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## Myofibroblasts Contribute to but are not Necessary for Wound Contraction

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### Introduction

Wound contraction facilitates tissue repair. The correct balance between too little contraction, which leads to non-healing wounds, and too much contraction, which leads to contractures, is important for optimal healing. Thus, understanding which cells cause wound contraction is necessary to optimize repair. Modified fibroblasts with smooth muscle (SM)-like features, including the expression of alpha SM actin (ACTA2), were first observed by Gabbiani et. al. in granulation tissue of healing wounds. This led him to suggest that myofibroblasts promote wound contraction and collagen production.<sup>1, 2</sup> Subsequent *in vitro* studies by Hinz et. al. demonstrated that myofibroblasts generate increased contractile force, as compared to fibroblasts, and that the mechanism is related to ACTA2 incorporation into stress fibers and increased focal adhesion size.<sup>3-6</sup> ACTA2 levels were found to associate with a fibroblasts ability to wrinkle a deformable substrate and enhance collagen gel contraction.<sup>7</sup>

The compelling hypothesis that myofibroblasts are essential for wound contraction is balanced by a counter hypothesis that myofibroblasts are not essential for wound contraction. Ehrlich et. al. found that human sacrococcygeal pilonidal sinus wounds contract without a high density of myofibroblasts being present, ACTA2 is absent in free floating

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collagen lattice contraction, full thickness excisional wounds in rats are capable of unwounded wound contraction in absence of myofibroblasts, and prevention of ACTA2 expression in rodents treated with vanadate does not alter wound contraction.<sup>8-12</sup> In 2002, Wrobel et al demonstrated that fibroblasts and myofibroblasts produce similar contractile forces.<sup>13</sup>

To determine the role of ACTA2 in wound contraction, we utilized human scar tissue, *Acta2* null (*Acta2*<sup>-/-</sup>) mice and murine open wound contraction model.<sup>14</sup> Human tissue was immunostained for ACTA2, beta cytoplasmic actin (ACTB), and gamma cytoplasmic actin (ACTG1). Human scar tissue showed increased expression of ACTA2, ACTB, and ACTG1. ACTA2 was focally expressed in clusters. ACTB and ACTG1 were widely, highly expressed throughout scar tissue. Wound contraction was significantly retarded in *Acta2*<sup>-/-</sup> mice, as compared to *Acta2*<sup>+/+</sup> controls. Control mice had increased epithelialization, cell proliferation, and neovascularization. *Acta2*<sup>-/-</sup> mice had lower levels of apoptosis, and fewer total numbers of cells. The smaller amount of collagen deposition and immature collagen organization in *Acta2*<sup>-/-</sup> mice demonstrate that the wounds were more immature. We conclude that the expression of ACTA2 is beneficial but not essential for wound contraction.

## Methods and Materials

### Human tissue

A total of 18 human scar samples with surrounding unwounded tissue were obtained following approved IRB protocols: 12 samples were obtained from First Affiliated Hospital of Sun Yat-sen University in accordance with IRB protocol, and 6 samples were obtained from Duke University Medical Center (DUMC) Department of Pathology. Samples were collected within 1 year of cicatrization. All the included samples exhibited obvious contracture (Supplementary figure 1). Samples were categorized according to patients' race, gender, age, and scar location (Table 1).

### Animals

Male and female *Acta2*<sup>+/+</sup>, *Acta2*<sup>+/-</sup>, and *Acta2*<sup>-/-</sup> mice (10–12 weeks old and weighing from 18–23g) were used in this study. The *Acta2*<sup>-/-</sup> mice used in this study were established by inserting the Pol2NeobpA cassette<sup>15</sup> into the +1 start site of the *Acta2* gene<sup>16</sup>. *Acta2*<sup>-/-</sup>, *Acta2*<sup>+/+</sup> and *Acta2*<sup>+/-</sup> mice from the same breeding colony were obtained from Warren E. Zimmer, Ph.D. at Texas A&M Health Science Center. All mice were monitored for signs of toxicity. The mice were housed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Duke University.

### Excisional Wounds and Gross Examination

All procedures were performed in accordance with a protocol approved by Duke University IACUC. Mice were anesthetized using gas anesthesia (oxygen, 2L/min, isoflurane, 2%). The back of was shaved with metallic clippers. The back was then sterilized using alcohol. Full-thickness excisional wounds were created using an 8mm biopsy punches (Miltex, York, Gibbstown, PA, USA) in the area between mice's scapular angles. The wounds were then covered by Tegaderm (Transparent Film Dressing Frame Style, 3M Health Care, St. Paul,

MN, USA). Dressings were changed daily for the first 4 days and then removed. Wounds were measured and photographed daily using Canon PowerShot A470 digital camera. Wound contraction was measured by gravitational planimetry and expressed as percentage of original wound size.

### Tissue collection

Mice were euthanized and tissue was collected. Collected tissue was cut into equal halves. One half was preserved in 10% formalin for histological analyses and the other half was immediately frozen in liquid nitrogen for additional analyses. Prior to staining, tissue sections were deparaffinized by warming at 65°C overnight, immersing in xylene for 15min, rehydrating with decreasing concentrations of ethanol in distilled water.

### Histological (HIS), Immunohistochemical (IHC) and immunofluorescence (IF) Analysis

For HIS analysis, hematoxylin and eosin (H&E, Sigma-aldrich, St. Louis, MO, USA) staining was performed following standard techniques. Masson's Trichrome staining was performed by use of Trichrome Stains kit (Sigma-aldrich), for collagen evaluation. The collagen index value was calculated as  $\text{collagen index} = (B + G)/(2R + B + G)$  for each pixel within the image (where R, G, and B represent the red, blue, and green pixel values, respectively). The value of the collagen index ranged from 0 for extremely red objects to 1 for completely blue-green objects. The average collagen index of 3 (HPF) images for each time point was graphed.

For IHC analysis, immunostaining was done. Briefly, to inhibit endogenous peroxidase activity, rehydrated sections were immersed in 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 10min to block endogenous peroxidase. Slides were rinsed with deionized water and then placed under retrieval solution (Target Retrieval Solution, Dako North America Inc. Carpinteria, CA, USA) in a water bath (98°C) to unmask antigens. After further tris-buffered saline (TBS, TBS Automation Washing Buffer, Biocare Medical, Concord, CA, USA) rinsing, sections were treated with 10% goat serum (Normal Goat Serum, Vector Laboratories, Burlingame, CA, USA) for 1 hour to block non-specific antibody binding. The primary antibody was incubated overnight at 4°C. The primary antibodies used included (1) for human scar tissue IHC: rabbit anti ACTA2 polyclonal antibody (1:100 dilution, Abcam, Cambridge, MA, USA), mouse anti ACTB monoclonal antibody (1:5000 dilution, Abcam), mouse anti ACTG1 monoclonal antibody (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA); (2) for mice wound tissue IHC: rabbit anti Ki67 monoclonal antibody (1:400 dilution, Thermo Scientific, Rockford, IL, USA), rabbit anti CD31 polyclonal antibody (1:200 dilution, Abcam). After washing with TBS, the slides were incubated with appropriate secondary antibody. The secondary antibodies used were: biotinylated goat anti rabbit IgG (1:200 dilution, Vector Laboratories), goat anti mouse IgG (1:200 dilution, Vector Laboratories) and horse anti mouse IgG (1:200 dilution, Vector Laboratories). The reactions were developed with an avidin-biotin complex reaction (Vector Laboratories).

