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Scratch Migration Assay and Dorsal Skinfold Chamber for In Vitro and In Vivo Analysis of Wound Healing

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To
Dr. Vineeta Bajaj
Science Editor
JoVE

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Dear Dr. Bajaj,

Many thanks for the assessment of our manuscript JoVE59608: "*In vitro* and *in vivo* analysis of wound healing by means of the scratch migration assay and the dorsal skinfold chamber". We are grateful to the editor and referees for their constructive comments and suggestions.

Attached we send the revised manuscript which has been prepared according to the guidelines listed in the Editorial comments and that includes complementary information addressing the points raised by the reviewers.

In the point by point response we try to give detailed answers to the editor's and reviewers' comments on our manuscript. Changes are highlighted in the revised manuscript.

We include a word file containing the filmable content (2.75 pages of the Protocol) that identifies the essential steps of the protocol for video production. As suggested by the reviewers Figure 1 is reorgamized and an image of the titanium chamber is included as Figure 2a in the revised manuscript.

Sincerely yours,

for the authors

Anouar Belkacemi

TITLE:

Scratch Migration Assay and Dorsal Skinfold Chamber for In Vitro and In Vivo Analysis of Wound Healing

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SUMMARY

Here, we present a protocol for an in vitro scratch assay using primary fibroblasts and for an in vivo skin wound healing assay in mice. Both assays are straightforward methods to assess in vitro and in vivo wound healing.

ABSTRACT

Impaired cutaneous wound healing is a major concern for patients suffering from diabetes and for elderly people, and there is a need for an effective treatment. Appropriate in vitro and in vivo approaches are essential for the identification of new target molecules for drug treatments to improve the skin wound healing process. We identified the β3 subunit of voltage-gated calcium channels (Cavβ3) as a potential target molecule to influence the wound healing in two independent assays, i.e., the in vitro scratch migration assay and the in vivo dorsal skinfold chamber model. Primary mouse embryonic fibroblasts (MEFs) acutely isolated from wild-type (WT) and Cavβ3-deficient mice (Cavβ3 KO) or fibroblasts acutely isolated from WT mice treated with siRNA to down-regulate the expression of the *Cacnb3* gene, encoding Cavβ3, were used. A scratch was applied on a confluent cell monolayer and the gap closure was followed by taking microscopic images at defined time points until complete repopulation of the gap by the migrating cells. These images were analyzed, and the cell migration rate was determined for each condition. In an in vivo assay, we implanted a dorsal skinfold chamber on WT and Cavβ3 KO mice, applied a defined circular wound of 2 mm diameter, covered the wound with a glass coverslip to protect it from infections and desiccation, and monitored the macroscopic wound closure over time. Wound closure was significantly faster in *Cacnb3*-gene-deficient mice. Because the results of the in vivo and the in vitro assays correlate well, the in vitro assay may be useful for the high-throughput screening before validating the in vitro hits by the in vivo wound healing model. What we have shown here for wild-type and Cavβ3-

deficient mice or cells might also be applicable for specific molecules other than Cavβ3.

INTRODUCTION

Skin wound healing starts immediately after the skin injury in order to restore the skin's integrity and to protect the organism from infections. The wound healing process goes through four overlapping phases; coagulation, inflammation, new tissue formation, and tissue remodeling[1]. Cell migration is crucial during these phases. Inflammatory cells, immune cells, keratinocytes, endothelial cells, and fibroblasts are activated at different time points and invade the wound area[2]. Methods to investigate wound healing in vitro and in vivo are of great interest not only to understand the underlying mechanisms but also to test new drugs and to develop new strategies aiming to ameliorate and accelerate skin wound healing.

To monitor and analyze cell migration, the scratch migration assay can be used. It is often referred to as in vitro wound healing assay. This method requires a cell culture facility[3]. It is a simple procedure, there is no need of high-end equipment and the assay can be performed in most cell biology laboratories. In this assay, a cell-free area is created by the mechanical disruption of a confluent cell monolayer, preferably epithelial- or endothelial-like cells or fibroblasts. Cells on the edge of the scratch will migrate in order to repopulate the created gap. Quantification of the decreasing cell-free area over time resembles the migration rate and indicates the time, which the cells need to close the gap. For this purpose, investigators can use either acutely isolated cells from WT mice or mice lacking a gene of interest[4], or immortalized cells available from reliable cell repositories. The scratch assay allows studying the influence of pharmacologically active compounds or the effect of transfected cDNAs or siRNAs on cell migration.

In vivo, wound healing is a complex physiological process, requiring different cell types including keratinocytes, inflammatory cells, fibroblasts, immune cells and endothelial cells in order to restore the skin's physical integrity as fast as possible[1]. Different methods to study in vivo wound healing have been developed and used in the past[5-8]. The dorsal skinfold chamber described in this article was previously used for wound healing assays[9]. It is used as a modified dorsal skinfold chamber preparation for mice. The modified skinfold chamber model has several advantages. 1) It minimizes skin contraction, which prevents observing the wound healing process and might influence wound repair in mice. 2) This chamber makes use of covering the wound with a glass coverslip, reducing tissue infections and desiccation, which could delay the healing process. 3) Blood flow and vascularization can be directly monitored. 4) It allows repetitive topical application of pharmacologically active compounds and reagents in order to treat the wound and accelerate healing[9,10].

We identified the β3 subunit of high voltage-gated calcium channels (Cavβ3) as a potential target molecule to influence skin wound healing using two independent protocols, i.e., the in vitro scratch migration assay and the in vivo dorsal skinfold chamber model. For the in vitro assay, we used primary fibroblasts, these cells do express the *Cacnb3* gene encoding the Cavβ3 protein but lack depolarization-induced Ca^{2+} influx or voltage-dependent Ca^{2+} currents. Here we describe a novel function of Cavβ3 in these fibroblasts: Cavβ3 binds to the inositol 1,4,5-trisphosphate receptor (IP3R) and constraints calcium release from the endoplasmic reticulum. Deletion of the *Cacnb3* gene in mice leads to increased sensitivity of the IP3R for IP3, enhanced cell migration and increased skin wound repair[4].

PROTOCOL

All experimental procedures were approved and performed in accordance with the ethics regulations and the animal welfare committees of Saarland and Saarland University.

1 Primary cell culture and siRNA transfection

NOTE: In the described method, primary fibroblasts are used. These cells play a crucial role in wound healing and tissue remodeling[11]. In this experiment, the *Cacnb3* gene, encoding the Cavβ3 subunit of high voltage-gated calcium channels[12] was down-regulated, thereby showing its role in cell migration in vitro and skin wound repair in vivo[4].

1.1 Preparation of siRNA: Before reconstituting the siRNAs, briefly centrifuge the tubes to ensure that the content is at the bottom. Reconstitute the siRNAs in 100 µL RNase-free buffer (100 mM potassium acetate, 30 mM HEPES, pH 7.5) provided by the manufacturer at a concentration of 20 µM. This is a stock solution of siRNAs.

1.2 Aliquot this stock solution at 10 µL per tube (20 µM concentration) and store at -20 °C until use.

1.3 Using an ultrafine permanent marker, mark a 6-well plate with a horizontal line at the bottom of each well in order to be able to always identify the same scratch region of interest and to follow its closure.

NOTE: 6-well culture plates were used in this assay because they are large enough, to provide enough space and flexibility to apply a consistent, reproducible and vertical scratch using a 200 µL pipette tip across the cell monolayer. If a limited number of cells are available, an alternative and probably the cost-efficient way would be to use 12- or 24-well culture plates.

1.4 Plate primary fibroblasts, isolated from the wild-type and β3-deficient mice[4], in a 6-well plate at a density of 5 x 10^5 cells/well in the presence of 2 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FCS).

NOTE: 5 x 10^5 cells per well has been established for 6-well culture plate and for primary mouse fibroblasts. Tests may be needed if using 12- or 24-well cell culture plates or other cell types, which could be different in size. Cells should be handled in a sterile environment such as biological safety cabinets class II.

1.5 Label the 6-well plate with the cell type, genotype, and the date.

1.6 Move the 6-well plate into the cell culture incubator and maintain cells at 37 °C and 5% CO_2 for 24 h.

1.7 The next day, take the plate out of the incubator, aspirate the cell culture medium out of the well, discard it and replace it with 2.25 mL fresh culture medium by adding it carefully against the wall of the well.

1.8 In order to transfect fibroblasts with siRNAs, use a lipid-based transfection reagent as recommended by the manufacturer.

1.9 For each transfection, label two microcentrifuge tubes. In the first one, add 9 µL of the transfection reagent and dilute it with 150 µL reduced serum medium. In the second tube, add 1.5 µL siRNA (*Cacnb3* siRNA-1, *Cacnb3* siRNA-2 or scrambled siRNA as a negative control) and dilute it with 150 µL reduced serum medium.

1.10 Add the diluted siRNA into the tube containing diluted transfection reagent and vortex for 2 s. Incubate the mixture for 5 min at 21 °C.

1.11 Label wells with either *Cacnb3* siRNA-1, *Cacnb3* siRNA-2 or scrambled siRNA. Add 250 µL of the siRNA-transfection reagent mixture dropwise to the cells.

1.12 Place the 6-well culture plate back into the incubator and keep cells at 37 °C and 5% CO_2 for 72 h.

1.13 In order to check the efficiency of *Cacnb3* gene silencing, collect transfected cells and perform immunoblot analysis as described previously[4].

2 In vitro wound healing assay (scratch migration assay)

2.1 Take the cell culture plate out of the incubator and examine the cells under the microscope using the 10x objective. Start with the scratch assay only when they have reached 100% confluency.

NOTE: For the accuracy and reproducibility, 100% confluency is a mandatory factor for starting the scratch migration assay. Therefore, it is important to seed the same number of cells into the culture wells, to examine each well for confluency and to apply the scratch at the same time point (day 0 confluency). Waiting for longer after the cells reach 100% confluency can evoke different responses.

2.2 Once the cell reaches 100% confluency, aspirate the culture medium out of the well and discard it.

2.3 Use a pipette tip (200 µL) to manually create a scratch vertical to the horizontal line marked at the bottom of the well, across the confluent cell monolayer in the middle of the well.

2.4 Rinse each well twice with 2 mL phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) to remove the released factors from damaged cells, loose cells, and debris from the scratched area. Add 2 mL of PBS carefully against the wall of the well to avoid detaching cells from the cell culture well.

