

# Healing and Normal Fibroblasts Exhibit Differential Proliferation, Collagen Production, $\alpha$ -SMA Expression, and Contraction

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**Abstract**—This study determines the differences in proliferation, collagen production,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) expression, and contraction between healing and normal fibroblasts. Transected and sham-operated rat medial collateral ligaments (MCL) were used to obtain healing and normal fibroblasts, respectively. It was found that healing fibroblasts in monolayer culture proliferated 1.4-fold faster at 48 h and had 1.7-fold greater protein expression of  $\alpha$ -SMA than normal fibroblasts. In addition, it was noted that the proliferation of healing fibroblasts in collagen gels was not significantly different from that of normal fibroblasts at 24 h, but it was at 48 h. Furthermore, in collagen gels, healing fibroblasts produced more type I collagen than normal fibroblasts and generated 1.6- and 1.7-fold larger contractile forces at 15 and 20 h, respectively, than their normal counterparts. Taken together, the results of this study show that healing fibroblasts possess a differential proliferation,  $\alpha$ -SMA protein expression, and contraction than normal fibroblasts.

**Keywords**—Fibroblast contraction,  $\alpha$ -Smooth muscle actin, Wound healing.

## INTRODUCTION

When injured, tissues usually heal with increased migration and proliferation of cells (e.g., fibroblasts) as well as formation and contraction of granulation tissue, where contraction serves to both reduce the size of the wound and promote tissue continuity.<sup>13</sup> In addition, healing tissue is characterized by the excessive production of collagen in the wound site.<sup>13,18,22,28</sup> Among those healing cells responsible for granulation tissue contraction and excessive collagen production are myofibroblasts, which are specialized fibroblasts that are transiently involved in the wound healing process.<sup>14,15,25</sup> These cells exert contractile forces to the surrounding extracellular matrix and deposit collagen at the wound site.<sup>13,25,29</sup> While these fibroblasts in the healing process repair and remodel the wounded tissue, those from normal tissue simply maintain the ma-

trix around them. Therefore, healing and normal fibroblasts may exhibit differences in phenotypic expression, such as cell proliferation, collagen synthesis, and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), which is a specific marker of myofibroblasts.<sup>4,5,13,16,17</sup> Identifying the differences between healing and normal fibroblasts responsible for these different functions can provide insight into the cellular and molecular mechanisms of tissue wound healing, and therefore aid in devising new therapeutic strategies to enhance the quality of healing tissues.

Healing fibroblasts likely proliferate faster than normal fibroblasts, because the fibroblasts at the injured area are lost or damaged due to injury<sup>27</sup>; therefore, larger numbers of these cells are needed to repair and remodel the wounded matrix. A previous study showed that fibroblasts derived from human periodontal ligaments from molar extractions were found to proliferate faster than those from an associated uninjured periodontal ligament following treatment with growth factors.<sup>20</sup> Also, while some degree of fibroblast contraction is necessary for wound closure, excessive fibroblast contraction can lead to the formation of scar tissue.<sup>6,26</sup> It has been suggested that during this period of excessive contraction, fibroblasts increase the expression of  $\alpha$ -SMA.<sup>1,17</sup> Furthermore, healing fibroblasts at the wound site need to rebuild the matrix and likely produce more collagen,<sup>7,12,22</sup> the major component of extracellular matrix in ligaments, than normal fibroblasts.

Therefore, the purpose of this study was to test the following hypotheses: (1) healing fibroblasts proliferate at a faster rate than normal fibroblasts; (2) healing fibroblasts produce a higher level of collagen than normal fibroblasts; (3) healing fibroblasts express a higher level of  $\alpha$ -SMA than their normal counterparts; and (4) healing fibroblasts exert greater contraction forces than normal fibroblasts. To test these hypotheses, the medial collateral ligament (MCL) of the rat was used as a model tissue to isolate healing and normal fibroblasts for cell culture experiments. The results showed that compared with normal fibroblasts, healing fibroblasts proliferated faster, produced more collagen,

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expressed a higher level of  $\alpha$ -SMA, and generated larger contraction forces.

