total of 200 cells were seeded in each well of six-well plates and cultured at 37°C in 5% CO_2. After ten days of culture, the cells were fixed with 4% paraformaldehyde in PBS for ten minutes and stained with crystal violet (0.5%). Only colonies with a diameter > 2 mm were included. The colony cell density was measured by randomly choosing two colonies > 2 mm that observed the clone centre under 40× magnification in each well, and the positive staining area calculated from six wells for each cell group. The number of colonies and colony cell density quantification were measured with ImageJ software (64-bit Java 1.6.0_24; NIH, Bethesda, Maryland).

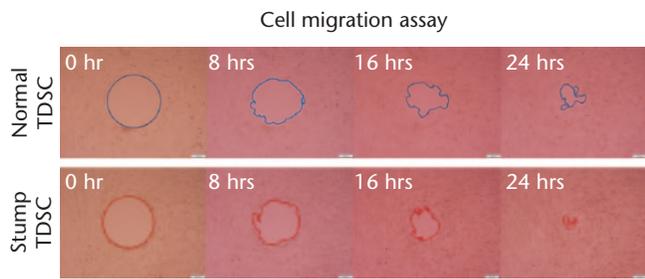Migration of TDSCs: We evaluated the changes in cell migration rate after tendon repair failure. The Radius
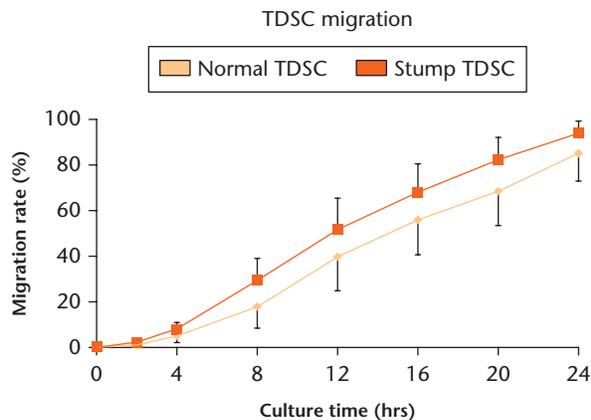
Cell migration assay
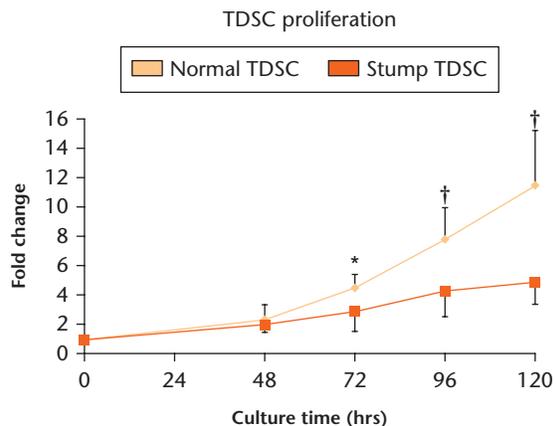

Fig. 1a

Fig. 1b

Fig. 1c

Cell migration and proliferation of stump and normal tendon-derived stem cells (TDSCs). a) The migration rate was calculated as follows: (initial wound area - residual wound area)/initial wound area (%). Scale bar = 200 μm. b) Chart showing that the migration rate of stump TDSCs had an upward trend compared with that of normal TDSCs, but without significant difference (n = 6). c) Chart showing that the stump TDSCs proliferated more slowly than normal TDSCs, with significant differentiation beginning from 72 hours (n = 9). The data are expressed as means (standard deviation). *$p < 0.05$; †$p < 0.01$ (unpaired Student's t-test).

24-Well Cell Migration Assay (Cell Biolabs, Inc., San Diego, California) was used to evaluate cell migration according to the manufacturer's instructions. Real-time cell migration status was observed under light microscopy by closing the round gap at regular intervals (at 2, 4, 6, 8, 12, 16, 20, and 24 hours). The migration rate was calculated as the following by ImageJ analysis software

(Fig. 1a): (initial wound area - residual wound area)/initial wound area (%).

Proliferation of TDSCs: We assessed the cell proliferation rate of stump TDSCs compared with that of normal TDSCs in order to investigate the differences in cell proliferation potential after tendon repair failure. A total of 2×10^4 P3 TDSCs were seeded in a 6 cm dish and cultured at 37°C in 5% CO_2. At 48, 72, 96, and 120 hours, these cells were detached from the dish with 0.25% trypsin and the cell number was counted using a haemocytometer. The cell proliferation rate was presented as a fold change of the calculated cell number at each timepoint, divided by the initial plating cell number.

Multi-differentiation assays: To identify the stem cell differentiation capabilities, the third-passage TDSCs were used for the osteogenic, adipogenic, and chondrogenic differentiation assays based on previously described protocols.[18,26,27] To investigate the osteogenic differentiation, 4×10^3 cells/cm² were plated in six-well plates and incubated at 37°C in 5% CO_2 with Dulbecco's Modified Eagle Medium (DMEM; Gibco; Thermo Fisher Scientific, Waltham, Massachusetts), and were replaced with osteogenic medium (StemPro Osteogenesis Differentiation Kit; Thermo Fisher Scientific) two days later. After 21 days' culture, the calcium nodules in mineralized deposits were stained with Alizarin Red dye. For the adipogenic differentiation, 1×10^4 cells/cm² were plated in six-well plates and incubated at 37°C in 5% CO_2 for two days, and were replaced with adipogenic medium (StemPro Adipogenesis Differentiation Kit; Thermo Fisher Scientific). After 14 days' culture, the presence of oil droplets was confirmed by Oil Red O staining. The positive staining area of Alizarin Red and Oil Red O were randomly measured under 200× magnifications from three dishes to quantify the results of osteogenic and adipogenic capability.

For chondrogenic differentiation, 1×10^6 cells/tube were centrifuged at $500 \times$ g for ten minutes to form a small pellet and then cultured in chondrogenic medium (StemPro Chondrogenesis Differentiation Kit; Thermo Fisher Scientific) at 37°C in 5% CO_2 for three weeks. At day 21, the pellet was fixed for histology with Alcian Blue staining. A RT-PCR of the chondrogenic genes collagen II and aggrecan was performed to assess the chondrogenic differentiation capability of TDSCs from stump and normal tendon (n = 6 with triplicate).