2.5 Add 2 mL of cell culture medium containing either 10% serum or 1% serum carefully to each well.

NOTE: It is recommended to perform the scratch assay under 10% serum and under 1% serum to confirm that the observed effect is caused by the cell proliferation and migration or by cell migration only.

2.6 Move the plate to the microscope stage and capture bright field images of the cell-free area (two areas per well) immediately after scratching (t=0h) at a 10x magnification using a light microscope. To image always the same region of the scratch, use the horizontal line, which was prepared in step (1.3), and take one image above this line and one image below this line. Save images as TIFF or JPEG.

2.7 Because the microscope stage does not maintain the cell growth condition, move the plate back to the cell culture incubator and keep the cells at 37 °C and 5% CO_2.

2.8 After 6, 10 and 30 h, move the plate to the microscope stage again and capture images the same way as described in step 2.6.

NOTE: These time points have been established for the described procedure and for primary fibroblasts. During the first pilot experiment, more time points were tested to see how fast fibroblasts repopulate the gap. Although 0, 6, 10 and 30 h are reasonable starting time points, the investigators should optimize and establish the appropriate time points for each application and for each cell type. The more accurate alternative, if available, would be to use time-lapse microscopy.

2.9 Using ImageJ[13], quantify the initial cell-free area (100%) and the remaining area after 6, 10 and 30 h (**Figure 1**). The percentage of scratch area repopulated by migrating cells is then calculated relative to the initial scratch area.

3 Analysis of the scratch area

3.1 Open ImageJ software[13].

3.2 Upload the first image as JPEG (e.g., 24-bit RGB images 1360x1024) by dropping the image into the ImageJ menu bar.

3.3 Select the **Freehand selections** button and mark the cell-free area

3.4 Click on **Analyze** and select **Measure**. A window with the results will appear containing the area value.

3.5 Transfer this value into an analysis spreadsheet.

3.6 Repeat steps 3.2-3.5 for each image from time point 0 h and then start again for the next time points 6, 10 and 30 h.

3.7 Calculate the percentage of scratch area repopulated by migrating cells after 6, 10 and 30 h for each scratch using the following equation:

$$\%\ of\ scratch\ area\ repopulated\ by\ migrating\ cells\ after\ xh = 100 - \left(\frac{b \cdot 100}{a}\right)$$

a = cell free area of the initial scratch, b = cell free area after 6 h

3.8 Calculate the mean and the standard error of the mean (S.E.M.) for the percentage of scratch area repopulated by migrating cells after 6 h. Show data as a column bar graph or a

scatter plot.

4 In vivo skin wound healing assay

NOTE: WT and wild-type or β3-deficient mice are used for this study.

4.1 One day before starting the experiment, autoclave all the surgical instruments, screws, nuts and titanium frames to be used for the skinfold chamber preparation.

NOTE: The titanium frame is composed of two symmetrical complementary halves and it has a circular observation window where the wound will be applied and followed by microscopy (see **Figure 2a**).

4.2 Anesthetize a wild-type or β3-deficient mouse (22-26 g body weight) by intraperitoneal (i.p.) injection of 0.1 mL saline/10 g body weight containing a mixture of ketamine (75 mg/kg body weight) and xylazine (25 mg/kg body weight). Check the depth of anesthesia by the lack of response to a toe pinch.

NOTE: This injection gives around 30 min surgical anesthesia and the depth of anesthesia must be controlled through the surgical procedure, by checking the reflexes of the mouse.

4.3 To avoid dryness or damage of the eyes, apply ophthalmic ointment to both eyes and repeat the application if necessary.

4.4 Carefully shave the mouse dorsum, using an electric shaver followed by the application of a depilation cream to the shaved area to remove any remaining hair. Take care not to injure the mouse skin. Leave the depilation cream for around 10 min to completely remove all hair.

4.5 Prepare the titanium chamber by taking one part of the symmetrical titanium chamber frame and fix the connecting screws with nuts on one side. These nuts will serve as a spacer to keep 400-500 µm between the two symmetrical parts of the chamber to avoid compression of blood vessels in the skin.

4.6 Remove the cream from the back of the mouse and clean the hair-free region with warm (35-37 °C) tap water.

4.7 Make sure that the place to perform surgery is clean, warm (37 °C) and humidified.

4.8 Disinfect the hair-free area of the mouse with 70 % (vol/vol) ethanol. Take a fold of the back skin of the mouse in front of a light source and position the middle line of the double layer of the skin where the titanium chamber will be implanted. After that, fix the skinfold with a polypropylene suture cranially and caudally and tighten the other side of the suture on a metal rack to lift the mouse folded skin. Adjust the height of the rack to allow the mouse to sit comfortably.

4.9 Implant the titanium chamber into the fold of the back skin of the mouse in a way to sandwich the folded dorsal skin layer between the two symmetrical halves of the titanium frame. Attach the first half of the titanium frame by polypropylene sutures on its superior

edge to the back of the dorsal skinfold.

NOTE: On the titanium frame, there are 8 holes on the superior edge (**Figure 2a**) and the folded skin should be well fixed by polypropylene sutures on each of the eight holes.

4.10 Before moving to the next step, check the reflexes of the mouse to make sure that the depth of anesthesia is maintained.

4.11 At the base of the skinfold, pass the two connecting screws, attached to the first half of the titanium chamber, to penetrate the skinfold from back to the front side. Make small incisions on the skin (using fine scissors) to help smooth penetration of the connecting screws.

4.12 Detach the mouse from the rack and place it on a lateral position. Put the second complementary half of the titanium chamber on top of the 3 connecting screws (see **Figure 2a**) and apply slight pressure with fingers in order to pass these screws through the second half of the titanium frame. Then, fix both symmetrical parts with stainless steel nuts.

4.13 Pay careful attention to the tightness of the screws at this step, since it might detach, if it is too loose. In contrast, if it is too tight, it will squeeze the skinfold, reduce blood flow and can lead to tissue impairment and necrosis.

NOTE: Nuts prepared in step 4.5 serve as a spacer to keep a distance of 400-500 µm between the two symmetrical halves of the titanium chamber. The nuts should be tightened until a slight resistance is felt.

4.14 Cut the remaining part of the screws using pliers.

NOTE: It is necessary at this step to use laboratory safety glasses for eye protection in case the screw comes off the wrong way.

4.15 Mark the wound area by a standardized biopsy punch (2 mm in diameter), in the center of the skin within the observation window (see **Figure 2a**) of the skinfold chamber in order to ensure reproducible wound sizes.

4.16 By using fine forceps and scissors, create a circular wound within the marked area by removing the complete skin with epidermis and dermis. The final wound area will be around 3.5-4.5 mm^2, see **Figure 2b.** Clean the wound with 0.5 mL sterile saline (0.9 % NaCl, 37 °C).

4.17 Cover the wound with a glass coverslip and fix this glass coverslip with a snap ring using the snap ring plier on the titanium chamber.

4.18 Immediately after finishing the surgical procedure, place the mouse on the imaging stage of a stereomicroscope equipped with a camera and take images (day 0) under illumination. Use the 40X magnification and save the images for future off-line analysis.

NOTE: The investigator should examine the images immediately after capture to ensure that quality is sufficient for future off-line analyses. The preparation of the skinfold chamber and performance of the skin wound takes around 30 minutes.

4.19 Keep the mouse at a warm place during recovery from anesthesia for at least 2 h. Thereafter, transfer mice in individual cages back to the animal facility (12 h light/dark cycle) and make sure that mice have access to food and water.

4.20 Three days post-wounding place the mouse in a mouse-restrainer and fix the restrainer on top of the imaging stage.

4.21 Place the stage under a stereomicroscope equipped with a camera. Take images under illumination with 40x magnification, record all pictures and save them for future off-line analyses

4.22 Repeat steps 4.20 and 4.21 again at day 6, 10 and day 14 post-wounding.

4.23 Use the wound images, for off-line analysis in ImageJ[13]. The wound area at day 0 is considered as 100 % and the wound closure over time is plotted relative to the initial wound area. Representative results are shown in **Figure 2c,d**.

4.24 Calculate the percentage (%) of the wound area at each time point using the following equation:

$$\%\ of\ wound\ area\ at\ t(x) = \left(\frac{b \cdot 100}{a}\right)$$

x: time point (day 0, 3, 10 or 14), *a*: wound area at day 0, *b*: wound area at time point *x*

REPRESENTATIVE RESULTS

The scratch assay was performed on a confluent cell monolayer of wild-type and β3-deficient MEFs (**Figure 1c**). After performing the "scratch" using a 200 µL pipette tip, cells from both genotypes migrate into the scratch area and close the gap. Images were taken after 6, 10 and 30 h (**Figure 1a**). Cell migration was quantified as the percentage (%) of scratch area repopulated by migrating cells 6 hours after performing the scratch. Migrating Cavβ3-deficient MEFs closed the scratch area significantly earlier than MEFs from wild-type mice (**Figure 1a,b**). To exclude any effect of cell proliferation, the scratch migration assay was performed in the presence of either 10% or 1% FCS. At 10% FCS both processes are present, the cell proliferation and migration, whereas at 1% FCS cell proliferation is minimized. Fibroblasts in 10% (**Figure 1b**, left) or 1% FCS (**Figure 1b**, right) showed a similar migration pattern, ruling out the possibility of cell proliferation contribution to the Cavβ3 observed phenotype. Cavβ3-deficient MEFs closed the gap significantly earlier than wild-type MEFs under both conditions. To confirm the Cavβ3-dependent effect observed in β3-deficient fibroblasts, wild-type fibroblasts were transfected with siRNA to down-regulate the Cavβ3 protein (**Figure 1e**). As a control for the down-regulation, immunoblots were performed to confirm the efficiency of the siRNA treatment. Two independent *Cacnb3* specific siRNAs (siRNA1 and siRNA2), and a scrambled siRNA (as a control) were used. Fibroblasts treated with the *Cacnb3*-specific siRNAs behave like β3-deficient fibroblasts (**Figure 1d**), i.e., the migration is increased in the absence of Cavβ3 protein (**Figure 1e**).

In vivo, the dorsal skinfold chamber was implanted (**Figure 2a,b**) and a defined circular wound of 2 mm diameter was generated on the shaved back (**Figure 2b**) of the wild-type and Cavβ3-deficient mice (8 animals per genotype, 8-12 weeks old and 22-26 g weight). The wound was

performed by removing the complete skin with epidermis and dermis. To compare skin wound healing between both genotypes, the wound area in the skin fold chamber was photographed directly after wounding (day 0) and then pictures were taken 3, 6, 10, and 14 days post wounding (**Figure 2c**). The sizes of the wounds were measured on these digital images and the wound area at a given day was expressed as the percentage (%) of the initial wound area (**Figure 2d**). Wound closure is increased in β3-deficient mice compared to wild-type controls. In contrast to the wild-type, the wound in β3-deficient mice was almost completely closed already after 10 days. At day 14 post-wounding, the wounds were completely closed in both genotypes (**Figure 2c,d**).