## MATERIALS AND METHODS

### *Cell Culture*

In the right knee of eight rats, a 2-mm gap injury was created in the MCL (IACUC #0210947). In the contralateral leg, a sham surgery was performed, which involved creating a skin incision, gently elevating the MCL, and finally closing the skin incision with sutures. The sham surgery allowed us to take into account any differences that may have resulted from the surgery instead of the injury itself. After 10 days, the rats were sacrificed, and "healing MCLs" from the right knees of the rats and "normal MCLs" from the left knees of the rats were harvested. The granulation tissue of the injured MCLs was clearly marked by differing matrix composition, as noted by a pinkish color and increased thickness. Only this area of the injured MCL and the 2-mm midsection of the sham-operated MCL were excised for culture experiments. Ten days was chosen because the healing tissue is in the proliferation phase.<sup>8</sup> Each MCL was washed twice with PBS, cut into small pieces in aseptic conditions, and then placed in a small Petri dish with 2 ml of Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin (P/S; Invitrogen). Cells from the MCL tissues grew out onto the Petri dishes and were then subcultured. The cells were grown as monolayer cultures in DMEM supplemented with 10% FBS and 1% P/S on plastic tissue-culture dishes in an atmosphere of 5% CO<sub>2</sub> and 100% humidity at 37°C. Note that cells from injured MCLs were considered as "healing" fibroblasts, whereas those from sham-operated MCLs were referred to as "normal" fibroblasts. Also, note that healing and normal fibroblasts of the same passage (less than five) and from the same rat were compared for each individual experiment in this study.

### *Fibroblast Proliferation Experiments*

Healing and normal fibroblasts were seeded into six-well plates at a density of  $3 \times 10^4$  cells per well for experiments done on cells plated in tissue-culture dishes. For experiments done on collagen gels, cells were seeded in a collagen gel solution [1:1:8 of 0.1 M NaOH;  $10 \times$  PBS; collagen type I (3.0 mg/ml; Cohesion Technologies Inc.)] at a ratio of  $3.75 \times 10^5$  cells in 0.75 ml of medium to 1.25 ml of collagen solution in wells of a six-well plate. The density of cells in collagen gels was the same that was used for the contraction experiments. The cells were grown in DMEM containing 10% FBS and 1% P/S for 24 and 48 h. Cell numbers were then measured by a standard MTT assay. This assay determines the degree of activation of cells by measuring the reduction of tetrazolium salt, the level of which is proportional to cell number.<sup>2,24,32</sup> Briefly, 200  $\mu$ l of MTT

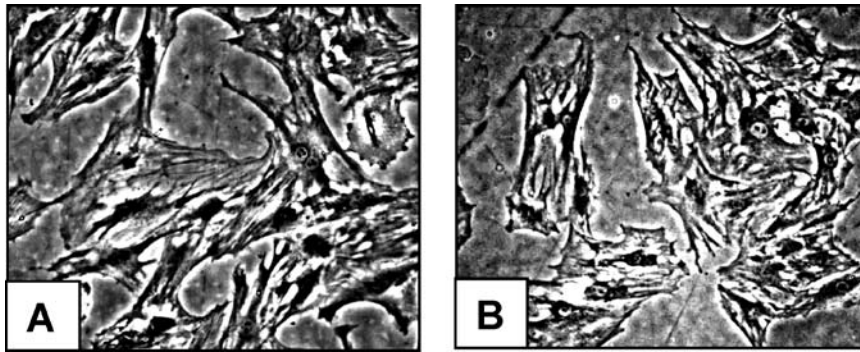
[tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] solution (5 mg/ml) was added to the appropriate wells. After 3 h of incubation, the wells were centrifuged for 5 min at 1900 rpm. The supernatant was extracted, and the insoluble product was dissolved with the addition of 1 ml extraction buffer, followed by incubation at 37°C overnight. The following day, 200  $\mu$ l samples of medium were removed from each well and pipetted into a 96-well plate in duplicate. Absorbance values for each sample were measured at 550 nm using a Spectra MAX 190 microplate spectrophotometer (Molecular Devices).

### *Assaying Collagen Production*

In contraction experiments, ascorbic acid was added to the medium to promote collagen production. Samples of medium were collected during the contraction experiments at 20 h to measure levels of collagen secretion in medium using the Procollagen Type I C-Peptide EIA Kit (Takara, Japan), which assesses collagen production by a standard sandwich ELISA method for procollagen type I carboxy-terminal peptide (PIP). The detection of collagen secretion using this method was first reported in 1974.<sup>30</sup> Briefly, in a 96-well plate that was precoated with a monoclonal antibody, an antibody-peroxidase conjugate solution, which is a secondary monoclonal antibody, was added to the appropriate wells. Either standard or sample was added to each well containing conjugate solution within 5 min. After 3 h of incubation at 37°C, the sample solution was removed, and the wells were washed four times with PBS. Next, a substrate solution was added to each well, and the plate was incubated at room temperature. After 15 min, a stop solution was mixed into each well. The color intensity of the solution in each well was proportional to the amount of PIP in the solution. The absorbance of the samples was read on a plate reader at 450 nm.