For IF analysis, Terminal deoxynucleotidyltransferase (TdT) dUTP nick end labeling (TUNEL) staining was performed by using In Situ Cell Death Detection POD Kit (Roche, IN, USA) according to the manufacturer's instructions. 4, 6-diamidino-2-phenylindole

(DAPI) counter stain was performed with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Labeled cells were visualized by use of a Nikon eclipse E600 microscope and images were captured with a Nikon DXM 1200 digital camera under the same setting. Morphometric evaluations were done from sections through the center of wounds in order to obtain the maximum wound area for evaluation. Measurements of epidermal thickness,<sup>17, 18</sup> collagen content,<sup>19</sup> positive immunostaining intensity<sup>20</sup> and counting<sup>21-23</sup> were performed with NIH ImageJ software, all the analyses were run as triplicates. To quantify non-muscle myosin II (isoform a, b, c, and ASMA) level of expression in scar vs. normal tissues by immunochemical stained image analysis, the 24-bit RGB images per region (300ppi) were converted to 8-bit gray value images with pixel intensity values ranging from 0 (black) to 255 (white) using NIH ImageJ software. An 8×8 mm sample region containing both scar and normal tissue was selected for analysis. The sample image was divided into an 8×8 grid (each box = 1mm<sup>2</sup>). A mean pixel intensity value for each box was measured using ImageJ. The middle 4 columns were excluded from analysis since the area contains both scar and normal tissues. As a result, 16 sample pixel intensity values were each obtained for normal and scar tissue. Statistical analysis was performed using JMP software (SAS Institute ver. 7). The means of the two samples were compared via Student's t-test. Differences were considered to be statistically significant at values of  $P < 0.01$ .

### Statistical Analysis

All data are presented as the mean and standard error of the mean of three independent experiments. Statistical differences were determined using Student's *t*-Test or one-way ANOVA with Bonferroni's post-hoc test. The difference was considered significant when the *p*-value was 0.05 or less.

## Results

### Expression of ACTA2, ACTB and ACTG1 in Unwounded Human Skin and Human Contracture Scar

In unwounded skin, expression of ACTA2 was absent in the epidermis and dermal fibroblasts, but expression was detected in SM cells of blood vessels and skin appendages, whereas ACTB and ACTG1 were expressed both in the epidermis and all the dermis including dermal fibroblasts (Figure 1A, upper row) of unwounded skin. In contracted scar, the epidermis was thickened and flattened, and the appendages were missing. ACTA2 expression in scar epidermis was absent. The expression of ACTA2 in scar dermis was detected in vascular SM cells and in cells located in the nodular structure in the deeper layers of the scar. ACTB and ACTG1 expression in scar epidermis was present similar to that in the unwounded skin epidermis, and the expression in the scar dermis was increased in comparison with ACTA2 (Figure 1B, upper row). The overall expression of ACTA2, ACTB and ACTG1 (Figure 1C) was found to be significantly increased in contracted scar tissue when compared with unwounded human skin tissue (1.8 fold for ACTA2, 2.5 fold for ACTB and ACTG1.  $P < 0.01$ ). Variation caused by patients' race, gender, age, scar location, and staining intensity of ACTA2, ACTB ACTG1 in unwounded tissue and scar were detected and summarized (Table 2).

### The Effect of ACTA2 Deficiency on Murine Wound Contraction

On day 2, compared with *Acta2*<sup>+/-</sup> mice (85.5±1.9%), relative wound area were significant larger in *Acta2*<sup>-/-</sup> mice (92.1±2.0%). There is no significant difference between *Acta2*<sup>+/+</sup> and *Acta2*<sup>+/-</sup> groups at days 3, 4 after injury, however; the impaired wound contraction in *Acta2*<sup>-/-</sup> mice in comparison with that of *Acta2*<sup>+/-</sup> mice still presented significant difference afterwards except day 9. In comparison with the wound size of the *Acta2*<sup>+/+</sup> mice (70.4±1.7%) the relative wound size on day 3 was significantly larger in *Acta2*<sup>-/-</sup> mice (78.9±2.1%) and the delay in wound contraction in *Acta2*<sup>-/-</sup> mice group was observed up to day 11. Finally, 11 days after injury, the wounds of *Acta2*<sup>-/-</sup> mice healed to the same degree as those of *Acta2*<sup>+/+</sup> and *Acta2*<sup>+/-</sup> mice (Figure 2). Collectively, the gross wound contraction was delayed in the absence of ACTA2.

### The Effect of ACTA2 Deficiency on Wound Evaluation

Microscopic assessment using H&E of wounds demonstrated that fibroblasts were the dominant cell type on days 7 and 11 in the wound area (Figure 3A). All the wounds in the mice were re-epithelized by day 11. Increased numbers of fibroblasts were arranged in parallel to the epidermis and were observed in the scar area. Follicular sebaceous glands were present at the wound edge and juxtaposed unwounded tissue, but absent in the scar. The scar showed increased epidermal thickness compared to surrounding unwounded tissue. (Figure 3B).

### The Effect of ACTA2 Deficiency on Collagen Deposition in Wound Sites

Masson's Trichrome staining showed more collagen surrounding the fibroblasts in the granulation tissue area on day 11 compared to day 7 in all groups. Wounds in *Acta2*<sup>+/-</sup> and *Acta2*<sup>-/-</sup> mice showed scant collagen deposition (Figure 4A). Our analysis<sup>19</sup> has demonstrated a decrease in collagen accumulation in *Acta2*<sup>+/-</sup> and *Acta2*<sup>-/-</sup> mice compared to that observed in *Acta2*<sup>+/+</sup> mice (Figure 4B).