FIGURE LEGENDS

Figure 1: In vitro scratch migration assay. (**a**) Representative images of cultures from wild-type (WT, left) and Cavβ3 KO (β3 KO, right) primary mouse embryonic fibroblasts (MEFs) immediately, 6, 10 and 30 h after performing a scratch. Images were converted into 8-bit gray scale, and the contrast, as well as brightness, were adapted to maximally visualize the cell free area. Analysis of the cell free area (% of scratch area repopulated by migrating cells) was performed on the original 24-bit RGB images. (**b**) Bar graphs showing percentages (%) of scratch area repopulated by migrating cells after 6 hours either in the presence of high (10 %, left) or low (1%, right) fetal bovine serum (FCS) in wild-type (black) and Cavβ3 KO (red) experiments (**c**) Immunoblot: Protein extracts from wild-type brain (50 µg per lane) and fibroblasts (MEFs, 100 µg per lane) using a Cavβ3 specific antibody. The β3 protein (55 kDa) is present in wild-type brain (used as a control), and fibroblasts, but is absent in protein extracts of Cavβ3-deficient brain and fibroblasts prepared from Cavβ3-deficient mice. (**d**) Summary of the percentage of scratch area repopulated by migrating cells after 6 hours in wild-type cells treated with either scrambled siRNA (control, black) or two independent *Cacnb3* siRNAs (siRNA1 and siRNA2, red open bars). (**e**) The corresponding immunoblot from the experiment in (**d**). Data are shown as mean ± SEM, p values were calculated using unpaired two-tailed Student's t-test. Panels **a** and **b** have been modified from [Belkacemi et al., 2018][4].

Figure 2: In vivo skin wound healing in mice. (**a**) Titanium frame interior side view showing one half of the titanium chamber and front side view showing the assembled titanium chamber with two symmetrical halves attached with screws and nuts. (**b**) Mouse after shaving the dorsal skin and mounting the dorsal skinfold chamber composed of two symmetric titanium frames (the weight of the titanium frame is around 2 g) and applying a circular wound (2 mm diameter). (**c**) Images of the wound directly after wounding (day 0) and 3, 6, 10, and 14 days post-wounding. The continuous process of wound closure, with complete epithelialization, is shown over 14 days in wild-type (WT, top) and Cavβ3 KO (β3 KO, bottom) mice. (**d**) At the time points indicated, the wound area was determined using a computer-assisted image analysis program and plotted as a percentage of the wound area immediately after injury at day 0 (mean ± SEM of n = 8, β3-KO mice and the corresponding wild-type control mice). P values were calculated using two-way ANOVA and Bonferroni's multiple comparisons test. Panels **c** and **d** have been modified from [Belkacemi et al., 2018][4].

DISCUSSION

In this manuscript, we describe an in vitro and in vivo wound healing assay and correlate the results obtained. For the in vitro assay, we used primary mouse fibroblasts[4,14,15] which play an important role in wound healing and tissue remodeling[11]. Other adherent cell types growing

as monolayers (e.g., epithelial cells, endothelial cells, keratinocytes) can be used as well. Plating the same number of viable and healthy cells and applying the scratch at the same degree of confluency is of paramount importance in order to obtain accurate and reproducible results. It is highly recommended to perform biological and technical replicates. In the present method, 6-well plates were used, but 12- or 24-well plates can be also used especially when cells are available only at limited numbers. In the case of siRNA treatment, immunoblot analysis after each set of experiments is mandatory to make sure that the target protein is efficiently down-regulated. Transfection reagent and time window should be tested and selected for each cell type before starting with the migration assay. In the case of fibroblasts and the *Cacnb3* gene, it took 3 to 4 days to reach the desired level of down-regulation. In contrast, the scratch migration assay needs shorter times (6 h to 24 h). To avoid high variability in the scratch size, it is mandatory that the same investigator applies the scratch for each set of experiments, that equal pressure is administered by the pipette tip and to keep the wound as much as possible vertical to the marked line at the bottom of the plate across the cell monolayer. Application of a mechanical scratch across the cell monolayer leads to the release of different cellular factors (e.g., ATP) from damaged cells into the extracellular space. These factors would induce paracrine signaling including Ca^{2+}-signaling in the neighboring cells, which in turn would influence cellular responses[16]. To avoid these effects, culture inserts can be used for plating cells and after removal of these inserts a cell-free gap is created without damaging the neighboring cells[17]. For high-throughput screening, the investigators might consider using instruments available on the market to create reproducible and consistent scratches in 96-well plates. To follow cell migration kinetics continuously over time, users can also consider using high-end commercial systems for automatic image capture. However, automated systems for scratch application and image capture are not always available because of high costs. A more accessible and cost-effective solution for time-lapse imaging would be for example using the system (ATLIS: an affordable time-lapse imaging and incubation system) described by Hernandez Vera and colleagues[18].

In the absence of any cell proliferation inhibitors, repopulation of the gap in the scratch migration assay is a combination of cell migration and cell proliferation. To monitor only cell migration, cell proliferation can be suppressed for example by treatment with either Actinomycin C[19] or Mitomycin C[20]. Adequate concentrations of these compounds must be carefully determined and tested to avoid the toxic effects of these compounds, which might affect the viability of the cells and their ability to migrate. As described in the present article, serum starvation or the reduction of the serum concentration in the culture medium is another way to reduce the effects of cell proliferation. Serum starvation is used in several other cell-based assays. It can induce a high number of cellular responses, which might interfere with the obtained results and interpretations[21]. Serum starvation must be carefully applied and its effect on the cell viability should be assessed before starting the experiment. In the present article, migration of primary fibroblasts in the presence of either 10% or 1% serum is shown (see **Figure 1b**). Migration rate, as expected, is slower at low concentrations of serum. However, β3-deficient fibroblasts migrate faster than wild-type cells at both conditions; low and high serum concentration.

The skin wound healing assay using the dorsal skinfold chamber is a relatively straightforward procedure to investigate skin wound closure over time in vivo. The implantation of the titanium dorsal skinfold chamber was described for the first time in rats[22]. Sorg et al. used this technique in SKH1-hr hairless mice to follow wound healing and formation of new blood

vessels[9]. The skinfold chamber model described in this article has many advantages over the classical wound healing assays performed on the dorsal skin, on the ear[23] or hind limb[7] of mice. Covering the wound area with a glass coverslip prevents infections and tissue trauma and limits desiccation of the wound. The observation chamber covered with the glass coverslip can be opened at any time during the healing process, allowing topical application of different pharmacologically active compounds (e.g., siRNA for Cavβ3 as solutions or ointments) and the chamber can be closed again. Murine skin wounds healing process is composed of both contraction and epithelialization[24]. Using the dorsal skinfold chamber in mice minimizes skin contraction and gives the opportunity to study mainly the epithelialization process. It provides also a clear window to observe and monitor the wound closure process. One disadvantage of the titanium chamber is that the mouse has to carry the titanium chamber, which has a weight of around 2 g (7.7% of the weight of a 26 g mouse), for 14 days. This might cause some discomfort for the mouse, although it seems that mice tolerate well this chamber and they are comfortable and can easily reach food and water. The skin wound healing model presented in this article can study only one wound per mouse. Other published methods[25,26] suggest applying two wounds per mouse which would reduce the number of animals needed for a study. It is of great importance to create a circular wound of similar size for all mice to get objective information as well as reliable and reproducible results. To take images of the wounds over time, mice were fixed on a mouse restrainer, placed on a stage, and the skinfold chamber is positioned under a stereomicroscope. Using the restrainer helps in avoiding anesthesia and minimizing stress for the mice. Mice can be sacrificed and tissues from the wounded area can be explanted and collected at different stages of the healing process (either after complete healing or at earlier time points) for histological analysis, RNA collection or protein biochemistry.

In summary, we have shown two techniques, an in vitro scratch assay using primary fibroblasts and an in vivo skin wound healing assay in mice. In both assays, wound healing/gap closure is increased in the absence of the Cavβ3 subunit of voltage-gated calcium channels. As with wild-type and Cavβ3-deficient mice or cells, both assays might well correlate in the absence or presence of other specific molecules.