Immunofluorescent staining of TDSCs: To investigate the stemness and tenogenic differentiation capability of the TDSCs, we used immunofluorescence and PCR to identify the stem cell markers (SRY-box 2 (Sox2), octamer-binding transcription factor 4 (Oct4), and homeobox protein Nanog (Nanog)),[18,24,25,28] as well as tenogenic markers (TNMD, TNC, and Scx). All cell slides were counterstained with cytoskeleton staining (phalloidin) and mounted with ProLong Gold Antifade Mountant with
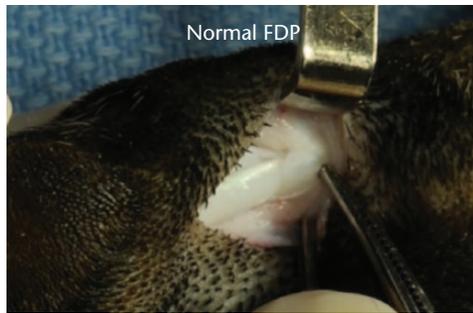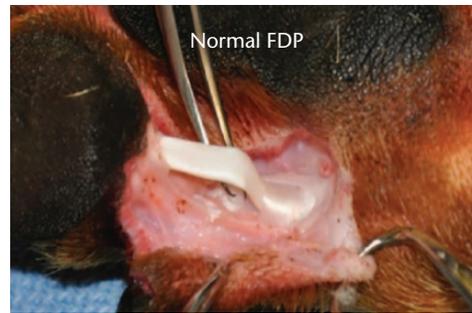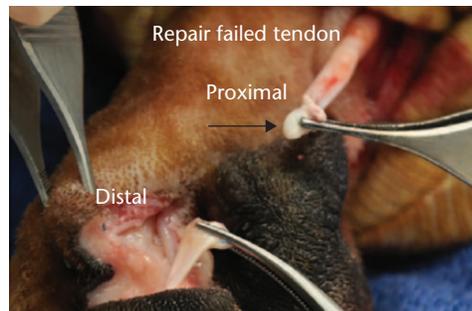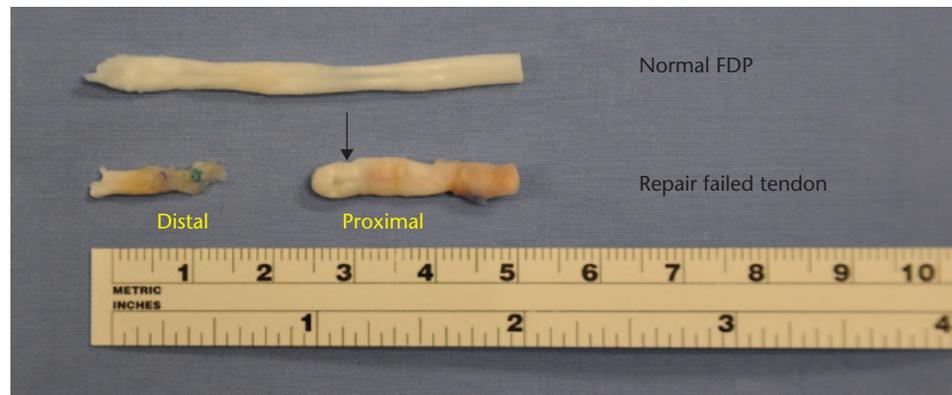
Fig. 2a

Fig. 2b

Fig. 2c

Fig. 2d

Fig. 2e

Representative images of: a) and b) normal flexor digitorum profundus (FDP) tendon; c) and d) failed-repair FDP tendon stump; and e) a comparison of the two. The failed-repair tendon was embedded within thickened synovium (indicated by the black arrow in Fig. 2c). The proximal failed-repair tendon showed a retracted, bowed pattern (indicated by the arrows in Figs 2d and 2e) compared with normal tendon.

4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). The antibodies used for immunofluorescent staining in this study are listed in Supplementary Table ii.

Gene expression of TDSCs: Gene expression in the TDSCs derived from stump and normal tendon was measured by RT-PCR for the following genes: collagen types I and III, collagen type II, aggrecan, MMP2, MMP3, MMP9, MMP13, stem cell markers (Sox2, Oct4, Nanog), and tenogenic markers (TNMD, TNC, Scx). The primers used in this study are shown in Table I.

Statistical analysis. Results were compared between stump and normal tendon tissue TDSCs using the unpaired Student's t-test. The data for flow cytometry, colony formation, cell migration, cell proliferation, and positive staining area are presented as means and standard deviations. The associated gene expression data are presented as means and standard errors. The significance level was set at $p < 0.05$ in all cases. All statistical analysis was performed using SPSS software version 20 (IBM Corp., Armonk, New York).

Results

Histological and gene expression changes in the tendon stump after repair failure. All repaired tendons were ruptured uniformly six weeks after primary surgery in all animals; the distal tendon stump adhered severely with scar tissue at the repair site. Compared with normal FDP tendon (Figs 2a and 2b), the proximal stump was covered with thickening synovium between zones II and III (Fig. 2c; black arrow). After opening the synovial sheath,
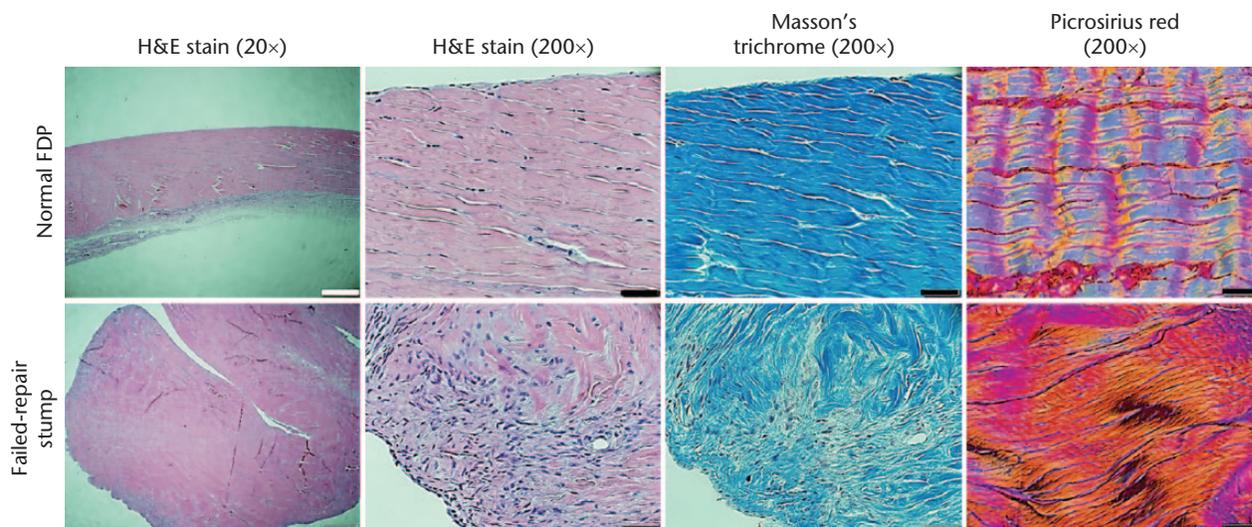
Fig. 3

Histological examination of failed-repair stumps and normal tendons. Histological staining with haematoxylin and eosin (H&E) and Masson's trichrome showed non-parallel, crumpled collagen fibres with cell accumulation in the failed-repair stump compared with the normal tendon with parallel collagen and few cells embedded. In addition, the irregular collagen arrangement in the stump tendon was confirmed by Picrosirius red staining. White bars = 500 μm; black bars = 50 μm. FDP, flexor digitorum profundus.

the stump was observed to be enlarged with a curved and bowed pattern (Figs 2d and 2e; arrows).