### The Effect of ACTA2 Deficiency on Cellularity

To further assess the granulation tissue area in the different mice, Ki67, a nuclear antigen expressed in proliferating cells, was used to evaluate the proliferative activity of the fibroblastic area (Figure 5A). The number of Ki67 positive cells in dermis of *Acta2*<sup>-/-</sup> mice wound was increased on day 7 and 11 compared to mice expressing ACTA2 (Figure 5B). Cellular apoptosis was assayed using TUNEL staining (Figure 6A), as apoptosis is a mechanism of myofibroblast resolution. The cellular apoptosis observed in the granulation tissue area of *Acta2*<sup>-/-</sup> mice was approximately half that compared to that observed in the granulation tissue area of *Acta2*<sup>+/+</sup> mice on day 7 and 11 (Figure 6B). DAPI staining was used to quantify cellularity. *Acta2*<sup>-/-</sup> mice had decreased number of cells in the granulation tissue area only on day 7 compared to that in mice expressing ACTA2 (Figure 6C). In summary, ACTA2<sup>+/+</sup> mice had a greater number of cells (as determined by DAPI) earlier in the wound repair process; however, due to the increased proliferation and decreased apoptosis in ACTA2<sup>+/-</sup> and ACTA2<sup>-/-</sup> mice, as observed on day 7, and relative reduction of proliferation and increase in apoptosis in ACTA2<sup>+/+</sup> mice, as observed on day 7, the level of cellularity in ACTA2<sup>+/-</sup> and ACTA2<sup>-/-</sup> mice eventually equaled that of ACTA2<sup>+/+</sup> mice

on day 11. These observations relate to wound size and may simply reflect the need to heal the wound.

### Neovascularization in *Acta2*<sup>-/-</sup> Mice

To assess neovascularization, CD31 immunostaining was performed (Figure 7A). Neovascularization relies upon smooth muscle cells, so mice deficient in ACTA2 could have defects in blood vessel formation that would affect healing. Compared to *Acta2*<sup>+/+</sup> mice, the granulation tissue areas of *Acta2*<sup>+/-</sup> and *Acta2*<sup>-/-</sup> mice had a surprisingly significantly greater number of vessels (Figure 7B). This result is consistent with previous mice wound contraction data and suggested that mice lack of ACTA2 expression have significant enhanced level of angiogenesis than that of *Acta2*<sup>+/+</sup> mice.

### Discussion

In our current study, the use of *Acta2*<sup>-/-</sup> mice allowed us to directly test the role of alpha smooth muscle actin expressing myofibroblasts in wound contraction. Wound contraction started after surgery, and the contraction observed in *Acta2*<sup>-/-</sup> mice was significant slower than that of *Acta2*<sup>+/+</sup> mice at all time points except day 2. The wound contraction difference between *Acta2*<sup>+/+</sup> and *Acta2*<sup>-/-</sup> mice peaked around day 7 post-injury and then slowed down afterwards. Nonetheless, control mice healed their wounds. Collectively, these results identify a role for ACTA2 in wound closure but not an absolute necessity.

The wound healing process is a stepwise sequence of overlapping events which often results in scar.<sup>24</sup> Persistent wound contraction after a wound has epithelialized leads to contractures, whereas delayed wound contraction leads to chronic non healing wounds.<sup>24</sup> Current theories posit that alpha smooth muscle actin expressing myofibroblasts are necessary for wound contraction.<sup>1</sup> Although myofibroblasts are clearly present in granulation tissue during wound closure and in pathological contracture tissues, questions have arisen as to whether they are essential for collagen/granulation tissue contraction.<sup>25</sup> It is clear that traction forces that are generated by fibroblasts as they migrate on a compliant substratum can reorganize collagen matrices.<sup>26</sup> In fact, in free-floating collagen lattices the resulting reduction in lattice diameter is due entirely to fibroblast traction forces, which are generated by fibroblast migration and contractility.<sup>27</sup> This is sufficient to result in wound closure, this makes the involvement of a specialized contractile cell unnecessary.<sup>9</sup>

The gross evaluation from our study showed that the wound contraction rate of *Acta2*<sup>-/-</sup> mice do not match that of *Acta2*<sup>+/+</sup> and *Acta2*<sup>+/-</sup> mice but the wounds still contract closed. This actin expression defect disturbs the balance of wound contraction and regeneration, which sequentially up regulates the proportion of dermal regeneration in wound closure process.<sup>28</sup> As present results demonstrate, although *Acta2*<sup>-/-</sup> mice showed impaired wound contraction, they have increased neovascularization and cell proliferation in the wound bed as compared to *Acta2*<sup>+/-</sup> and *Acta2*<sup>+/+</sup> mice. The increase level of neovascularization in *Acta2*<sup>-/-</sup> mice implies that the absence of ACTA2 did not intervene the developing and formation of new blood vessels, which complies with the previous finding of Ehrlich HP et al that the development of ACTA2 in fibroblasts requires signals different from those required for these structures to appear in SM cells.<sup>12</sup>

Wound contraction and scar contracture share a similar mechanism of action, namely, the contraction of the granulation bed.<sup>3</sup> Our current study started with IHC analysis of human contracture scar. Except blood vessels, ACTA2 was detected in the nodular structure found in contracture scar samples, which is consistent with previous reports.<sup>29, 30</sup> It appears that the existence of the particular nodules structure may have a relationship with the gross contractile characteristic of these samples<sup>30</sup>. The expression of ATCB and ACTG1 in scar was significant higher in comparison with that in unwounded tissue. In contrast with the local, nodular expression of ACTA2, ATCB and ACTG1 were widely expressed throughout the scar. The proportional difference in actin isoform expression within scar brought up the same questions proposed by a sizeable body of studies<sup>8, 9, 11, 12, 25, 31-33</sup> concerning the controversial role of ACTA2: (a) is the contribution of ACTA2 expression necessary for contractile force generation and tissue remodeling? (b) Will ACTB and ACTG1 expression sufficiently compensate the function of ACTA2?

In addition, there are variations in different investigations concerning the ACTA2 expression level in scar tissue.<sup>29, 30, 34</sup> Possible explanations for this inconsistency are: (a) differences in the sources of samples related with species, patients with demographic distribution, (b) differences in the samples inclusion and exclusion criteria, (c) percentages of scar in different stages of repair process, (d) differences in the evaluation methods and (e) sizes of sample population and observer variance. Our current research focuses on determining the relationship between actin isoforms and wound contraction. We observed a significantly higher expression of each actin in scar tissue, consistent with reports of Ehrlich HP et al<sup>30</sup>, our laboratories previous work<sup>20</sup>, Tian Y et al.<sup>35</sup> and Wang XQ et al.<sup>34</sup>

While our manuscript was in preparation, Tomasek et al<sup>36</sup> reported on wound healing in the *Acta2*<sup>-/-</sup> mouse. Analogous to our study, he found that ACTA2 expression was not necessary for wound closure. He reported that alpha smooth muscle actin null fibroblasts become more contractile in response to TGF- $\beta$  and that compensatory increases in actin isoforms such as, cardiac muscle  $\alpha$ -actin, cytoplasmic  $\beta$ -actin, cytoplasmic  $\gamma$ -actin, skeletal muscle  $\alpha$ -actin, and smooth muscle  $\gamma$ -actin could explain normal wound contraction rates. Differential expression of actin isoforms enabled non alpha smooth muscle actin isoform expressing myofibroblasts to form. He also found a statistically different rate of wound contraction between animals, but not at all time points, as we observed. We believe the discrepancies can be attributed to: 1) smaller wound sizes in his study, and 2) fewer time points to closure; meaning he may have missed statistically significant differences at more time points. Nevertheless, our work is in agreement with his that alpha smooth muscle actin is not necessary for wound closure.