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

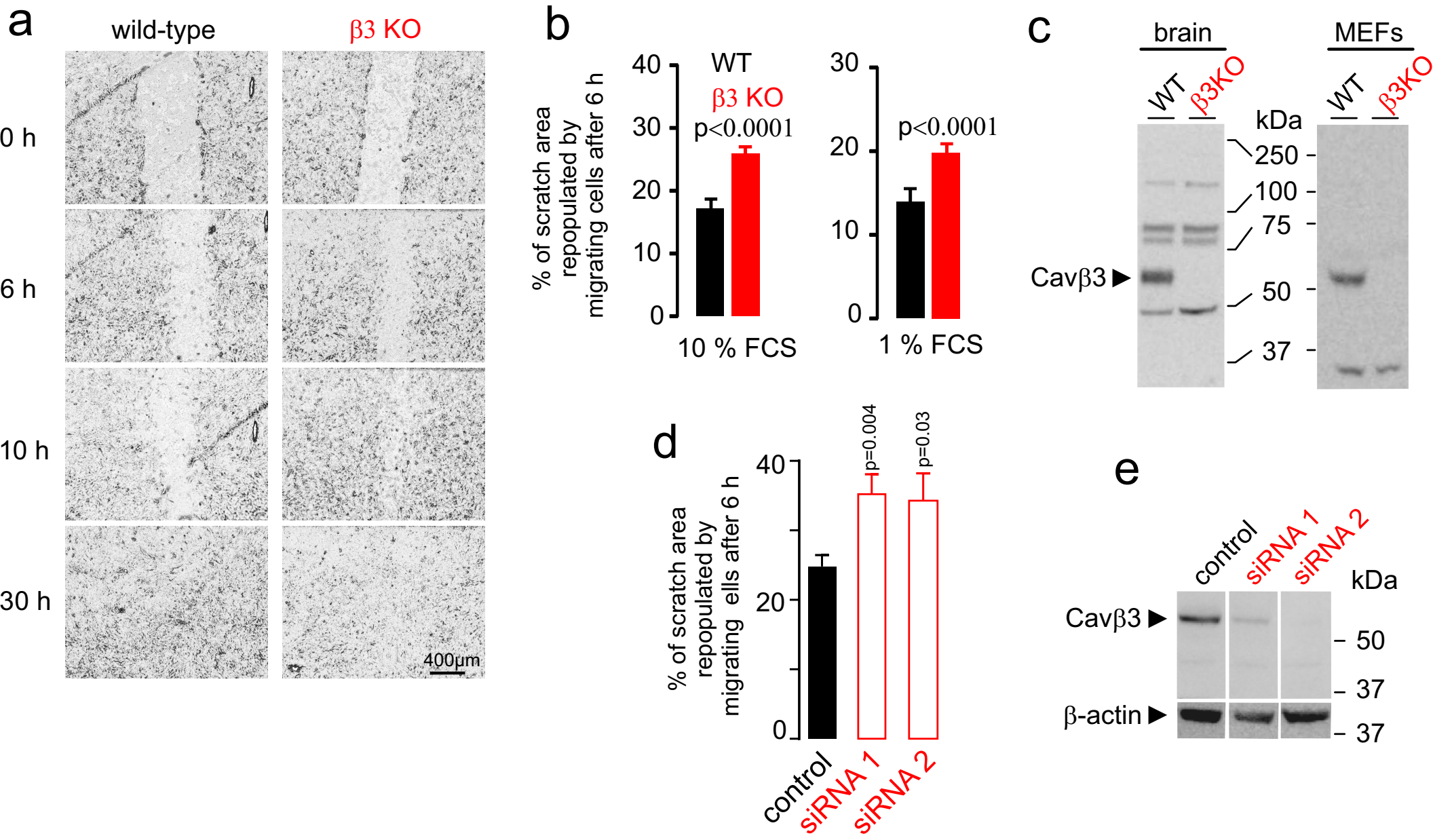
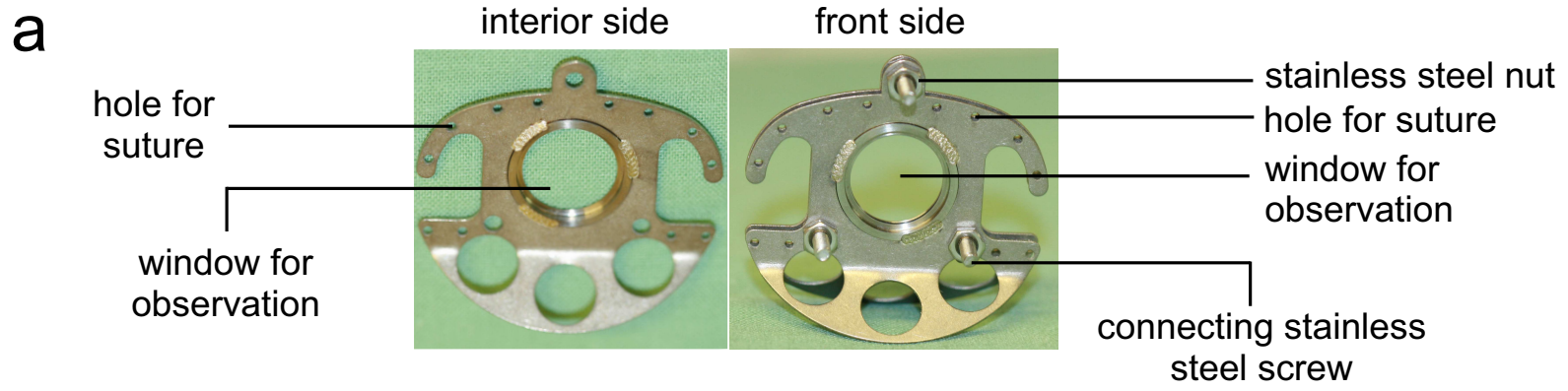
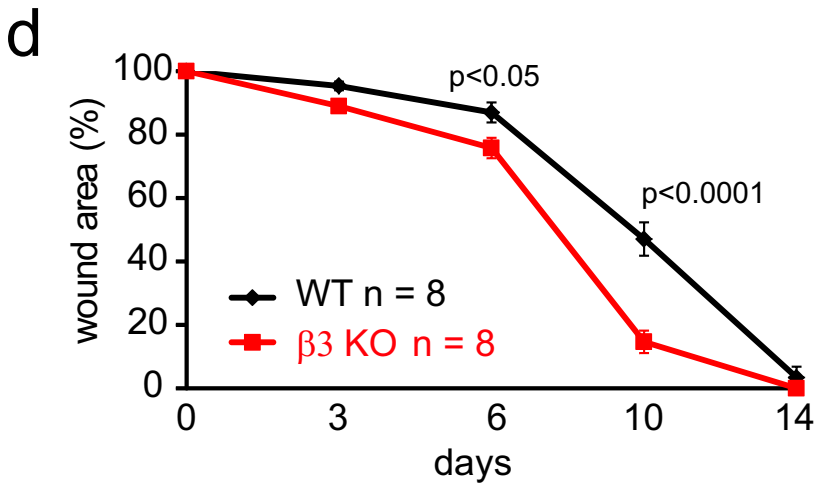
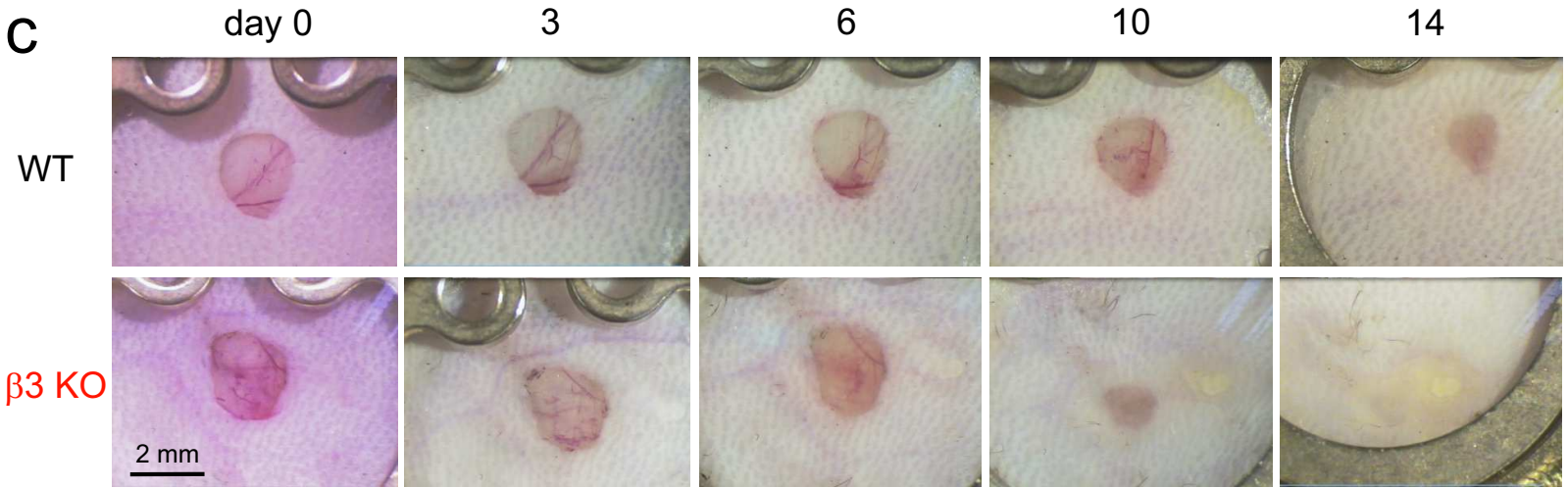


Figure 2







Name of Material/ Equipment	Company	Catalog Number
0.9 % NaCl		
1 ml syringes	BD Plastipak	303172
6 well plate	Corning	3516
Biopsy punch	Kai Industries	48201
Cacnb3 Mouse siRNA Oligo Duplex (Locus ID 12297)	Origene	SR415626
Depilation cream		
Dexpanthenol 5% (BEPANTHEN)	Bayer	3400935940179.00
Dihydroxylidinothiazine hydrochloride (Xylazine)	Bayer Health Care	
Dulbecco's Modified Eagle Medium (DMEM)	Gibco by life technologies	41966-029
Fetal bovine serum	Gibco by life technologies	10270-106
Hexagon full nut		
Ketamine hydrochloride	Zoetis	
Light microscope	Keyence, Osaka, Japan	BZ-8000
Lipofectamine RNAiMAX Transfection Reagent	Thermo Fisher Scientific	13778075
Micro-forceps		
Micro-Scissors		
Mouse restrainer	Home-made	
Normal scissors		
Objective	Nikon	plan apo 10x/0.45
Opti-MEM	Gibco by life technologies	51985-026
polypropylene sutures		
Screwdriver		
Slotted cheese head screw		
Snap ring		
Snap ring plier		
surgical microscope with camera	Leica	Leica M651
Titanium frames for the skinfold chamber	IROLA	160001
Wire piler		

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2 mm

any depilation cream
(BEPANTHEN)
Rompun 2%

KETASET
Similar microscopes might be used

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We introduced the following note before starting the protocol:
“Note: In the described method, primary fibroblasts are used. These cells play a crucial role in wound healing and tissue remodeling. We downregulated the Cacnb3 gene, encoding the Cavβ3 subunit of high voltage gated calcium channels, because it is shown to play a crucial role in cell migration in vitro and in skin wound repair in vivo.”

11. 2.5: before taking the image include a statement saying the plate was moved to the microscope stage. Also include whether or not the microscope stage maintained the cell growth condition. What kind of microscope is used and how are the images taken?
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2.7 Because the microscope stage does not maintain cell growth condition, move the plate to the cell culture incubator and keep the cells at 37 °C and 5 % CO2.
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- Yes we used eye ointment and this is introduced in the protocol and in the material list.
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17. Please move the ethics statement before the start of the protocol section.
Has been done.

18. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.
All figures are discussed and referenced in the Representative Results and Protocol.

19. Please expand the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
Representative Results are expanded, and the text refers to all figures.

20. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.
Title included. Symbols and abbreviation are described.

21. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Copyright permission to reuse is obtained and uploaded with the revised version. Figures are cited appropriately.

22. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Points in (a-e) are now considered in the revised version.

23. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. Please sort the table alphabetically.
Removed and sorted in alphabetic order.

24. Please reword lines 71-74 as it matches with previously published literature.
Line 71-74 has been rephrased.

Reviewers' comments:

Reviewer #1:

The manuscript contributes to the field by describing an integrated in vitro and in vivo approach to visualizing wound healing. Overall, the written methods do not include enough information for implementation; though the video version may contain more detail, it is still important to document the missing information.