### *Measuring $\alpha$ -SMA Levels*

The protein levels of  $\alpha$ -SMA by healing and normal fibroblasts were measured using a standard Western blot technique. Cellular proteins were prepared by extracting cells from confluent plastic tissue-culture dishes with a mammalian protein extraction reagent (Pierce) containing antipain, leupeptin, chymostatin, and pepstatin for proteinase inhibition. Total protein concentration was determined using a BCA protein assay (Pierce). Equal amounts of total protein (5  $\mu$ g) from healing and normal fibroblasts were loaded on a 10% Tris-HCl gel for electrophoresis, and the separated proteins on the gel were then transferred to a nitrocellulose membrane. After blocking the membrane for 2 h in a 5% fat-free milk/PBS-Tween-20 solution,  $\alpha$ -SMA was probed with an anti-actin  $\alpha$ -smooth muscle monoclonal antibody (Sigma Aldrich). The membrane was rinsed three times for 15 min each with PBS-Tween and then incubated with a secondary antibody, a peroxidase conjugated goat



**FIGURE 1.** The morphology of healing and normal rat MCL fibroblasts. (A) Healing fibroblasts and (B) normal fibroblasts. It is apparent that the morphology of the healing and normal fibroblasts remain similar even after several passages.

antimouse secondary antibody (Jackson ImmunoResearch Laboratories Inc.). The membrane was developed using an ECL detection kit (Amersham Biosciences UK Limited). Finally, X-OMAT Kodak films were exposed to the blots, and the density of the bands was estimated using Quantity One software (BioRad Laboratories).

#### *Fibroblast Contraction Experiments*

Contraction was measured using a multistation culture force monitor (CFM) system that was developed previously in our laboratory.<sup>9</sup> Briefly, the collagen solution was prepared by combining 0.1 M NaOH, 10 × PBS, and collagen type I (3.0 mg/ml; Cohesion Technologies Inc.) in a ratio of 1:1:8. Cells were grown on plastic tissue-culture dishes until about 90% confluence. The MCL fibroblast populated collagen gels (FPCGs) were prepared by adding  $1.5 \times 10^6$  cells in 3 ml of medium to 5 ml of the collagen solution (final collagen concentration = 1.5 mg/ml) in a silicone dish of dimensions 9 cm × 3 cm × 1 cm, with a porous vyon bar at each end. The vyon bar at one end was attached to a cantilever beam, while the vyon bar at the other end was fixed to the silicone dish. Following 10 min of incubation, 7 ml of medium was added to each FPCG, which was then attached to the CFM system. As the collagen gel contracted, the mobile vyon bar deflected the cantilever beam and a Labview program recorded a corresponding change in voltage. This change in voltage corresponded to a force measurement through a previous calibration. Data was collected for 10 s every 10 min at 10 Hz for a period of up to 24 h. The 100 data points collected at each time point were then averaged to yield a representative force measurement at that time point.

#### *Statistics*

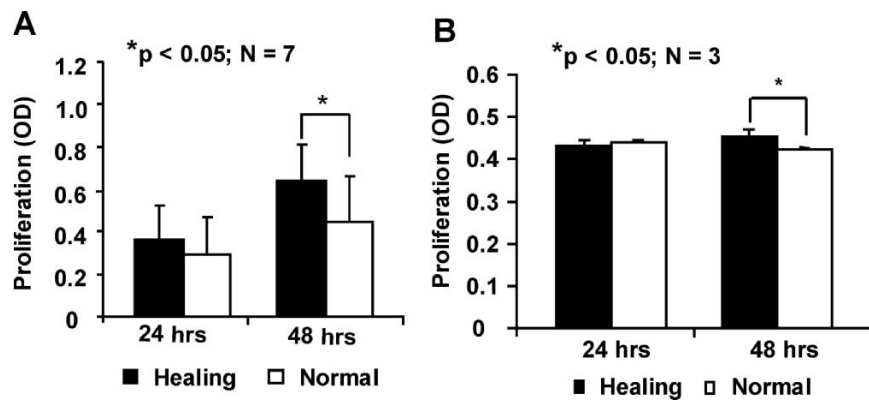
A student's *t*-test, unpaired or paired wherever appropriate, was used for statistical analysis of differences in cell proliferation, collagen production,  $\alpha$ -SMA expression, and contraction forces between healing and normal fibroblasts.