## Conclusion

The present study has demonstrated that alpha smooth muscle actin expressing myofibroblasts contribute to but are not necessary for wound contraction. Although *Acta2*<sup>-/-</sup> mice showed impaired wound contraction, they have increased neovascularization and cell proliferation in the wound bed as compared to *Acta2*<sup>+/-</sup> and *Acta2*<sup>+/+</sup> mice. Compensatory increases in actin isoform expression may explain why wound contraction still occurs until wound closure. Thus, we posit that targeting contractile elements different than actin

isoforms; yet, shared by myofibroblasts and fibroblasts (e.g. non muscle myosin II) may be a necessary approach for promoting healing of chronic wounds or reducing scar contractures.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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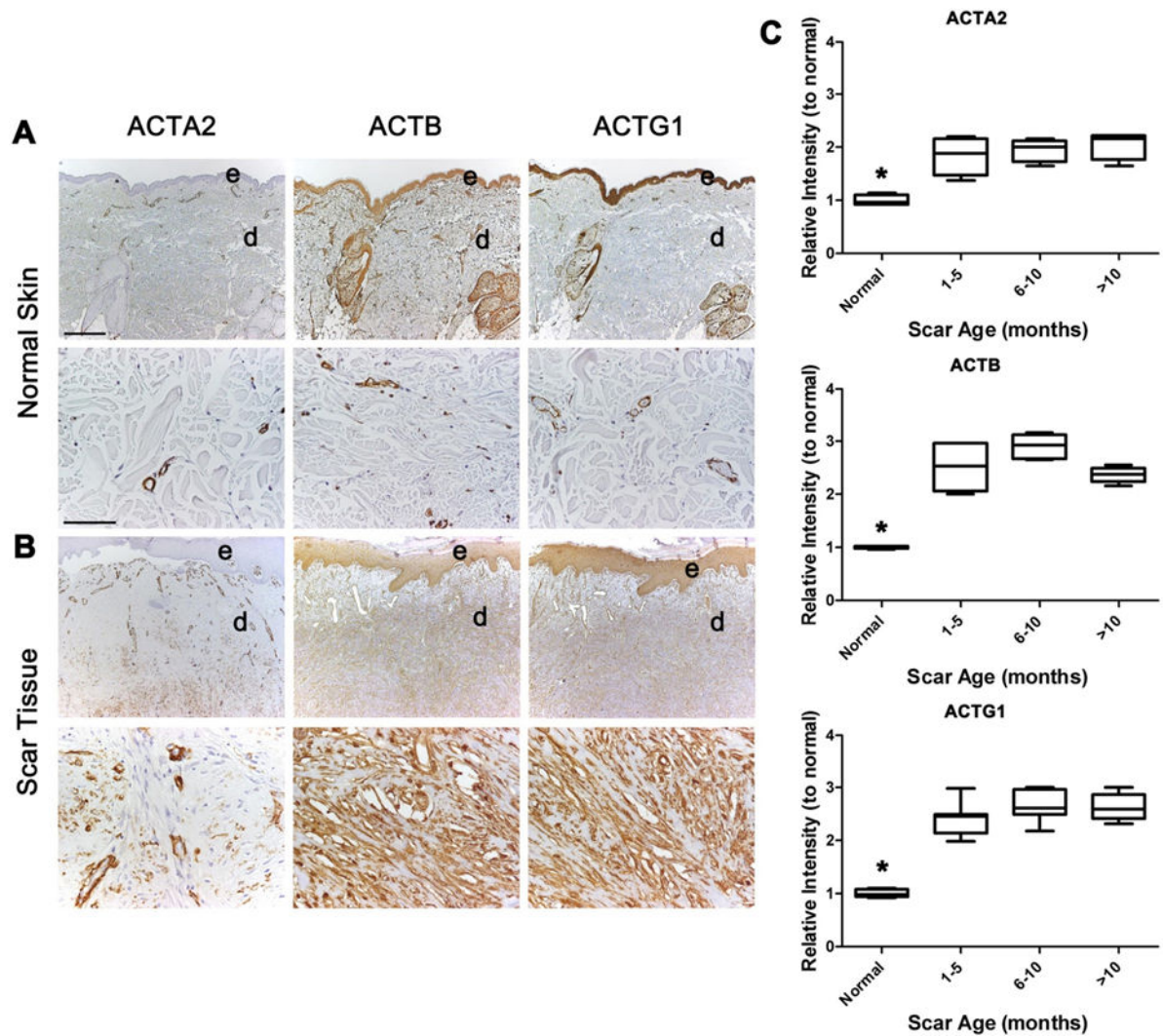
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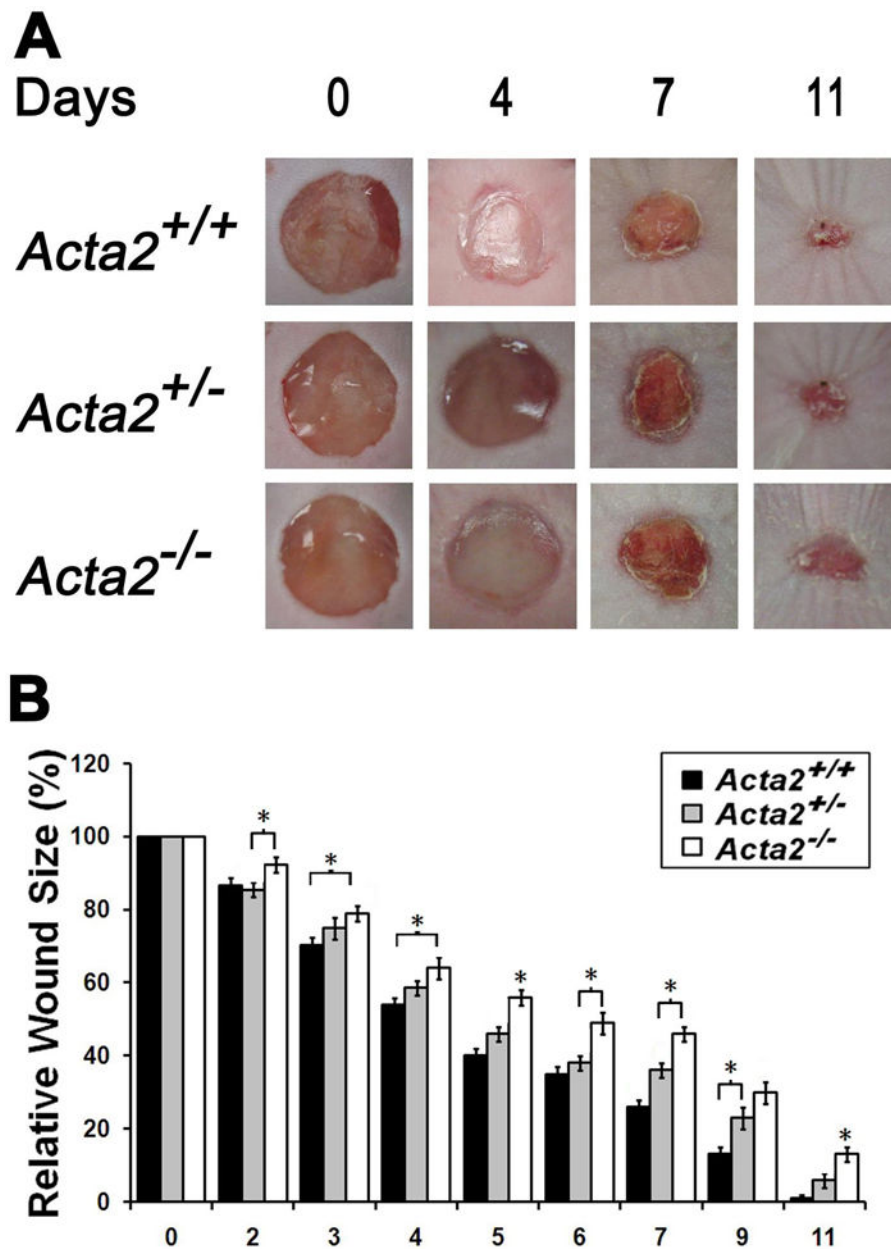
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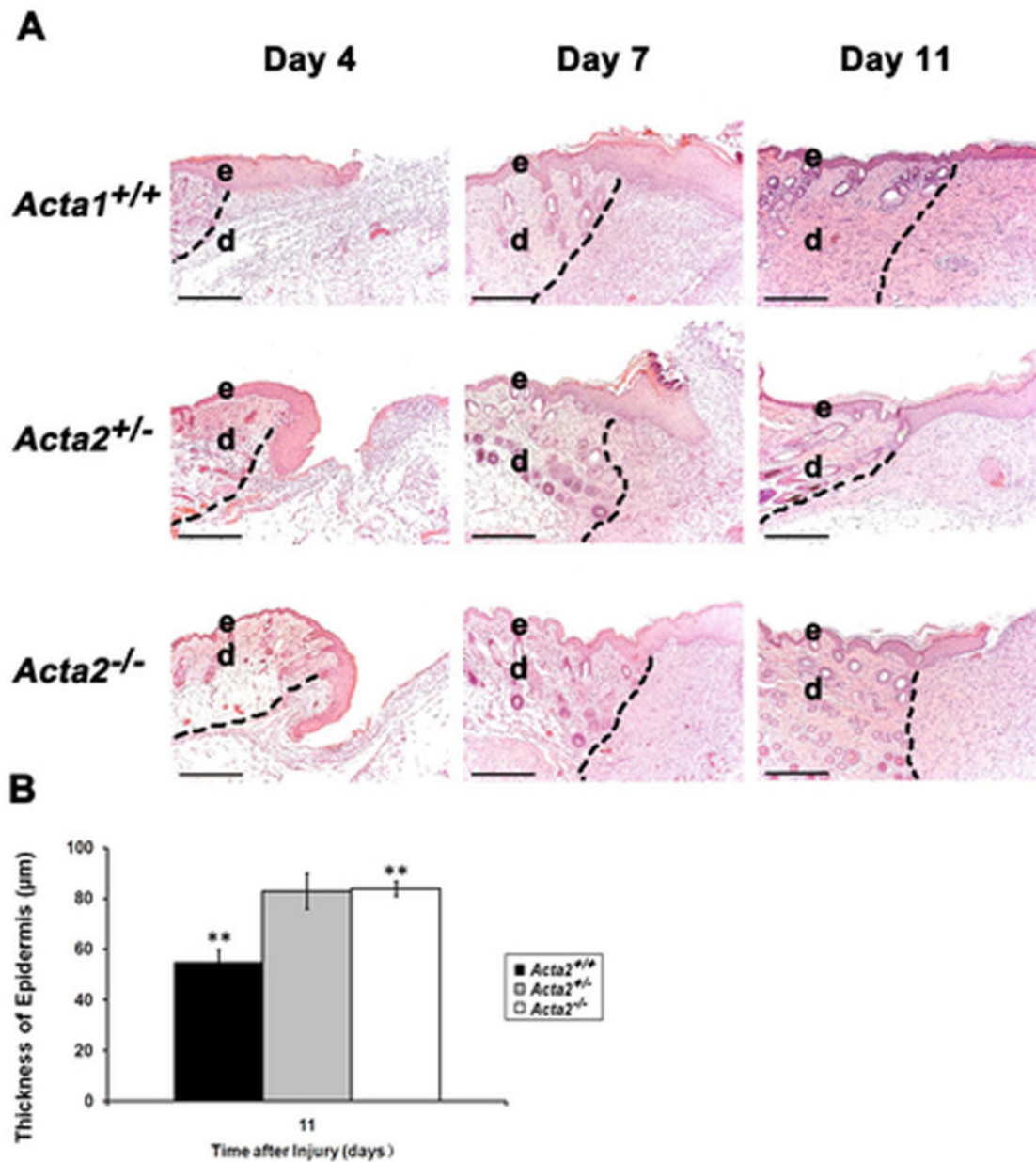