Suggestions for improvement:
1. Provide more information about the image acquisition for the in vitro and in vivo assays
a. Bright-field, phase, DIC etc? Any filters in the light path to increase contrast?
b. Objective and NA
c. **Camera**
d. Model of the microscope and/or whether it is homebuilt

Information has been included in step 2.6 for the in vitro assay and in step 4.19 for the *in vivo* assay. Model of the microscope, objective and numerical aperture have been introduced in the material list.

More information is needed for the image analysis.
e. Format of images (non-compressed TIF for example)?
f. Any pre-processing
g. How are the images thresholded? Intensity, edge-based detection etc
h. How is the area measured over time
i. How are the images calibrated spatially
- Image format has been included: JPEG 24-bit RGB images (1360x1024)

- For the manuscript, (Figure 1a) images were converted into 8-bit gray scale, and the contrast as well as brightness were adapted to maximally visualize the cell free area, especially for the printed version. Analysis of the cell free area (% of scratch area repopulated by migrating cells) were performed on the original RGB images.

- Measurements of the % of scratch area repopulated by migrating cells is now described in the protocol 3.1 to 3.8

2. ImageJ is open source and it is critical that researchers cite them as requested by the people who lead this effort: Citation instructions here: https://imagej.net/Citing
Has been now cited correctly.

3. ImageJ not Image J (that is, no sapce between the "e" and the "J").
Has been corrected.

4. Related to the following:
32 apply a scratch on a confluent cell monolayer and follow gap closure by taking microscopic
33 images at defined time points until complete repopulation of the gap by the migrating cells.
Without direct real-time monitoring, the exact time point is not known to any precise extent as the scratches are monitored at 6, 10 and 30 hours. Let the reader know that the time points depend on the application and must be established for every preparation, though these time points are reasonable starting points.
The following sentences are now introduced after step 2.8 to highlight this point:
"These time points have been established for the described procedure and for primary fibroblasts. During the first pilot experiment, more time points where tested to see how fast fibroblasts repopulate the gap. Although 0, 6, 10 and 30 hours are reasonable starting time points, the investigators should optimize and establish the appropriate time points for each application and for each cell type. The more accurate alternative -if available- would be to use time-lapse microscopy"

5. Related to the lines below:
39...Because the results of both assays
40 correlate well, the in vitro assay may be useful for high-throughput screening before validating
41 the in vitro hits by the in vivo wound healing model.

Manual experiments are acceptable as described here. Yet it is important to give the reader the whole picture- if they are starting out in this area, there are a number of more efficient approaches.
As the authors bring up high-throughput screening using the in vitro scratch assay, it is important to mention how this could be achieved, from high-end commercial systems to more accessible and cost effective solutions, as shown here:

We addressed this point now in the discussion:
"For high-throughput screening, the investigators might consider using instruments available on the market to create reproducible and consistent scratches in 96-well plates. To follow cell migration kinetics continuously over time, and to avoid keeping employees in the laboratory for longer than 8 hours per day, users can consider also using high-end commercial systems for automatic image capture. These automated systems for scratch application and image capture are not always available because of high costs. A more accessible and cost effective solution for time-lapse imaging would be for example using the system "ATLIS" described by Hernandez Vera and colleagues (A Modular and Affordable Time-Lapse Imaging and Incubation System Based on 3D-Printed Parts, a Smartphone, and Off-The-Shelf Electronics, https://doi.org/10.1371/journal.pone.0167583)"

6. Some mention should be made about how/why the time points were selected. Why time points separated by 6, 10 hr and 30 hr? Although long experimental days are common, it is important to give a reader a sense of alternate and more efficient approaches as the 10 hr time point sometimes means that a student has to be working 10.5 hour days. Suggest checking whether time-lapse automated stage options are available in the local community of the reader.

We agree with the reviewer; using these time points means that the student has to stay longer in the lab. One of the authors performed these experiments by himself and it was doable. Taking images does not take very long time, although this depends on how many samples you have. Suggestion of time-lapse automated stage options is mentioned in the Note after step 2.8.

7. A note about the plate selection. Why 6-well plates be used when it is more cost effective to use smaller wells?
This point is now addressed after step 1.3:

"Six-well culture plates were used in this assay because they are large enough, to give sufficient space and flexibility, to apply a consistent, reproducible and vertical scratch using a 200 µl pipette tip across the cell monolayer. If a limited number of cells is available, an alternative and probably more cost efficient way would be to use 12- or 24-well culture plates"

8. The images in Figure 1 are confusing. Are they meant to represent the data as they are acquired at the microscope? They have low bit depth and it is hard to make out details. That is, the images do not resemble the natural grey scale as would be expected from standard white-light techniques. If the images are carried out in bright-field to aid the image segmentation, that this should be mentioned explicitly as in "the images are acquired using bright-field because it makes the image segmentation into cells and background i.e. the wound simpler."
Attached for the reviewer are now the original JPEG 24-bit RGB images (1360x1024) without any contrast modification (Fig. R1). For the manuscript, (Figure 1a) images were converted into 8-bit gray scale, and the contrast as well as brightness were adapted to maximally visualize the cell free area, especially for the printed version. Analysis of the cell free area (% of scratch area repopulated by migrating cells) were performed on the original RGB images.

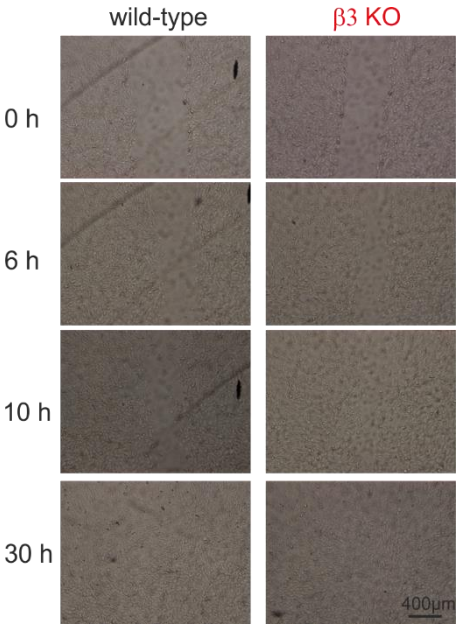

***Figure R1**: Original RGB images of the 8-bit gray scale images used for figure 1a in the manuscript.*

9. Please confirm that the western blot presentation adheres to ethical guidelines as specified here: http://crosstalk.cell.com/blog/common-pitfalls-in-figure-preparation
Note that I ask this as a matter of course when I don't see this in the journal guidelines (apologies if I missed this in the JoVE guidelines).
Yes, we confirm and we show the complete uncropped western blots for the reviewer below (Figure R2):

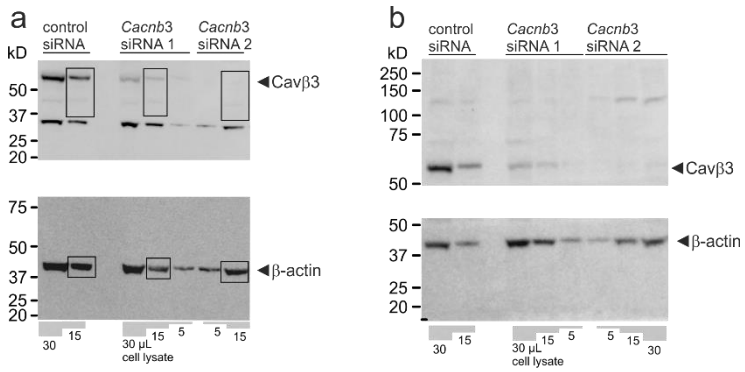

***Figure R2:** Immunoblot of protein lysates from mouse embryonic fibroblasts (MEFs) transfected with Cacnb3 siRNA1, Cacnb3 siRNA2 or scrambled siRNA as a control. immunoblots were probed with the anti Cavβ3 antibody (home-made 828) and the anti β-actin as a loading control. The boxes highlight the areas cropped for the immunoblot (Figure 1 e) in the manuscript.*

10. Why is y axis reported as a migration rate. "Migration rate" implies units of distance over time. Here the results are reported as % of the wound area and thus it would be hard to reproduce from lab to lab. Again, as this is a methods paper, good practice would be to report the wound healing in a way that could be reproduced by other labs. For example, the area of the wound could be measured and then the % filling in could be reported at a time point.
We agree with the reviewer and the Y axis in the migration is corrected into *"% of scratch area repopulated by migrating cells after 6 hours"*

11. Why then are 3 time points required if only one time point is reported?
At time point 10 hours the cell-free area was almost closed and it was not easy to mark this area and calculate the % of repopulation. Therefore, we decided to calculate only the 6 hours.

12. What was the rationale for targeting the beta3 subunit of the Cavbeta3 channel?
Related to the following lines:
Here we identify the β3 subunit of

27 voltage-gated calcium channels (Cavβ3) as a potential target molecule to influence wound
28 healing in two independent assays, i.e. the in vitro scratch migration assay and the in vivo
29 dorsal skinfold chamber model.
Although the methodology is in the spotlight, a 1-2 sentences to provide context on the choice of this channel. Why is it important in tissue healing?
This we have published previously: Belkacemi et a., 2018 Cell Reports. In fact we started with the *in vitro* scratch assay to characterize Cavβ3 protein-deficient fibroblasts compared to fibroblasts from wild-type mice as controls. Because the in vitro scratch assay is also dubbed "*in vitro* wound healing assay" we wanted to know, whether there is any correlation with *in vivo* wound healing at all. We introduce the following sentences in the introduction:
"We identified the β3 subunit of high voltage-gated calcium channels (Cavβ3) as a potential target molecule to influence skin wound healing using two independent protocols, i.e. the in vitro scratch migration assay and the in vivo dorsal skinfold chamber model. For the in vitro assay, we used primary fibroblasts, These cells do express the Cacnb3 gene encoding the Cavβ3 protein but lack depolarization-induced Ca^{2+} influx, or voltage-dependent Ca^{2+} currents. We described a novel function of Cavβ3 in these fibroblasts: Cavβ3 binds to the inositol 1,4,5-trisphosphate receptor (IP3R) and constrains calcium release from the endoplasmic reticulum. Deletion of the Cacnb3 gene in mice leads to increased sensitivity of the IP3R for IP3, enhanced cell migration and increased skin wound repair"

13. Accelerated should be changed to increased or decreased in this paper, depending on the context. Acceleration is the rate of change of velocity per unit of time and this has not been measured in this paper.
Accelerated is now changed into *"increased"*.