The significance level was set at 0.05. Note that the error bars in the figures are standard deviations.

## RESULTS

Healing and normal MCL fibroblasts were grown in culture, and they did not show an apparent difference in morphology (Fig. 1). However, healing and normal fibroblasts in tissue-culture dishes proliferated at a different rate. Although at 24 h, healing fibroblasts appeared to proliferate faster than normal fibroblasts, a significant difference in proliferation rate was found only at 48 h, when the proliferation of healing fibroblasts was 1.4 times faster than that of normal fibroblasts [Fig. 2(A)] ( $p < 0.05$ ). Similarly, when fibroblasts were seeded in collagen gels, proliferation of healing fibroblasts was not significantly faster than normal fibroblasts at 24 h [Fig. 2(B)], but only at 48 h ( $p < 0.05$ ). Furthermore, healing fibroblasts produced an average of 10% more collagen in medium than normal fibroblasts (Fig. 3) ( $p < 0.05$ ).

In addition to faster proliferation and increased collagen production, immunofluorescence results (not shown) revealed that healing fibroblasts expressed greater  $\alpha$ -SMA throughout the entire population compared to normal fibroblasts. The results were confirmed by Western blotting [Fig. 4(A)]. Quantitatively, on average, healing fibroblasts expressed 1.7-fold more  $\alpha$ -SMA protein compared to normal fibroblasts [Fig. 4(B)] ( $p < 0.05$ ). Furthermore, it was found that healing MCL fibroblasts produced much larger contractile forces than normal MCL fibroblasts. Specifically, at 24 h, the maximum contraction of healing fibroblasts was  $\sim 160$  dyne, whereas it was  $\sim 78$  dyne for normal fibroblasts [Fig. 5(A)]. The difference between the healing and normal fibroblast contraction was not due to the possible difference in cell numbers, because the difference between the healing and normal fibroblast contraction was seen at less than 5 h, and the proliferation of healing fibroblasts in collagen gels was also not significantly different from that of normal fibroblasts at 24 h.

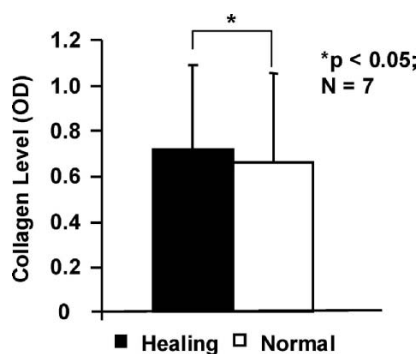


**FIGURE 2.** The proliferation of healing and normal rat MCL fibroblasts. At 24 h, cell numbers either in tissue-culture dishes (A) or collagen gels (B) were not significantly different; however, at 48 h, the number of healing fibroblasts was significantly greater than that of normal fibroblasts. Note, however, that the fibroblast proliferation rate in collagen gels (B) was much slower than that in tissue-culture dishes (A).

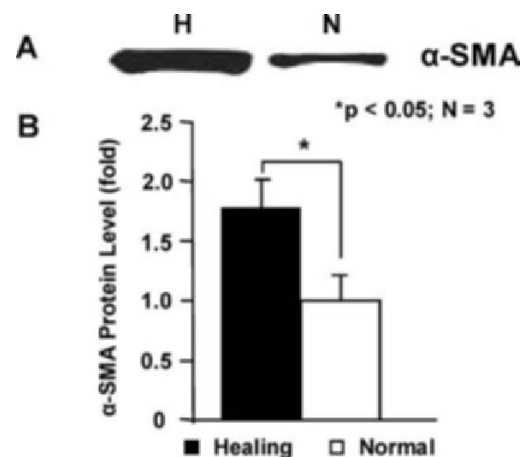
Overall, the contraction forces changed with time. Initially, there was only a slightly larger contraction of healing fibroblasts compared to normal fibroblasts, as seen at 10 h. However, as the cells continued to contract the collagen gel, healing fibroblasts showed 1.6 and 1.7 times greater contraction forces than their normal counterparts at 15 and 20 h, respectively [Fig. 5(B)] ( $p < 0.05$ ). On average at 20 h, the healing fibroblasts produced 1.2 nN/cell of contractile force and the normal fibroblasts produced 0.73 nN/cell of contractile force. The trend of larger contraction forces produced by healing fibroblasts compared to normal fibroblasts was consistently seen in four separate experiments.