**Figure 1.**

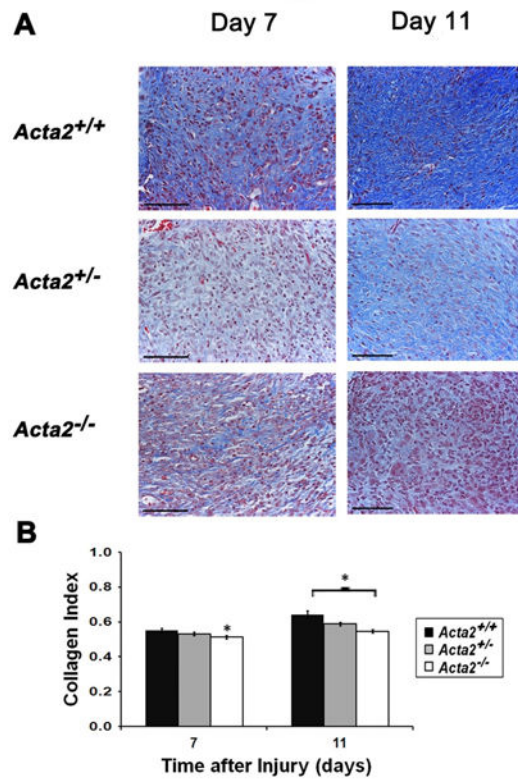
ACTA2, ACTB and ACTG1 expression in the remodeling phase of repair. Representative human unwounded skin (A) Scar specimens (B) investigated by IHC staining are shown to reveal the expression pattern of ACTA2, ACTB and ACTG1. Scale bars, 400 $\mu$ m for 4 $\times$  (upper panel of A and B) and 100 $\mu$ m for 40 $\times$  (lower panel of A and B) images, e, epidermis; b, dermis. C: The staining intensity of human unwounded skin tissue and contraction scar over time was quantified. Data are expressed as mean $\pm$ SEM. \* $P < 0.05$ , tested by one-way ANOVA with Bonferroni's post-hoc test, performed on 6–10 months vs. >10 months scar for ACTB expression.



**Figure 2.** Skin wound healing of *Acta2*<sup>+/+</sup>, *Acta2*<sup>+/-</sup> and *Acta2*<sup>-/-</sup> mice. A: Representative macroscopic views from wounded mice are shown at day 0, 3, 7 and 11 post wounding. All pictures were taken at the same distance. B: The fraction of wound area at indicated time point in comparison to the original wound area (quantified as described in *Material and Methods* section) was plotted and shown. Values are represented as mean±SEM (n=25, 31 and 38 for *Acta2*<sup>+/+</sup>, *Acta2*<sup>+/-</sup> and *Acta2*<sup>-/-</sup> mice group). \**P*<0.05, tested by one-way ANOVA with Bonferroni's post-hoc test, performed on *Acta2*<sup>+/+</sup> vs. *Acta2*<sup>+/-</sup>, *Acta2*<sup>+/-</sup> vs. *Acta2*<sup>-/-</sup>, *Acta2*<sup>+/+</sup> vs. *Acta2*<sup>-/-</sup>, respectively.

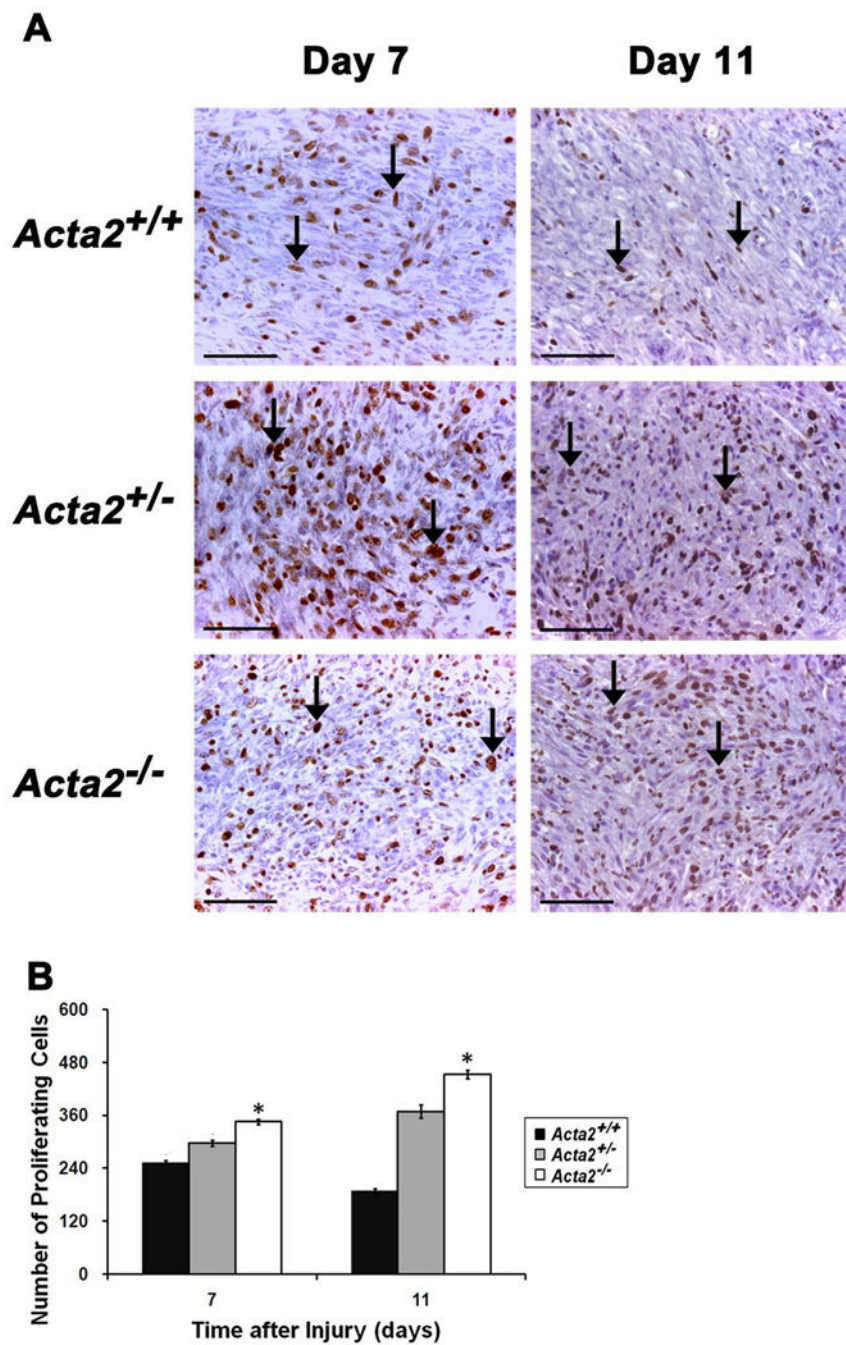


**Figure 3.** Histological evaluation of wounded skin. A: H&E staining was performed at day 4, 7, 11 after injury. Representative results from *Acta2<sup>+/+</sup>*, *Acta2<sup>+/+</sup>* and *Acta2<sup>-/-</sup>* mice are shown. The dotted lines indicate the margin areas of the wound. Scale bar, 200 $\mu\text{m}$  for 10 $\times$  images, e, epidermis; b, dermis. B: Epidermal thickness was measured in *Acta2<sup>+/+</sup>*, *Acta2<sup>+/+</sup>* and *Acta2<sup>-/-</sup>* mice at day 11 after injury. Data are expressed as mean $\pm$ SEM (n=3 per genotype). \* $P$ <0.05 tested by one-way ANOVA with Bonferroni's post-hoc test, performed on *Acta2<sup>+/+</sup>* vs. *Acta2<sup>+/+</sup>*, *Acta2<sup>+/+</sup>* vs. *Acta2<sup>-/-</sup>*, respectively.