14. Related to the lines here:
269 ....At the end of the wound healing
270 experiment, mice will be sacrificed and tissues can be collected at different stages for
271 histological analysis, RNA collection or protein biochemistry.
Mention the wound size vs. the imaged area for the vivo work. Some description about how to ensure that the wound area signal is not overwhelmed by the background tissue signal is needed for the assays. For the histology, how can the reader choose the appropriate field to examine in the tissue?
We tried to make it clear for the reader now:
"Mice can be sacrificed and tissues from the wounded area can be explanted and collected at different stages of the healing process (either after complete healing or at earlier time points) for histological analysis, RNA collection or protein biochemistry."

15. If a circular wound is used in vivo why was a scratch wound used in vitro as the geometry of the scratch can influence the physiological response?
We are aware that the *in vivo* environment is more complex than *in vitro*. The *in vitro* scratch assay resembles a limited model for wound healing since it informs about migration of one cell type in a dish. The scratch assay does not represent the *in vivo* physiological situation but it is a simple model for studying migration and wound healing *in vitro*.

16. The lines here
261...The
262 observation chamber covered with the glass coverslip allows topical application of different
263 reagents at any time during the healing process.
Describe how the topical application can be done using this in vivo window.
The following short explanation is introduced in the discussion:
"The observation chamber covered with the glass coverslip can be opened at any time during the healing process, allowing topical application of different pharmacologically active compounds (for example siRNA for Cavβ3 as solutions or ointments; we are currently applying for permission to do such experiments) and the chamber can be closed again".

17. "Western" is "western" though this may be a nit-picky point
See below:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5240824/

Thank you for the comment, but please check "Transparency Is the Key to Quality" by Amanda J. Fosang and Roger J. Colbran, an editorial of the THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 50, pp. 29692–29694, December 11, 2015.

We replaced "Western" by immunoblot throughout.

18. Referring to the lines below:
110 1- Examine the cells under the microscope: Start with the scratch (step 3) only when they
111 reach 100 % confluence.
For reproducible results, emphasize that the wound should be made at the same time point; that is, day 0 confluence vs day 2 after confluence can evoke different responses.
The following sentences are introduced after step 2.1:
"Note: For accuracy and reproducibility, 100 % confluency is a mandatory factor for starting the scratch migration assay. Therefore, it is important to seed the same number of cells into the culture wells, to examine each well for confluency and to apply the scratch at the same time point (day 0 confluency). Waiting longer after the cells reach 100 % confluency can evoke different responses".

19. How deep is the skin punch wound? Model of biopsy punch needed in the Material/Equipment listing.
The type of punch is included in the Material list.
We used the biopsy punch (2 mm in diameter; pfm Medical, Köln, Germany) to mark the region of the wound. Then the wound was created by removing the complete skin with epidermis and dermis with the subcutaneous tissue remaining, using fine Scissors and forceps.

20. Related to the following lines:
164....Pay
165 careful attention to the tightness of the screws at this step, because if it is too loose; it
166 might detach. If it is too tight; it will reduce blood flow and can lead to necrosis of the
167 tissue.
Can the authors provide some guidance here based on their experiences" The reader is cautioned yet it is hard to know how to tell if it is too loose or too tight. Describe what to look for in the blood flow, for example.
Is there a spacer or other external gauge that could be applied esp. if it can avoid cutting off the end of the screw? See comment 21.

The following note has been introduced after step 4.13:
"Note: Nuts prepared in step 4.6 serve as spacer to keep a distance of 400-500 µm between the two symmetrical halves of the titanium chamber. The nuts should be tightened until a slight resistance is felt".

21. Related to the following line:
168 12- Cut the remaining part of the screws using a plier
Some mention of eye protection that can withstand the recoil if the screw comes off the wrong way.
The following note is introduced after step 4.14.
" It is necessary at this step to use laboratory safety glasses for eye protection in case the screw comes off the wrong way."

22. Refers to the lines below

- Place the Plexiglas stage under a stereomicroscope and take images with different 188 magnifications

It is important to specify the magnifications here and to remind the reader to analyze the data immediately after the first imaging system to ensure that the data are of sufficient quality for analysis.

Magnifications are introduced in step 4.19.

This note is introduced after step 4.19:

"The investigator should examine the images immediately after capture to ensure that the data are of sufficient quality for the off-line analysis".

Reviewer #2:

Manuscript Summary:

The manuscript describes two techniques to analyse the wound healing process: the scratch migration assay and the dorsal skinfold chamber, in vitro and in vivo, respectively. Despite these techniques are not innovative, the visualization of both experimental procedures and their detailed explanation could help researchers to use them in a much better way.

Major Concerns:

In the discussion, the authors explain the importance of inhibitors, such as Mitomycin C, to monitor cell migration in wound healing assay. Did they test its effect in their experiments?

We did not try neither Actinomycin C nor Mitomycin C in our experiments, and we mentioned them in the discussion as a possibility to inhibit cell proliferation. We also suggest to carefully determine adequate concentrations of these compounds to avoid cell toxicity. We used serum starvation to inhibit cell proliferation.

This sentence in the discussion is reworded to make it clear for the reader that it is a suggestion:

"To monitor only cell migration without the influence of cell proliferation, cell proliferation can be suppressed for example by treatment with either Actinomycin C or Mitomycin C. Adequate concentrations of these compounds must be carefully determined and tested to avoid toxic effects of these compounds, which might affect the viability of the cells and their ability to migrate.".

Figure 1 is not easy to understand. Please, reorganize it. I would suggest to separate panels c, d and e.

Figure 1 is reorganized as suggested.

The β-actin should be shown also in figure 1 c. Red β3-KO is not indicated in brain. Why?

A new western blot is shown in Figure 1b. The antibody for Cavβ3 decorates proteins not related to Cavβ3 in protein lysates from wild-type and Cavβ3-dedicient mice. The amount of these additional proteins, the identity of which is not known, were used as loading controls.

In figure 1d, it seems that the use of siRNA1 is more efficient in promoting migration; in fact the authors show a p value lower than siRNA2. However, this result does not seem to correlate with the light band shown for β3 protein. Please explain it.

For scratch migration, we have used 8 wells for each condition (scrambled, siRNA1 and siRNA2). Two extra wells (for each condition) were collected for immunoblot analysis to control the siRNA efficiency. We agree with the reviewer, that in the Western blot shown, it seems that the siRNA2 is more efficient than siRNA1. We believe this finding rather be caused by efficacy of the transfection procedure and this is the reason why it is recommended to use independent siRNA preparations and transfections. We also regularly observe similar differences when transfecting cells with protein tagged with fluorescent proteins: Although transfection was always done according to the same protocol, we observe slight differences in the transfection efficiency from one dish to another dish, although they have been treated in the same way in parallel.

In figure 1 e: Why is the molecular weigth of 37 kDa close to the band of protein β3?

Shown are two independent immunoblots (Figure R2 below): β actin is a 41.7 kDa protein (calculated Mr), Cavβ3 a 54.6 kDa protein (calculated Mr): Both proteins run in the SDS PAGE according to their Mrs.

Was the loaded amount of siRNA1 equal to siRNA2? The bands of β-actin are different from each other.

The volume of cell lysate applied into the gel is equal, but it seems from the beta-actin band that the amount of protein for the siRNA1 is a bit less than the amount of siRNA2.

We include in the following Figure for the reviewer containing the complete uncropped western blot.

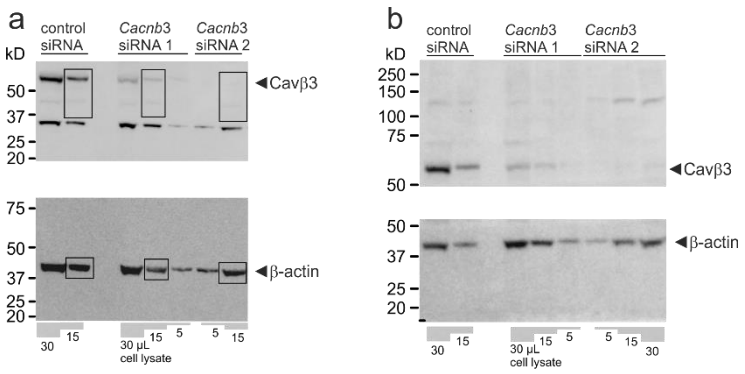

Figure R2: *Immunoblot of protein lysates from mouse embryonic fibroblasts (MEFs) transfected with Cacnb3 siRNA1, Cacnb3 siRNA2 or scrambled siRNA as a control. Immunoblots were probed with the anti Cavβ3 antibody (home-made 828) and the anti β-actin as a loading control. The boxes highlight the areas cropped for the immunoblot (Figure 1 e) in the manuscript.*

Minor Concerns:
In Figure 1 is MEFs the abbreviation of Mouse Embryonic Fibroblasts? please indicate it in brackets at lines 29 and 86.
Figure 2 "ko" is "KO".
That's correct, MEFs is the abbreviation of Mouse Embryonic Fibroblasts and this is now mentioned.

Avoid the use of personal pronouns.
Has been done now throughout the protocol.

Since the authors analyse and monitor cell migration, it would be helpful to indicate are other methods for studying the in vitro wound healing process in a highly reproducible manner (see PMID: 29608162; PMID: 27671059).
This point now is indicated in the revised manuscript and the methodological articles are cited.

Line 128 "The rate of migration is than calculated" "than" is "then"
Line 149 replace "is be clean" with "is clean"
Line 211 "Cavβ3 mice" is "Cavβ3-deficient mice"?
Line 218 "arround" is "around"
Has been corrected.

In vitro and *in vivo* analysis of wound healing by means of the scratch migration assay and the dorsal skinfold chamber

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KEYWORDS
Wound healing, scratch migration, dorsal skinfold chamber, fibroblasts, siRNA transfection, Cavβ3

SUMMARY

Here, we present a protocol for an *in vitro* scratch assay using primary fibroblasts and for an *in vivo* skin wound healing assay in mice. Both assays are straightforward methods to assess *in vitro* and *in vivo* wound healing.