## DISCUSSION

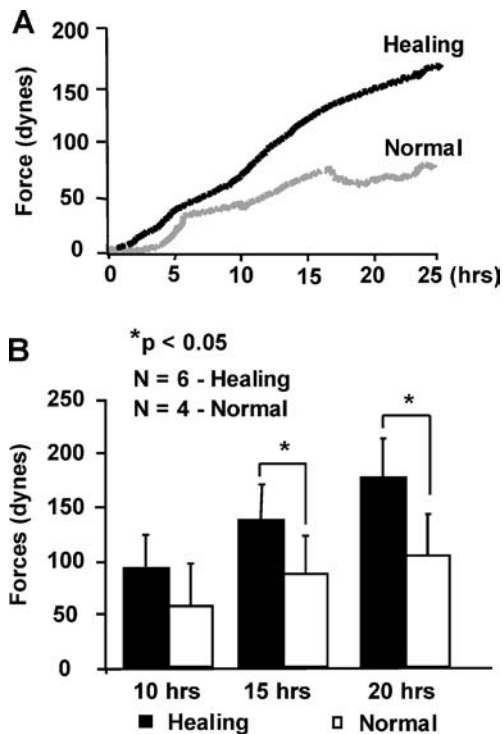
This study showed that in monolayer culture, healing fibroblasts proliferated faster than normal fibroblasts at 48 h, which is similar to results found from periodontal ligaments.<sup>20</sup> Also, compared to normal fibroblasts, healing fibroblasts produced a higher level of type I collagen in medium, expressed a greater level of  $\alpha$ -SMA, and generated larger contraction forces. Intuitively, the results appear reasonable and are consistent with previous studies, because healing and normal fibroblasts have a different environment *in vivo*, and therefore they should function differently.<sup>22</sup> Previous studies in ligaments have found collagen type I



**FIGURE 3.** Collagen production by healing rat MCL fibroblasts was higher than that of normal fibroblasts. Note that a total of five separate experiments were performed to measure collagen levels in cell medium at 20 h. Also, the values are group means  $\pm$  SD and are shown for illustrative purposes only; statistical analysis was performed on the differences in collagen values between healing and normal fibroblasts.



**Fig. 4.** Healing and normal fibroblasts display differential protein expression of  $\alpha$ -SMA. (A) A representative Western blot result of  $\alpha$ -SMA protein expression. (B) Quantitative analysis of  $\alpha$ -SMA protein levels showed that healing fibroblasts displayed significantly greater protein expression than normal fibroblasts. Note that H and N represent healing and normal fibroblasts, respectively.



**Fig. 5.** Healing rat MCL fibroblasts exhibited differential contraction compared to their normal counterparts. (A) A typical contraction curve of healing and normal fibroblasts. (B) The difference in contraction forces between healing and normal fibroblasts increased with culture time. Differences in contraction between healing and normal fibroblasts were seen at less than 5 h.

expression to differ between healing and normal ligaments on an mRNA level, as well.<sup>7</sup> In addition,  $\alpha$ -SMA protein expression has been shown to be upregulated in a healing environment.<sup>10</sup> Further, this study was able to quantify contraction forces between these healing and normal fibroblasts. Fibroblasts in healing ligaments need to repair the injured matrix around them, whereas those in normal ligaments maintain the existing matrix. Therefore, higher levels of proliferation rate, collagen synthesis,  $\alpha$ -SMA protein expression, and contraction of healing fibroblasts accelerate the wound repairing process.

This study showed that healing fibroblasts expressed a higher protein expression level of  $\alpha$ -SMA, which is a specific marker of myofibroblasts.<sup>13,17</sup> Since equal amounts of total protein were loaded onto gels for  $\alpha$ -SMA protein expression analysis, cell number was not a factor responsible for the difference between healing and normal fibroblasts in  $\alpha$ -SMA protein expression. Therefore, expressing significantly more  $\alpha$ -SMA protein, healing fibroblasts function more like myofibroblasts, which are known to produce greater contractile forces in healing tissue.<sup>13</sup> Myofibroblasts are initially observed in the wound tissue on the fourth to sixth day, and they remain ac-

tive until the second to third week.<sup>19</sup> The higher levels of  $\alpha$ -SMA protein expression may be responsible for the larger contraction seen in this study, because increasing  $\alpha$ -SMA protein expression increases cell contraction.<sup>17</sup> Previous studies have shown granulation tissue to have a combination of residential and migrating fibroblasts and these migrating fibroblasts become differentiated into myofibroblasts.<sup>33</sup> Future studies should look into other markers of these specialized cells, such as ED-A fibronectin.<sup>31</sup>