**Figure 4.**

Representative wound section following Masson's Trichrome staining. A: Collagen deposition and organization in days 7 and 11. Blue area represents collagen. B: Collagen deposition results are shown as mean±SEM (n=3 per genotype). \* $P < 0.05$  tested by one-way ANOVA with Bonferroni's post-hoc test, performed on *Acta2*<sup>+/+</sup> vs. *Acta2*<sup>-/-</sup> for day 7 mice tissue, on *Acta2*<sup>+/+</sup> vs. *Acta2*<sup>+/-</sup>, *Acta2*<sup>+/+</sup> vs. *Acta2*<sup>-/-</sup> for day 11 mice tissue, respectively. Scale bars, 100µm for 40× images.



**Figure 5.** Proliferation activity in wound dermis. A: Day 7 and day 11 *Acta2*<sup>+/+</sup>, *Acta2*<sup>+/-</sup> and *Acta2*<sup>-/-</sup> mice wound specimens were evaluated by staining with anti-Ki67 antibody. Representative photos are shown and Ki67-positive cells are indicated by black arrows. Level of cell proliferation was determined by the number of positive stained nuclei in wound dermis. Scale bars, 100µm for 40× images. B: Quantified data are represented as means ±SEM (n=3 per genotype). \* *P*<0.01 tested by one-way ANOVA with Bonferroni's post-hoc

test, performed on *Acta2*<sup>+/+</sup> vs. *Acta2*<sup>+/-</sup>, *Acta2*<sup>+/-</sup> vs. *Acta2*<sup>-/-</sup>, *Acta2*<sup>+/+</sup> vs. *Acta2*<sup>-/-</sup>, respectively.

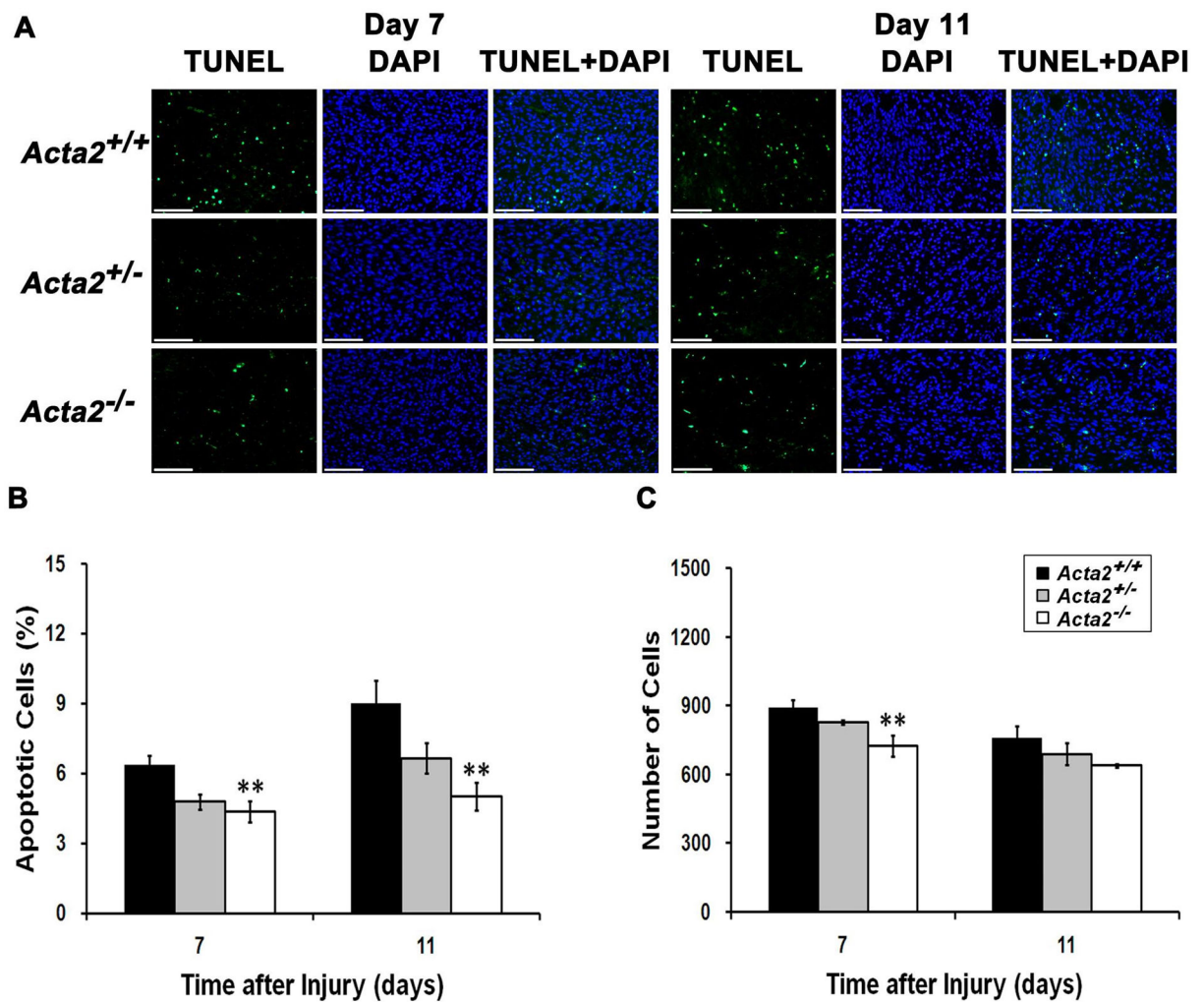
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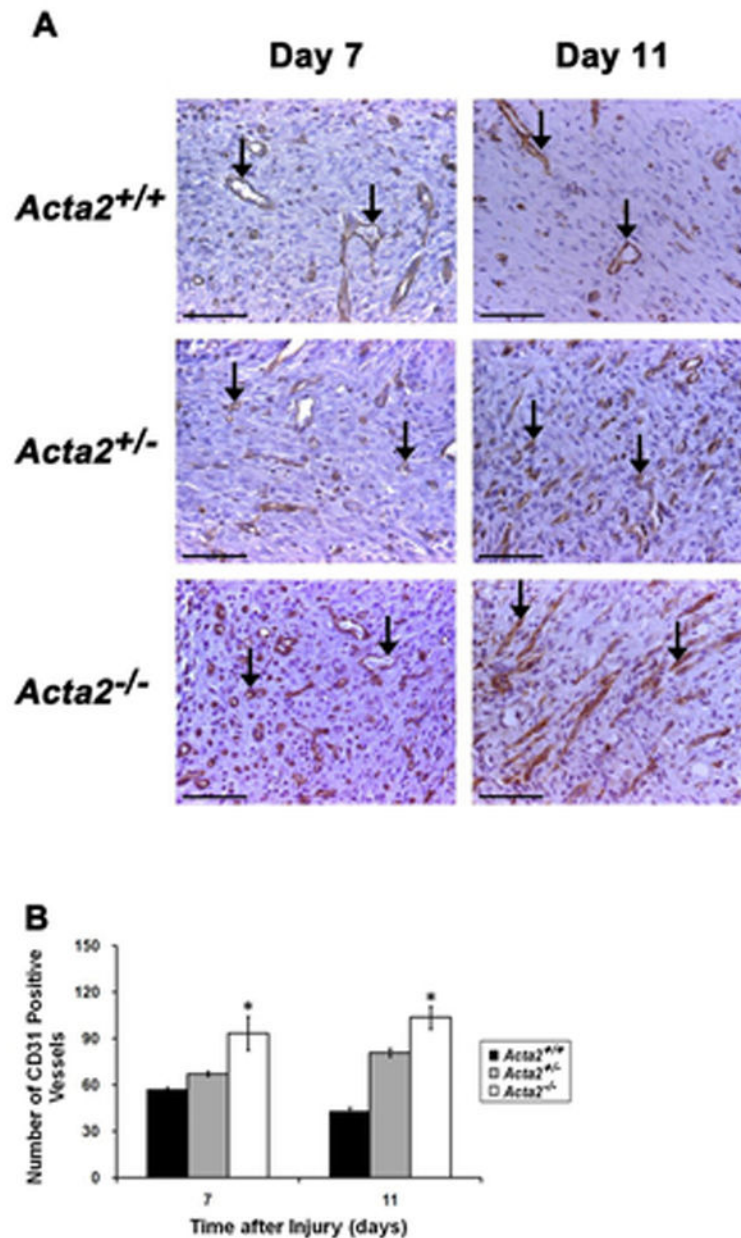
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**Figure 6.**