The stump tissue had non-parallel, crumpled fibre alignment with cellular accumulation compared with the normal tendon, which showed parallel, uniform collagen fibre alignment, with few cells (Fig. 3). Collagen fibres visualized by Picrosirius red staining showed that the failed tendon resulted in thicker collagen fibres than did the normal tendon (Fig. 3).

Gene expression in stump and normal FDP tendon tissues: Gene expression in the failed-repair tendon stump showed significantly higher collagen I and III gene expression than normal tendon (Fig. 4a). Collagen II gene expression was significantly lower in the stump tendon group than in the normal tendon group, but aggrecan expression showed similar levels in both groups (Fig. 4a). The stump tissue showed higher gene expression of MMPs (Fig. 4b), growth factors (VEGF, PDGF, IGF), and tenogenic markers (TNC, FBN) than normal tendon (Fig. 4c). Scleraxis gene expression was significantly lower in stump tissue than in normal tendon (Fig. 4d).

Isolation and characterization of TDSCs from stump and normal tendon. The result showed higher levels of CD31 and significantly higher levels of CD34, CD44, and CD90 on stump TDSCs than on normal TDSCs (Fig. 5), which indicated that the stump TDSCs present with mesenchymal stem cell and haematopoietic cell markers.

Colony formation: After ten days with initial 200 cell plating, the P1 and P3 normal and stump TDSCs formed colonies (Fig. 6a). The colony number in stump TDSCs was not significantly different to that in normal TDSCs at both P1 and P3 passages (Fig. 6b). However, colony cell density in P1 and P3 stump TDSCs was significantly lower

than in the normal TDSC group (Figs 6c and 6d). These results revealed that the stump TDSCs kept the similar self-renewal ability but they had significantly decreased cell proliferation capability compared with normal TDSCs.

The migration rate in the stump TDSCs was not significantly different in normal and stump TDSCs, although there was a trend towards an increased rate in stump TDSCs (Fig. 1b). However, the proliferation rate of stump TDSCs was significantly lower than that of normal TDSCs after 72 hours (Fig. 1c).

Osteogenesis differentiation: After 21 days of osteogenic induction, the culture medium of stump and normal TDSCs showed orange-red staining by Alizarin Red (Fig. 7a). There was no significant difference in the positive staining area of the culture medium between stump and normal TDSCs (Fig. 7d).

Adipogenesis differentiation: After 14 days of adipogenic induction, fat droplets were found on microscopy in both the stump and normal TDSC medium (Fig. 7b). Under Oil Red O staining, the stump TDSC medium showed lower adipogenesis capability, with a significantly less positive staining area than that of the normal TDSC medium (Fig. 7e).

Chondrogenesis differentiation: After chondrogenesis induction, the pellets of both normal and stump TDSCs showed positive Alcian Blue staining (Fig. 7c). The PCR results showed that stump TDSCs had slightly lower collagen II expression and significantly lower aggrecan gene expression (Fig. 7f).

Stem cell and tenogenic marker identification: Both normal and stump TDSCs demonstrated positive immunofluorescence staining for stem cell markers (Sox2,
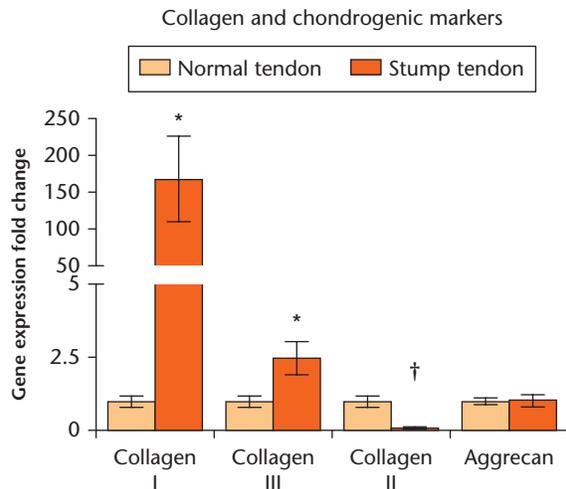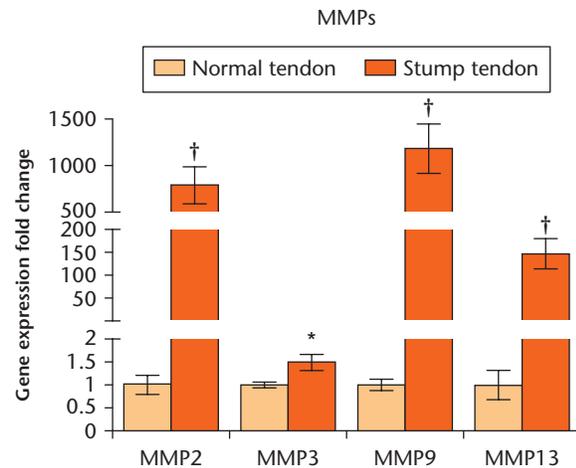
Collagen and chondrogenic markers