ABSTRACT

Impaired cutaneous wound healing is a major concern for patients suffering from diabetes and for elderly people, and there is a need for efficient treatment. ~~Thus,~~ Appropriate *in vitro* and *in vivo* approaches are essential for the identification of new target molecules for drug treatments to improve the skin wound healing process. ~~Here we identify~~We identified the β3 subunit of voltage-gated calcium channels (Cavβ3) as a potential target molecule to influence wound healing in two independent assays, i.e. the *in vitro* scratch migration assay and the *in vivo* dorsal skinfold chamber model. ~~We use~~ Primary mouse embryonic fibroblasts (MEFs) acutely isolated from wild~~-~~-type (WT) and Cavβ3-deficient mice ~~(~~β(Cavβ3 KO) or fibroblasts acutely isolated from WT mice treated with siRNA to down-regulate expression of the *Cacnb3* gene, encoding Cavβ3~~. We apply~~, were used. A scratch was applied on a confluent cell monolayer and ~~follow~~the gap closure was followed by taking microscopic images at defined time points until complete repopulation of the gap by the migrating cells. These images ~~are~~were analyzed and the cell migration rate ~~is~~was determined for each condition. In ~~addition~~an *in vivo* assay, we ~~implant~~implanted a dorsal skinfold chamber on WT and Cavβ3 KO mice, ~~apply~~applied a defined circular wound of 2 mm diameter, ~~cover~~covered the wound with a glass coverslip to protect it from infections and desiccation, and ~~monitor~~monitored the macroscopic wound closure over time. ~~Gap~~Wound closure ~~/ wound healing is~~was significantly ~~accelerated~~faster in *Cacnb3*-gene-deficient mice ~~and in β3 KO fibroblasts or WT fibroblasts transfected with siRNA targeted to β3.~~. Because the results of ~~both~~the *in vivo* and the *in vitro* assays correlate well, the *in vitro* assay may be useful for high-throughput screening before validating the *in vitro* hits by the *in vivo* wound healing model. What we have shown here for wild~~-~~-type and Cavβ3-deficient mice or cells might also be applicable for specific molecules other than Cavβ3.

INTRODUCTION

Skin wound healing starts immediately after skin injury in order to restore the skin's integrity and to protect the organism from infections. The wound healing process goes through four overlapping ~~stages~~phases; coagulation, inflammation, new tissue formation and tissue remodeling[1]. Cell migration is crucial during these ~~stages~~phases. Inflammatory cells, immune cells, keratinocytes, endothelial cells and fibroblasts are activated at different time points and invade the wound area[2]. Methods to investigate wound healing *in vitro* and *in vivo* ~~wound healing~~ are of great interest not only to understand the underlying mechanisms, but also to test new drugs and to develop new strategies aiming to ameliorate and accelerate skin wound healing.

To monitor and analyze ~~and monitor~~ cell migration ~~in~~, the scratch migration assay~~,~~ can be used. It is often referred to as "*in vitro* wound healing~~", is a simple and relatively inexpensive assay~~". This method requires a cell culture facility[3]. It ~~does not require~~is a simple procedure, there is no need of high-end equipment and the assay can be performed in most cell biology laboratories. In this assay, a cell-free area is created by mechanical disruption of a confluent cell monolayer ~~of cells~~, preferably epithelial- or endothelial-like cells or fibroblasts. Cells on the edge of the scratch will migrate in order to repopulate the created gap. Quantification of ~~this~~the decreasing cell-free area over time resembles the migration rate and indicates the time, which the cells need to close the gap. For this purpose, ~~one~~investigators can use either acutely isolated cells from WT mice or mice lacking a gene of interest[4], or immortalized cells available from reliable cell repositories ~~or commercial sources. This method also~~. The scratch assay allows studying the influence of pharmacologically active compounds or the effect of transfected cDNAs or siRNAs on cell migration.

In vivo, wound healing is a complex physiological process, requiring different cell types including keratinocytes, inflammatory cells, fibroblasts, immune cells and endothelial cells in order to restore the skin's physical integrity as fast as possible[1]. Different methods to study *in vivo* wound healing have been developed and used over the past[5-8]. The dorsal skinfold chamber ~~we describe~~described in this article was originally modified for wound healing assays by Sorg et al.[9]. It applies a modified dorsal skinfold chamber preparation to mice. The ~~advantages of this~~ modified skinfold chamber model ~~are manifold. First, it prevents~~ has several advantages: 1) It minimizes skin contraction, which ~~impedes following~~prevents observing the wound healing process and might influence wound repair in mice. ~~Second, it allows~~2) This chamber makes use of covering the wound with a glass coverslip, ~~curtailing~~ reducing tissue infections and desiccation ~~and tissue trauma~~, which ~~might~~could delay the healing process. ~~Third, wound healing can be continuously followed under the microscope, as shown in Figure 2b. Fourth,~~3) Blood flow and vascularization can be directly monitored. ~~Fifth,~~4) It allows repetitive topical application of pharmacologically active ~~drugs~~compounds and reagents in order to treat the wound and accelerate healing[9, 10].

We identified the β3 subunit of high voltage-gated calcium channels (Cavβ3) as a potential target molecule to influence skin wound healing using two independent protocols, i.e. the *in vitro* scratch migration assay and the *in vivo* dorsal skinfold chamber model. For the *in vitro* assay, we used primary fibroblasts, These cells do express the *Cacnb3* gene encoding the Cavβ3 protein but lack depolarization-induced Ca^{2+} influx, or voltage-dependent Ca^{2+} currents. We described a novel function of Cavβ3 in these fibroblasts: Cavβ3 binds to the inositol 1,4,5-trisphosphate receptor (IP3R) and constrains calcium release from the endoplasmic reticulum. Deletion of the *Cacnb3* gene in mice leads to increased sensitivity of the IP3R for IP3, enhanced cell migration and increased skin wound repair[4].

PROTOCOL

~~1.~~ All experimental procedures were approved and performed in accordance with the ethic regulations and the animal welfare committees of Saarland and Saarland University.

1 Primary cell culture and siRNA transfection

Note: In the described method, primary fibroblasts are used. These cells play a crucial role in wound healing and tissue remodeling[11]. We down-regulated the Cacnb3 gene, encoding the Cavβ3 subunit of high voltage-gated calcium channels[12], because we could show that it plays a crucial role in cell migration in vitro and skin wound repair in vivo[4].

1.1 Preparation of siRNA: Before reconstituting the siRNAs, briefly centrifuge the tubes to ensure that the content is on the bottom. Reconstitute the siRNAs in 100 µl RNase-free buffer, provided by the manufacturer and containing 100 mM potassium acetate, 30 mM HEPES, pH 7.5 to prepare 20 µM stock solution of siRNAs.

1.2 Freeze 10 µL siRNA (20 µM concentration) aliquots at -20 °C until use.

~~1. Here we use primary fibroblasts, which were isolated from wild type and β3 deficient mice as described previously[4].~~

1.3 Using an ultrafine permanent marker, mark a 6-well plate with a horizontal line at the bottom of each well in order to be able to always identify ~~later~~ the same scratch region of interest and to follow ~~the~~its closure ~~of the scratch.~~.

Note: Six-well culture plates were used in this assay because they are large enough, to give sufficient space and flexibility, to apply a consistent, reproducible and vertical scratch using a 200 µl pipette tip across the cell monolayer. If a limited number of cells is available, an alternative and probably more cost efficient way would be to use 12- or 24-well culture plates.

1.4 Plate ~~cells~~ primary fibroblasts, isolated from wild-type and β3-deficient mice[4], in a 6-well plate at a density of 5 x 10^5 cells/well in the presence of 2 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FCS).

Note: Cells should be handled in a sterile environment such as biological safety cabinets class II.

1.5 Label the 6-well plate with the cell type, genotype and the date.

Note: 5 x 10^5 cells per well has been established for 6-well culture plate and for primary mouse fibroblasts. Tests may be needed if using 12- or 24-well cell culture plates or other cell types, which could be different in size.

~~1.5~~1.6 Move the 6-well plate into the cell culture incubator and maintain cells at 37 °C and 5 % CO_2 for 24 hours.

~~1.6~~1.7 Next day, take cell out of the incubator, aspirate cell culture medium out of the well, discard it and replace it with 2.25 mL fresh culture medium by adding it carefully against the wall of the well.

1.8 In order to transfect fibroblasts with siRNAs, ~~we use the Lipofectamine RNAiMAX Transfection Reagent and follow~~the transfection reagent, listed in the materials table, was used following the manufacturer´s protocol.

Note: Other transfection reagents might work equally effective but have not been tested by us.

~~1.7~~1.9 For each well prepare the following~~.~~: Tube (1~~): dilute~~) contains 9 µL of the ~~Lipofectamine RNAiMAX Transfection Reagent~~ transfection reagent diluted in 150 µL Opti-MEM~~.~~ and tube (2~~): Dilute~~) contains 1.5 µL siRNA (*Cacnb3* siRNA-~~A~~1, *Cacnb3* siRNA-~~B~~2 or scrambled siRNA as a negative control) diluted in 150 µL Opti-MEM.

~~1.8~~1.10 Add diluted siRNA tube (2) ~~to~~into diluted ~~Lipofectamine RNAiMAX~~ transfection reagent tube (1) and vortex for 2 s.

~~1.9~~1.11 Incubate the mixture for 5 min at 21 °C.

1.12 Label wells with either *Cacnb3* siRNA-1, *Cacnb3* siRNA-2 or scrambled siRNA.

~~1.10~~1.13 Add 250 µL of the siRNA-~~RNAiMAX~~transfection reagent mixture dropwise to the cells.

~~Incubate~~

~~1.11~~1.14 Place the 6-well culture plate back into the incubator and keep cells at 37 °C and 5 % CO_2 for 72 h.

~~1.12~~1.15 In order to control the efficiency of *Cacnb3* gene silencing, collect transfected cells and perform ~~Western blot~~immunoblot analysis as described previously[4]. ~~A representative result is shown in Figure 1c and e.~~

2 ~~2~~ *In vitro* wound healing assay (Scratch migration assay)

2.1 Take the cell culture well palate out of the incubator and examine the cells under the microscope using 10X objective: Start with the scratch ~~(step 3)~~assay only when they have reached 100 % confluency.

Note: Note: For accuracy and reproducibility, 100 % confluency is a mandatory factor for starting the scratch migration assay. Therefore, it is important to seed the same number of cells into the culture wells, to examine each well for confluency and to apply the scratch at the same time point (day 0 confluency). Waiting longer after the cells reach 100 % confluence.confluency can evoke different responses".

2.12.2 Aspirate culture medium out of the well and discard it.

~~Scratch manually the cells using~~

2.22.3 Use a pipette tip (200 µL) to manually create a ~~smooth scratch over~~scratch vertical to the horizontal line marked at the bottom of the well, across the confluent cell monolayer in the middle of the well.