Apoptosis at wound sites. A: TUNEL staining of wound sections were used for apoptosis detection (green) and DAPI staining was performed to visualize the total cell number (blue). Scale bars, 75 $\mu$ m for 20 $\times$  images. Cell counting was performed at 20 $\times$  magnification. Quantification of apoptosis ratio was achieved by plotting the number of TUNEL positive cell against that of DAPI positive cells (B). Wound cellularity was quantified by counting the number of DAPI positive cells (C). Data are represented as means  $\pm$  SEM (n=3 per genotype). \* $P$ <0.05 tested by one-way ANOVA with Bonferroni's post-hoc test, performed on *Acta2*<sup>+/+</sup> vs. *Acta2*<sup>-/-</sup>, respectively.



**Figure 7.** Angiogenesis in mice skin after wounding. A: Day 7, 11 *Acta2*<sup>+/+</sup>, *Acta2*<sup>+/-</sup> and *Acta2*<sup>-/-</sup> mice wound sections were evaluated by staining with anti-CD31 antibody. Representative micrographs are shown and CD31-positive vessels were indicated by black arrows. Scale bars, 100 $\mu$ m for 40 $\times$  images. B: Levels of vascularization were determined by the number of vessels positive stained for CD31 per high power field in wound area. Data are represented as means $\pm$ SEM (n=3 per genotype). \**P*<0.05 tested by one-way ANOVA with Bonferroni's post-hoc test, performed on *Acta2*<sup>+/+</sup> vs. *Acta2*<sup>-/-</sup> for day 7 mice tissue, on *Acta2*<sup>+/+</sup> vs. *Acta2*<sup>+/-</sup>, *Acta2*<sup>+/-</sup> vs. *Acta2*<sup>-/-</sup>, *Acta2*<sup>+/+</sup> vs. *Acta2*<sup>-/-</sup> for day 11 mice tissue, respectively.

**Table 1**

Patient demographic of selected scar samples. A total number of 18 skin lesions with typical contraction scar and surrounding unwounded skin between 1–12 months were matched according to patients' race, gender, age, scar location and scar ages.

GROUP		N
All		18
Race	Caucasian	6
	Asian	12
Gender	Male	12
	Female	6
Age (Years)	<20	8
	>20	10
Scar Location	Head& trunk	9
	Upper extremities	3
	Lower extremities	6
Scar Ages	1–5 months	7
	6–10 months	6
	>10 months	5

**Table 2**

ACTA2, ACTB and ACTG1 expression comparison among groups. Expression levels of ACTA2, ACTB and ACTG1 in human contraction scar were measured by IHC staining intensity analysis. Values are indicated as mean  $\pm$  SEM, for groups matched according to patients' race, gender, age, scar location and scar ages, \* $P < 0.01$  tested by one-way ANOVA with Bonferroni's post-hoc test, performed on ACTA2 vs. ACTB, ACTA2 vs. ACTB, respectively. For groups matched according to tissue type, \* $P < 0.01$  tested by Student's *t*-Test, performed on unwounded skin tissue vs. scar tissue.

<i>Group</i>	<i>P-value</i>		
	<b>ACTA2</b>	<b>ACTB</b>	<b>ACTG1</b>
<i>All</i>	52.0 $\pm$ 1.7*	107.5 $\pm$ 3.7	104.3 $\pm$ 2.9*
<b><i>Race</i></b>			
<i>Caucasian</i>	49.5 $\pm$ 4.0	100.7 $\pm$ 7.7	98.2 $\pm$ 5.7
<i>Asian</i>	53.3 $\pm$ 1.6	110.8 $\pm$ 3.9	107.4 $\pm$ 3.0
<b><i>Gender</i></b>			
<i>Male</i>	51.6 $\pm$ 2.2	110.1 $\pm$ 4.5	103.6 $\pm$ 3.1
<i>Female</i>	52.8 $\pm$ 2.8	102.2 $\pm$ 6.5	105.8 $\pm$ 6.4
<b><i>Age (years)</i></b>			
<i>&lt;20</i>	52.9 $\pm$ 1.9	111.7 $\pm$ 5.2	105.5 $\pm$ 4.1
<i>&gt;20</i>	51.3 $\pm$ 2.7	104.1 $\pm$ 5.2	103.4 $\pm$ 4.2
<b><i>Scar location</i></b>			
<i>Head&amp; trunk</i>	50.8 $\pm$ 4.5	103.7 $\pm$ 8.9	103.8 $\pm$ 6.9
<i>Upper extremities</i>	52.1 $\pm$ 3.2	114.6 $\pm$ 2.3	103.4 $\pm$ 9.1
<i>Lower extremities</i>	53.8 $\pm$ 2.2	109.5 $\pm$ 6.8	105.6 $\pm$ 4.1