Fig. 4a

MMPs

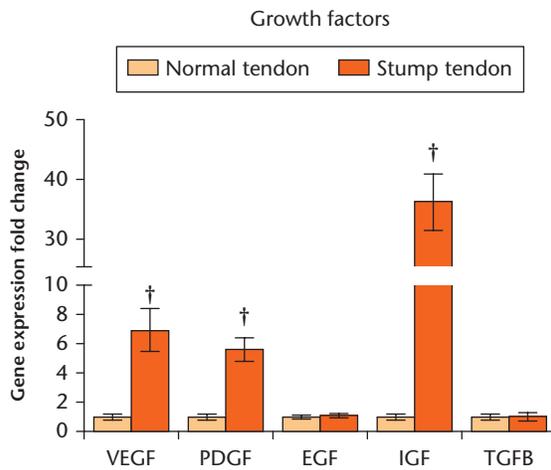
Fig. 4b

Growth factors
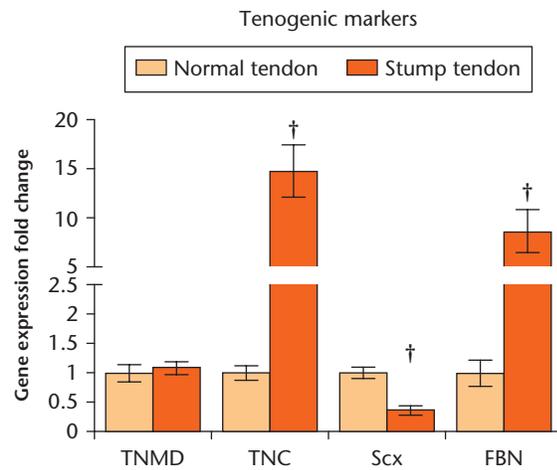

Fig. 4c

Tenogenic markers

Fig. 4d

Charts showing gene expression of failed-repair stumps and normal tendons for: a) collagen and chondrogenic markers; b) matrix metalloproteinases (MMPs); c) growth factors; and d) tenogenic markers. The stump tissue showed significant higher gene expression of collagen I, collagen III, MMP2, MMP9, MMP13, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), tenascin C (TNC), and fibronectin (FBN). The stump tissue also showed significantly lower gene expression of collagen II, MMP3, and scleraxis (Scx). Data expressed as means and standard errors of six samples (n = 6) in triplicate and normalized to normal tendon. *p < 0.05; †p < 0.01 (unpaired Student's *t*-test). EGF, epidermal growth factor; TGFB, transforming growth factor beta; TNMD, tenomodulin.

Oct4, Nanog; Fig. 8) and tenogenic markers (TNMD, TNC, Scx; Fig. 9). The PCR results showed significantly higher Sox2 and Oct4 gene expression (Fig. 8d), as well as significantly higher TNMD and Scx gene expression (Fig. 9d), in stump TDSCs compared with normal TDSCs.

Gene expression comparison: Stump TDSCs had significantly higher expression of collagen III, but no differences in collagen I expression (Fig. 10). Evaluation of MMPs revealed that MMP2 was significantly increased in the stump TDSCs, whereas MMP3 and MMP13 were significantly lower. No differences were found with MMP9 expression (Fig. 10).

Discussion
To characterize the cellular and molecular changes in the tendon stump due to disuse or repair failure is important in order to understand the healing capacity between tendon stump and graft after tendon reconstruction. In this study, the failed-repair tendon stump presented increased cellularity with high growth factor gene expression. The stump TDSCs showed both mesenchymal stem cells and haematopoetic cell markers, which showed high stemness and tenogenic capability. To our knowledge, this study is the first to investigate both the stump tissue and cellular changes in the flexor tendon after repair failure.

Growth factors are critical in stimulating synthesis of extracellular matrix, cell migration, proliferation, and differentiation during tendon healing.[9,29-32] In a chicken FDP repair model, Chen et al[29] found high expression of connective tissue growth factor, VEGF, IGF-1, and TGF-β in the healing period within three weeks of tendon repair. In