The large contractile forces that are generated by healing fibroblasts may also be responsible for higher levels of collagen production by these cells, since contracting fibroblasts in anchored collagen gels increased collagen production compared to relaxed fibroblasts in free-floating gels.<sup>23</sup> Other studies have also shown that higher contraction forces correlate to increased collagen synthesis.<sup>21,23</sup> Therefore, the result that healing fibroblasts produced larger contraction forces than normal fibroblasts may explain why these cells also produced higher levels of collagen than normal fibroblasts. Although previous studies have shown that collagen type III is increased in scar tissue,<sup>3</sup> this study only measured the amounts of collagen type I secreted in media. Future studies will explore possible differences in the expression of other proteins, such as the production of collagen type III, as well as proteoglycans, between healing and normal fibroblasts.

This study revealed not only differences between healing and normal MCL fibroblasts on a protein level, but also on a functional level, that is, the contractile forces generated by healing and normal fibroblasts. It is interesting to note that the shape of the curve produced by the healing fibroblasts is slightly different than that produced from the normal fibroblasts, in that the contraction from the normal fibroblasts seems to plateau between 15 and 20 h, whereas the curve from the healing fibroblasts continues to increase. This may be due to the differing inherent contractile abilities for the two groups of cells. While healing fibroblasts work at closing the wound and regenerating the matrix for some time following injury, the normal fibroblasts do not need such a great contractile ability to maintain the relatively quiescent tissue. It is also important to note that the increase in contraction forces produced by the healing fibroblasts seen in this study was not due to an increase in cell number. This is because differences between healing and normal fibroblasts in contraction forces were consistently seen in all cell contraction experiments at less than 5 h, and cell numbers were not significantly different until 48 h. Excessive fibroblast contraction contributes to scar formation in injured tissues, such as wounded ligaments,<sup>26</sup> and results in inferior structural and mechanical properties of the healing tissue. Therefore, it is important to find ways to reduce the excessive contraction by healing fibroblasts, which may in turn decrease excessive collagen production by these cells. Downregulation of  $\alpha$ -SMA protein expression in healing

fibroblasts using RNAi technology<sup>11</sup> may be an effective approach to reduce fibroblast contraction and minimize impairments to tissue function due to scar formation.

This study also shows that even after passages up to five times, fibroblasts derived from healing MCLs are still markedly different from normal fibroblasts. Since passaging cells likely causes cell de-differentiation, the level of differential protein expression/secretion, proliferation rates, and contraction between healing fibroblasts and normal fibroblasts observed in this study could be even larger *in vivo*. Also, because of the differential “phenotypic expression” between healing and normal fibroblasts, the results of this study emphasize the need to use healing fibroblasts in the study of cellular and molecular mechanisms of tissue wound healing as well as in tissue engineering of injured ligaments.

Note that this study used a sham-operated ligament as a control. The reason for this was to account for the effects of the surgery that was performed on MCLs. Therefore, the differences that were seen between the healing and normal fibroblasts were due to the injury itself and not the surgery. It is also important to note a few limitations of this study. First, this study used fibroblasts from only one time point of the wound healing process, that is, 10 days post-injury. Future studies should investigate kinetic changes at different healing time points in the gene and protein expression of healing fibroblasts compared to normal fibroblasts. Second, this study did not investigate the effect of different passages on  $\alpha$ -SMA protein expression and contraction of fibroblasts. It should be interesting to see how  $\alpha$ -SMA protein expression and fibroblast contraction change with cell passages in future studies. Future studies should also look into the possible differences between healing and normal fibroblasts in response to growth factors (e.g., TGF- $\beta$ 1 and PDGF) and cytokines (e.g., IL-1 and TNF), all of which affect tissue wound healing. Finally, the effects of external mechanical loading, a factor affecting healing and normal ligaments *in vivo*, on healing and normal fibroblasts should be investigated in future studies.

In summary, this study shows that healing and normal fibroblasts from MCLs exhibit differential expressions in their proliferation, collagen production,  $\alpha$ -SMA protein expression, and contraction. Future studies should study the possible differential responses of healing fibroblasts and normal fibroblasts to growth factors/cytokines under mechanical loading conditions.

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