Fig. 5a

Fig. 5b
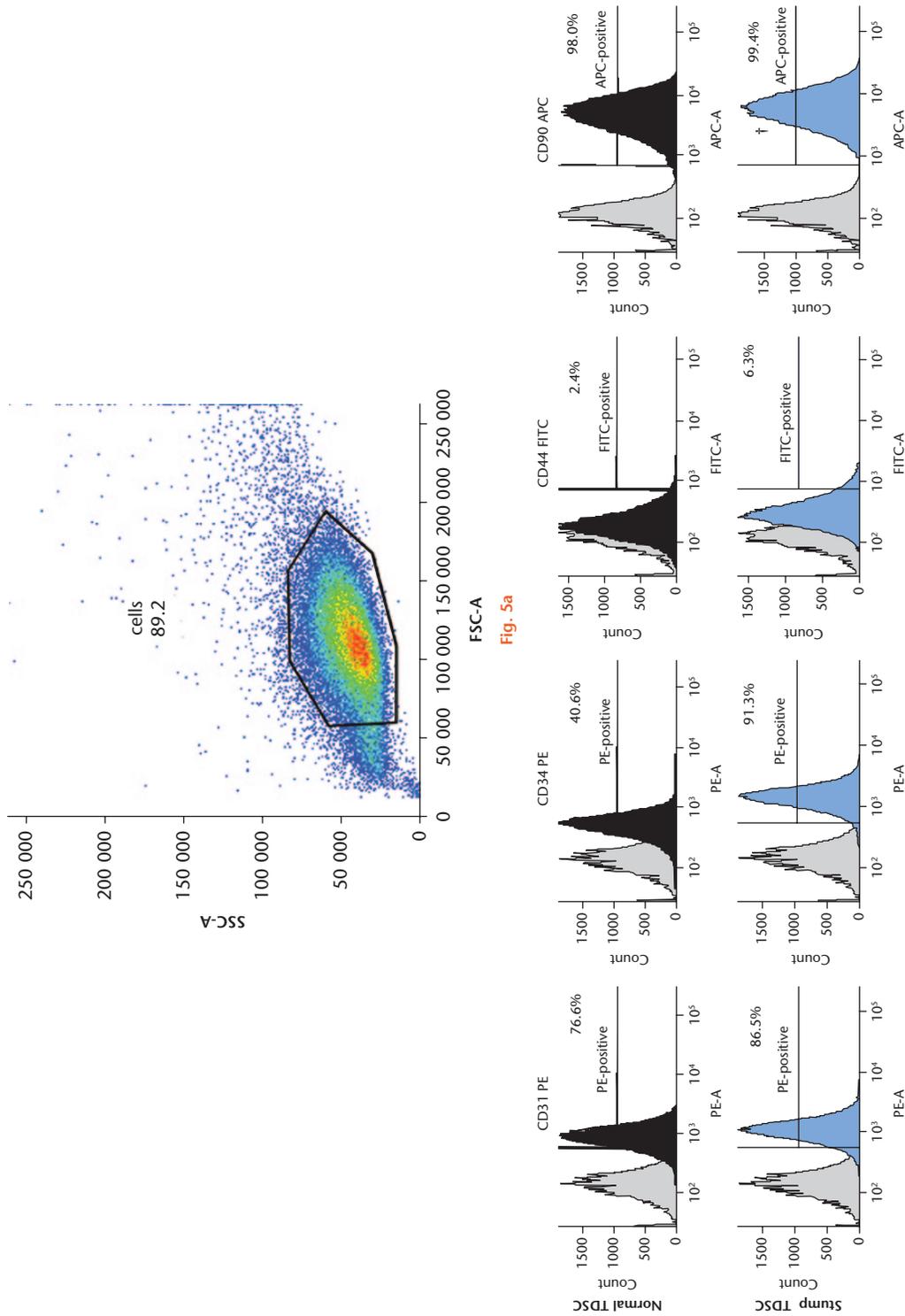
a) Representative gating parameters of tendon-derived stem cells (TDSCs). b) Flow cytometry analysis of haematopoietic and endothelial markers (CD31 and CD34) and mesenchymal stem cell markers (CD44 and CD90) in stump and normal TDSCs. The stump TDSCs showed significantly higher expression of CD34, CD44, and CD90 cell markers than normal TDSCs. Data are expressed as means (n = 6). *p < 0.05; †p < 0.01 (unpaired Student's t-test). SSC, side scatter; FSC, forward scatter; PE, phycoerythrin; FITC, fluorescein isothiocyanate; APC, allophycocyanin.
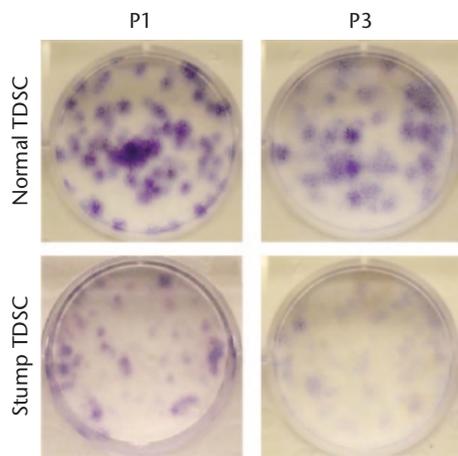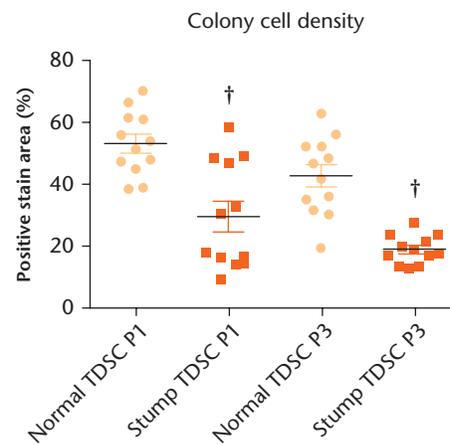
Fig. 6a
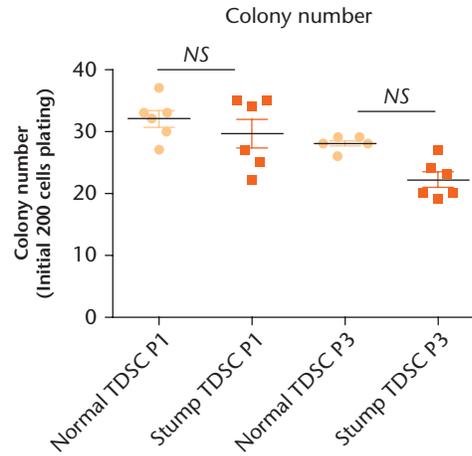
Fig. 6b
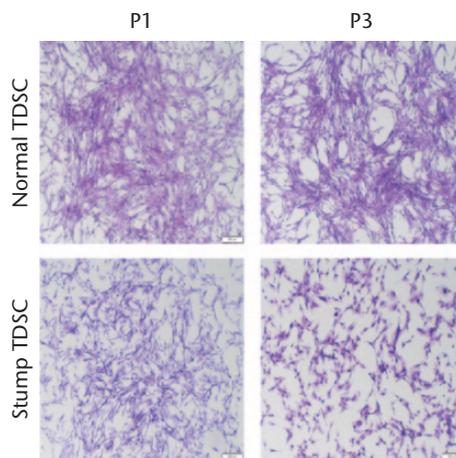
Fig. 6c

Fig. 6d

a) Colony-forming assay and b) colony cell density of stump tendon-derived stem cells (TDSCs). c) Colony-forming assay and d) colony cell density of normal TDSCs. At ten days with initial 200 cells plating, the colony number of normal and stump TDSCs was not significantly different in P1 and P3 passages. The colony cell density measured at the centre of the clone in stump TDSCs was significantly lower than in the normal TDSC group at both P1 and P3. Data are expressed as dot plot of six samples (n = 6). †p < 0.01 (unpaired Student's t-test). NS, no significant difference.

our study, histological examination from stump tissue six weeks after primary repair failed showed many tenocytes embedded with crumpled, disoriented, and coarse collagen fibres. The stump tissue highly expressed collagen I, collagen III, MMPs, and TNC genes compared with normal tendon. Significantly higher gene expression of VEGF, PDGF, and IGF was also found in the stump tissue compared with normal tendon tissue. These results indicate that the stump tendon tissue still possesses the capability for tissue repair and high regeneration, although the tissue has been disused for six weeks. Under compression conditions, the tendon expresses a high concentration of cartilage-like molecules (aggrecan and type II collagen), which leads to fibrocartilage formation in the extracellular matrix, to withstand the compression force.[33,34] Rui et al[35] found that the tendon cells isolated from a collagenase-induced failed healing patellar tendon exhibited higher osteogenic and chondrogenic

expression, which contributed to the pathological chondro-ossification process, compared with normal healthy tendon. In our study, neither the tissue level nor the cellular level revealed higher chondrogenic properties compared with normal tendon tissue and cells. This suggests that the stump tissue and cells reduced the chondrogenesis significantly under the failed-repair conditions with the loss of tensile load.

In our study, we successfully isolated TDSCs from the stump tissue. The stump TDSCs showed self-renewal with clonogenic capability, multi-differentiation, significantly higher stem cell marker (Sox2, Oct4) and tenogenic marker (TNMD, Scx) expression, and a similar migration rate but lower proliferation capability compared with normal TDSCs. In general, human TDSCs express CD44 and CD90 (stem cell markers) but not CD34 and CD31 (endothelial or haematogenic cell markers).[22,25,36] In our study, the flow cytometry result showed
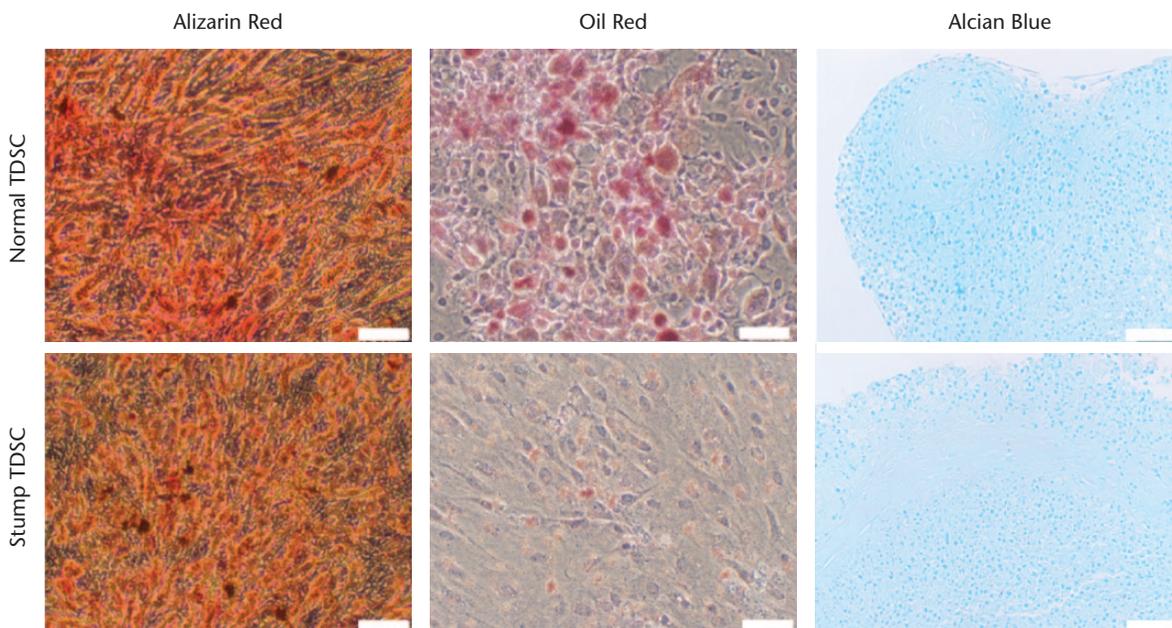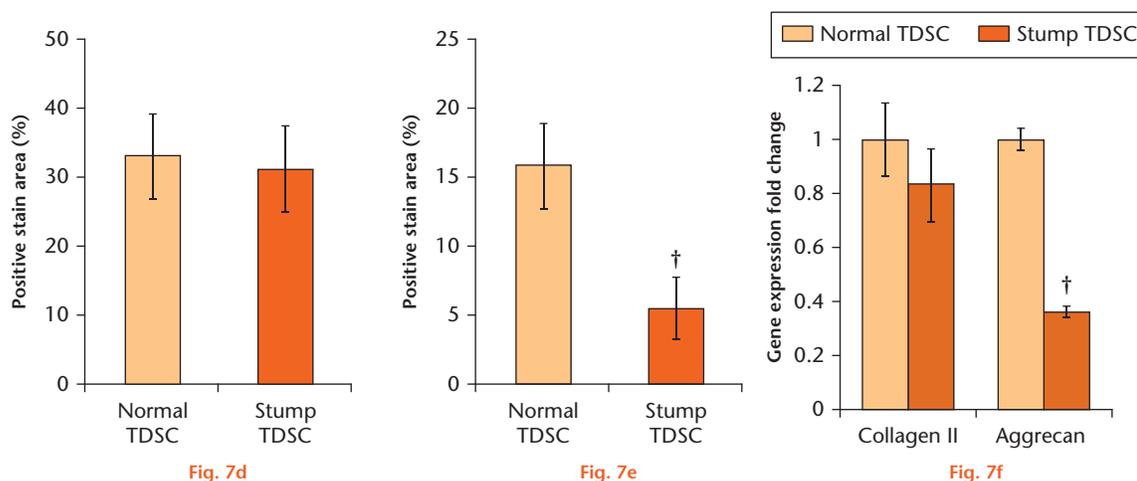
Fig. 7a **Fig. 7b** **Fig. 7c**

Fig. 7d **Fig. 7e** **Fig. 7f**

Multi-differentiation assays in stump and normal tendon-derived stem cells (TDSCs). a) and d) Osteogenic mineralization was seen in both the stump and normal TDSC culture medium after Alizarin Red staining, with no significant difference in positive staining area between stump and normal TDSCs. Data are means and standard deviations (n = 9). b) and e) Analysis of fat droplet formation in normal and stump TDSC culture medium after Oil Red O staining showed a significantly lower positive staining area in stump TDSCs than in normal TDSCs. Data are means and standard deviations (n = 9). The positive staining area of osteogenesis and adipogenesis were randomly measured three areas under 200× magnification from three dishes. c) and f) After induction of chondrogenesis, positive Alcian Blue staining was seen in pellets of normal and stump TDSCs. Gene expression of aggrecan was significantly lower in stump TDSCs than in normal TDSCs, but gene expression of collagen type II was not significant. Data are expressed as means and standard errors of six samples (n = 6) in triplicate and normalized to normal TDSC. †p < 0.01 (unpaired Student's *t*-test). White bars = 50 μm; black bars = 100 μm.

high expression of CD31 and significantly higher CD34, CD44, and CD90 cell markers in TDSCs harvested from ruptured canine FDP tendon. The possible causes for high CD31 and CD34 expression in our cultured cells may relate to different species, different donor tendon area, and cell migration from surrounding tissue or the ruptured stump in the repair phase. Matsumoto et al[12] found more CD34+ stem cells at the human ACL rupture site and concluded that these vascular-derived stem cells may contribute to ligament regeneration. Mifune et al[13] revealed that intra-articular injection of the human

ACL-derived CD34⁺ cells increased the tendon-bone interface healing strength through enhancement of neovascularization and osteointegration in a rat ACL reconstruction model. In an ACL partial tear animal model, a combination injection of mesenchymal stem cells and haematogenic stem cells was effective in the acceleration of ACL healing.[37] Interestingly, we found increased cells expressing progenitor/stem cell markers CD44 and CD90 in the stump TDSCs. These cells may serve as an important population of undifferentiated progenitor cells that can differentiate into tenocytes. Hence, our findings
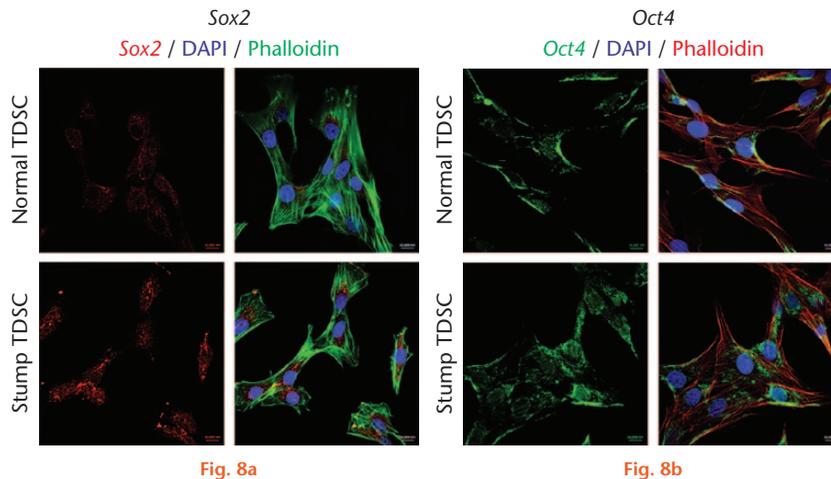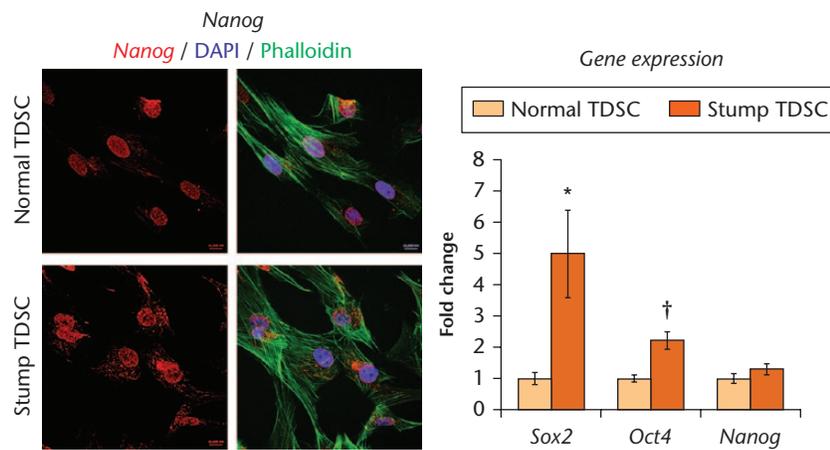
Fig. 8a

Fig. 8b

Fig. 8c

Fig. 8d

Representative images of immunofluorescent staining and gene expression of stem cell markers in stump and normal tendon-derived stem cells (TDSCs): a) SRY-box 2 (Sox2); b) octamer-binding transcription factor 4 (Oct4); and c) homeobox protein Nanog (Nanog). d) The result showed that Sox2 and Oct4 gene expression, but not Nanog, was significantly higher in stump TDSCs than in normal TDSCs. The TDSCs were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue nucleus) and phalloidin cytoskeleton staining. The polymerase chain reaction data are expressed as means and standard errors of six samples (n = 6) in triplicate and normalized to normal TDSC. *p < 0.05; †p < 0.01 (unpaired Student's t-test). Bars = 10000 nm.

indicated that both the mesenchymal stem cells and haematopoietic cells in our stump tendon tissue preserved the stemness and tenogenesis capabilities that would stimulate revascularization and healing in the implanted graft.

The healing stages after tendon injury include inflammatory, reparative, and remodelling stages.[7-9,38] In the early healing stage, inflammatory cells migrate into the injury site with increasing vasoactive and chemotactic factors to enhance angiogenesis and tenocyte proliferation. A few days to weeks after tendon repair, tenocytes migrate and synthesize type III collagen. The remodelling phase occurs within a few weeks of injury and lasts for months or even a year. During this phase, cellularity and collagen synthesis decrease with a decline in tenocyte metabolism. The expression of MMP9 and MMP13 increases within two weeks; MMP2 and MMP3 increase until four weeks after tendon repair.[39] In contrast with normal tendon healing, the six-week failed-tendon stump

tissue and cells after repair failure still exhibited hypercellularity, increased collagen synthesis, and MMP expression, which indicated that the stump tissue and cells were in an early repair stage. The results of MMP expression in stump TDSCs was variable, showing higher MMP2 but lower MMP3 and MMP13. We speculate that the cells from disused stump tendon expressed in tendon remodelling have a different profile to that of normal tendon tissue. In this work, there is no evidence that the remodelling phase has taken place six weeks after injury in the failed repaired tendon stump, which may be related to the disuse that either delays or prevents tendon remodelling. In the stump tissue, Scx gene expression is lower than in normal tendon tissue. The differences in Scx expression may reflect the stage of tendon repair healing, and the surrounding vascular and inflammatory cells infiltrated into the stump tissue. Scx is largely seen in earlier developmental programming but does not appear to be associated strongly with repair.[40] After TDSC isolation
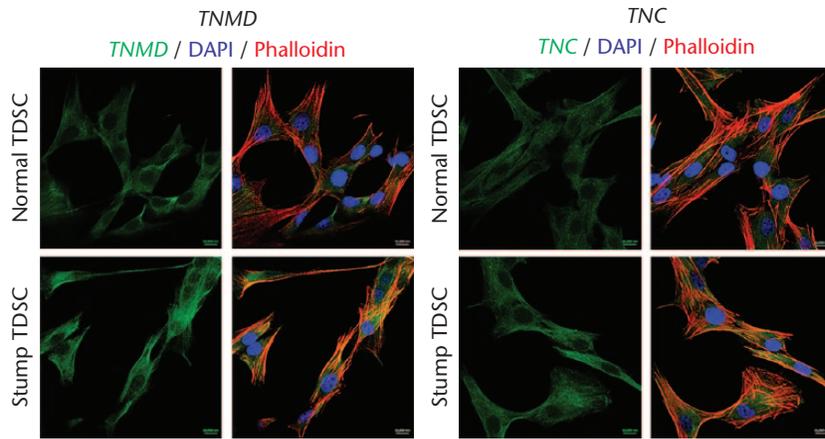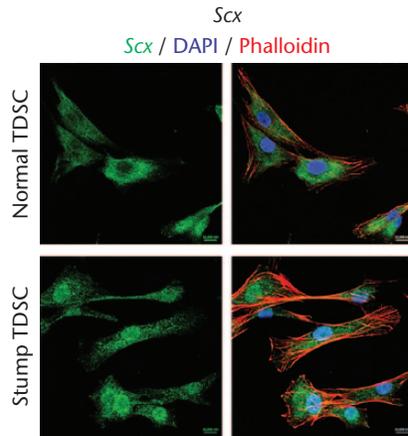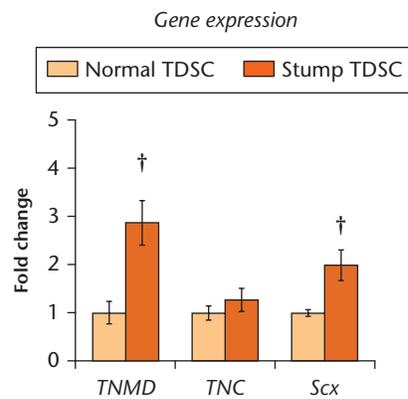
Fig. 9a

Fig. 9b

Fig. 9c

Fig. 9d

Representative images of immunofluorescent staining and gene expression of tenogenic markers in stump and normal tendon-derived stem cells (TDSCs): a) tenomodulin (TNMD); b) scleraxis (Scx); and c) tenascin C (TNC). d) The result revealed that TNMD and Scx gene expression, but not TNC, was higher in stump TDSCs than in normal TDSCs. The polymerase chain reaction data are expressed as means and standard errors of six samples (n = 6) in triplicate and normalized to normal TDSC. *p < 0.05; †p < 0.01 (unpaired Student's *t*-test). Bars = 10 000 nm.
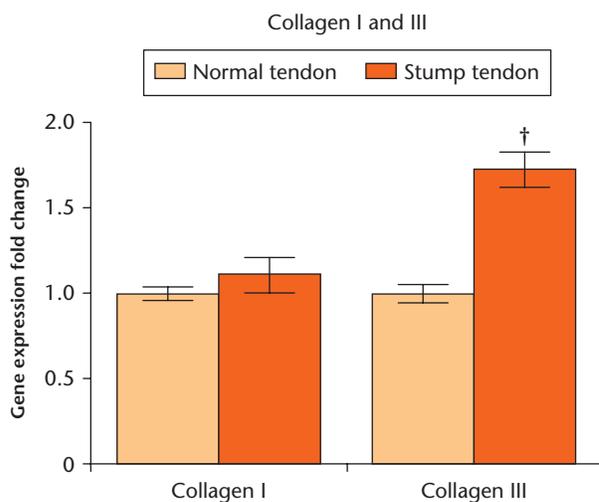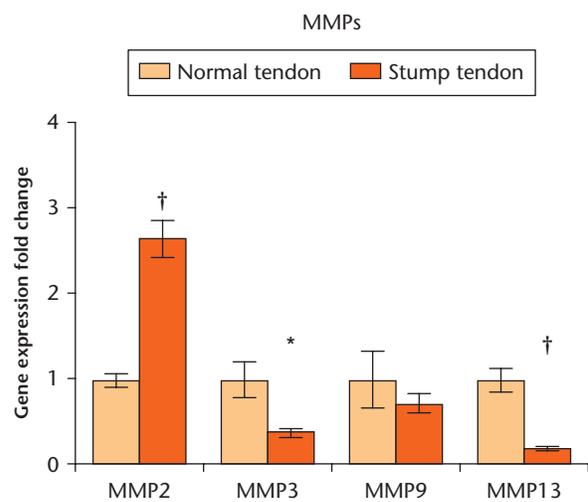

Fig. 10a

Fig. 10b

Gene expression of collagens I and III and matrix metalloproteinases (MMPs) in stump and normal tendon-derived stem cells (TDSCs). a) The stump TDSCs had significantly higher collagen III gene expression than normal TDSCs, but not collagen I. b) Stump TDSCs had significantly higher MMP2 gene expression than normal TDSCs but significantly lower MMP3 and MMP13 gene expression than normal TDSCs. The polymerase chain reaction data are expressed as means and standard errors of six samples (n = 6) in triplicate and normalized to normal TDSC. *p < 0.05; †p < 0.01 (unpaired Student's *t*-test).

and culture from the stump tissue, the stump TDSCs showed significantly higher Scx gene expression than the normal TDSCs, indicating that there were progenitor cells in the tendon stump that might have a potential for tenogenesis.

Histologically, we observed hypercellularity at the tendon stump six weeks after injury. From the cell culture study, we found that the normal TDSCs continued to proliferate compared with limited proliferation in the stump TDSCs, with significant difference after 72 hours of culture (Fig. 1). Although the colony-forming unit decreased in the stump TDSCs compared with the normal TDSCs without significant difference, the cell density in colonies was significantly lower in the stump TDSCs (Fig. 6). These findings indicated that the cell proliferation capability was reduced due to decreased cellular activities and productivities of the disused tendon after repair failure. However, the actual mechanism is unknown and needs to be investigated.

Clinically, a ruptured tendon can be directly repaired a few weeks after injury if the tendon stumps can be connected for healing. In general, the injured and disused tendon stump loses the elasticity with hypercellular (fibrotic) characters after repair failure.[10] We studied the stump tissue six weeks after repair failure and confirmed the tendon regeneration capability in these stump tissues. From the results of our study, failed repair tendon could be re-repaired (revision) at least six weeks after repair failure as the disused tendon still has regenerative potential if the tendon stumps can be stretched. However, the clinical problem is that six weeks after repair rupture, it will be very difficult to reconnect the tendon stumps due to tendon/muscle contracture. In such cases, a tendon graft will be indicated. The important clinical relevance is that, when the tendon graft is considered, the surgeons should keep in mind that the failed tendon stumps do have regenerative potential. Therefore, limited debridement of the residue stump would be recommended during either tendon repair or reconstruction in clinical practice.

This study provided us with evidence of the regeneration capability of stump tendon tissue and cells, and the fundamental knowledge to develop the methods of using the stump tissue and cells to enhance repaired tendon regeneration. There are several limitations to our study. First, the results are based on a canine model, in which the tendons may not have the same characteristics as flexor tendons in humans. Second, we do not know the exact mechanism for the lower proliferation rate in stump TDSCs. Third, the study tissue and cells were harvested six weeks after the initial repair; the associated changes in cell characteristics at earlier timepoints are unknown. Finally, the cell viability at the injury site was not assessed. These limitations need to be studied in the future before clinical application of stump tissue and cells for tendon regeneration.

In conclusion, the failed-repair stump tissue increased cellularity with significantly higher gene expression of growth factors compared with the normal tendon tissue, which preserved the tissue viability with healing potential. The TDSCs from the ruptured FDP tendon stumps were identified and characterized with higher stem cell and tenogenic markers, indicating that they have the ability to move further down the tendon lineage and enhance tendon healing. Therefore, the tendon stumps could be used as a recycling biomaterial for tendon regeneration in secondary grafting surgery.

Supplementary Material

ë   A table showing CD markers antibodies used in this study, as well as a table showing antibodies used for immunofluorescent staining in this study.

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