~~Wash cells~~

2.32.4 Rinse each well twice with 2 mL phosphate-~~-~~buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH7.4 to remove ~~the remaining~~released factors from damaged cells, loose cells, and debris ~~in~~from the scratched area. Add the 2 mL PBS carefully against the wall of the well to avoid detaching cells from the cell culture well.

2.42.5 Add 2 mL cell culture medium containing either 10 % serum or 1 % serum carefully to each well.

~~Take~~

Note: It is recommended to perform the scratch assay under 10 % serum and under 1 % serum to confirm that the observed effect is caused by cell proliferation and migration or by cell migration only.

2.52.6 Move the plate to the microscope stage and capture bright field images of the cell-free area (two ~~pictures~~areas per well) immediately after scratching (t=0h) at a 10X magnification using a light microscope ~~equipped with a camera. Take images above and below the~~ . To image always the same region of the scratch, use the horizontal line, which ~~you~~was prepared ~~before~~in step (1.3), and take one image above this line and one image below this line. Save images as TIFF or JPEG.

2.62.7 Because the microscope stage does not maintain cell growth condition, move the plate back to the cell culture incubator and keep the cells at 37 °C and 5 % CO_2.

~~Take images again~~

2.8 After 6, 10 and 30 h~~. This~~, move the plate to the microscope stage again and capture images the same way as described in step 2.6.

Note: These time ~~frame has~~points have been ~~determined~~established for the described procedure and for primary fibroblasts ~~and~~ . During the first pilot experiment, more time points where tested to see how fast fibroblasts repopulate the gap. Although 0, 6, 10 and 30 hours are reasonable starting time points, the investigators should ~~be optimized for each other~~optimize and establish the appropriate time points for each application and for each cell type. The ~~line on the bottom of the 6-well plate is used as reference to recognize the same region of interest at each time point~~more accurate alternative -if available- would be to use time-lapse microscopy.

2.9 Using ~~Image J,~~ImageJ[13], quantify the initial cell-free area (100 %) and the remaining area after 6, 10 and 30 hours. The ~~rate~~percentage of ~~migration~~scratch area repopulated by migrating cells is ~~than~~then calculated relative to the initial scratch area. ~~Typical result~~

3 Analysis of the scratch area

3.1 Open ImageJ software[13].

3.2 Upload the first image as JPEG (e.g. 24-bit RGB images 1360x1024) by dropping the image into the ImageJ menu bar.

3.3 Select the "Freehand selections" button and mark the cell-free area

3.4 Click on "analyze" and select measure. After clicking "Measure", a window with the results will appear containing the area value.

3.5 Transfer this value into an excel sheet.

3.6 Repeat steps 3.2-3.5 for each image from time point 0h and then start again for the next time points 6, 10 and 30 hours.

3.7 Calculate the percentage of scratch ~~images and statistical analysis~~area repopulated by migrating cells after 6, 10 and 30 hours for each scratch using the following equation:

$$\%\ of\ scratch\ area\ repopulated\ by\ migrating\ cells\ after\ xh = 100 - \left(\frac{b \cdot 100}{a}\right)$$

a = cell free area of ~~fibroblasts migration rate are shown in Figure 1a~~the initial scratch, b~~, d.~~ = cell free area after 6 hours

3.8 ~~3.~~ Calculate the mean and the standard error of the mean (S.E.M.) for the percentage of scratch area repopulated by migrating cells after 6 hours. Show data as column bar graph or scatter plot.

~~3~~4 *In vivo* skin wound healing assay

~~*All experimental procedures were approved and performed in accordance with the ethic regulations and the animal welfare committees of Saarland and Saarland University.*~~

~~3.1~~4.1 One day before starting the experiment, autoclave surgical instruments, screws, nuts and titanium frames to be used for the skinfold chamber~~, screws and nuts~~ preparation.

Note: The titanium frame is composed of two symmetrical complementary halves and it has a circular observation window where the wound will be applied and followed by microscopy (see Figure 2 a).

~~3.2~~4.2 Anesthetize a wild-type or β3-deficient mouse (22-26 g body weight) by intraperitoneal (i.p.) injection of 0.1 mL saline/10 g body weight containing a mixture of ketamine (75 mg/kg body weight) and xylazine (25 mg/kg body weight).

Note: This ~~anesthesia~~injection gives ~~you~~ around 30 minutes surgical anesthesia and the depth of anesthesia has to be controlled ~~over time~~through the surgical procedure, by checking the reflexes of the mouse.

4.3 To avoid dryness or damage of the eye, apply ophthalmic ointment to both eyes and repeat application if necessary.

~~3.3~~4.4 Carefully shave the mouse dorsum, using ~~a shaving machine~~an electric shaver followed by application of a depilation cream~~.~~ to the shaved area to remove any remaining hair. Take care not to injure the mouse skin.

4.5 The depilation cream needs around 10 min to ~~remove~~ completely ~~the~~remove all hair. ~~During this time, take~~

~~3.4~~4.6 Prepare the titanium chamber by taking one part of the symmetrical titanium chamber frame, and fix ~~on one side~~ the connecting screws with nuts on one side. These nuts will serve as spacer to keep a distance of 400-500 µm between the two symmetrical parts of the chamber ~~and~~ to avoid compression of blood vessels ~~on~~in the skin.

~~3.5~~4.7 Remove the cream from the back of the mouse and clean the hair-free region with warm (35-37 °C) tap water.

~~3.6~~4.8 Make sure that the surgery place is ~~be~~ clean, warm (37 °C) and humidified.

~~3.7~~4.9 Disinfect the hair-free area of the mouse with 70 % (vol/vol) ethanol ~~and then, take the sandwiched skinfold and by using~~. Take a fold of the back skin of the mouse in front of a light source ~~decide~~ and position the middle line of the double layer of the skin where ~~to implant~~ the titanium chamber~~.~~ will be implanted. After that, fix the skinfold ~~then~~ with a ~~prolene~~polypropylene sutures cranially and caudally and ~~hang~~tighten the other side of the suture on a metal rack~~.~~ to lift the mouse folded skin. Adjust the height of the rack to allow the mouse to sit comfortably.

4.10 Implant the titanium chamber into the fold of the back skin of the mouse in a way to sandwich the folded dorsal skin layer between the two symmetrical halves of the titanium frame. Attach ~~by prolene~~the first half of the titanium frame by polypropylene sutures ~~the titanium frame prepared in (step 4)~~ on its superior edge to the back of the dorsal skinfold.

Note: On the titanium frame, there are ~~8~~eight holes on the superior edge (Figure 2 a) and the ~~skinfold~~folded skin should be well fixed by ~~prolene~~polypropylene sutures on each of the eight holes.

~~3.8~~4.11 Before moving to the next step ~~10;~~, check reflexes of the mouse to make sure that the depth of anesthesia is maintained.

~~3.9~~4.12 At the base of the skinfold, pass the two connecting screws ~~prepared in (step 4)~~, attached to the first half of the titanium chamber, to penetrate the skinfold from back to the front side. ~~Making~~Make small incisions on the skin (using fine scissors ~~can~~) to help smooth penetration of the connecting screws.

~~Attach~~

~~3.10~~4.13 Detach the mouse from the rack and place it on a lateral position. Put the second ~~symmetrical part~~complementary half of the titanium chamber ~~directly~~ on top of the 3 connecting screws (~~prepared on step 4~~see Figure 2 a) and ~~fix it then~~apply slight pressure with fingers in order to pass these screws through the second half of the titanium frame. Then, fix both symmetrical parts with stainless steel nuts. Pay careful attention to the tightness of the screws at this step, ~~because~~since it might detach, if it is too loose~~; it might detach.~~. In contrast, if it is too tight~~;~~: it will squeeze the skinfold, reduce blood flow and can lead to tissue impairment and necrosis ~~of the tissue~~.

Note: Nuts prepared in step 4.6 serve as spacer to keep a distance of 400-500 µm between the two symmetrical halves of the titanium chamber. The nuts should be tightened until some slight resistance is felt.

~~3.11~~4.14 Cut the remaining part of the screws using ~~a plier~~pliers.

~~In the center of the skin bordered by the observation window of the skinfold chamber,~~

Note: It is necessary at this step to use laboratory safety glasses for eye protection in case the screw comes off the wrong way.

4.15 Mark the wound area by a standardized biopsy punch (2 mm in diameter), in the center of the skin within the observation window (see Figure 2a) of the skinfold chamber in order to ensure reproducible wound sizes.

~~3.12~~4.16 By using fine forceps and scissors, create a circular wound within the marked area by removing the complete skin with epidermis and dermis. The final wound area will be around 3.5-4.5 mm^2, see Figure 2 b.

~~3.13~~4.17 Clean the wound with 0.5 mL sterile saline (0.9 % NaCl, 37 °C~~)~~).

~~3.14~~4.18 Cover the wound with a glass coverslip and fix this glass coverslip with a snap ring using the snap ring plier on the titanium chamber.

~~3.15~~4.19 Immediately after finishing the surgical procedure, place the mouse on ~~a homemade Plexiglas platform and take pictures (day 0) under~~ the imaging stage of a stereomicroscope equipped with a camera and take images (day 0) under illumination. Use the 40X magnification, and save the images for future off-line analysis.

Note: ~~This~~The investigator should examine the images immediately after capture to ensure that quality is sufficient for future off-line analyses. The preparation of the skinfold chamber and performance of the skin wound takes around 30 minutes.

~~3.16~~4.20 Keep the mouse at a warm ~~area~~place during recovery from anesthesia ~~and post-surgically~~ for at least 2 hours. Thereafter, transfer mice in individual cages back to the animal facility~~.~~ (12 hours light/dark cycle) and make sure that ~~the mouse has enough~~mice have access to food and water.

~~1- Keep mice at individual cages.~~

~~3.17~~4.21 Three days post-wounding, place the mouse in a ~~Plexiglas~~ mouse-restrainer~~.~~ and fix the restrainer on top of the ~~Plexiglas~~imaging stage.

~~3.18~~4.22 Place the ~~Plexiglas~~ stage under a stereomicroscope ~~and take images with different magnifications.~~equipped with a camera. Take images under illumination with 40X magnification, record all pictures and save them for future off-line analyses

~~3.19~~4.23 Repeat ~~step 20~~steps 4.21 and ~~21~~4.22 again at day 6, 10 and day 14 post-wounding.

~~3.20~~4.24 Use the wound images ~~taken on step 21~~, for off-line analysis ~~using Image J.~~in ImageJ[13]. The wound area at day 0 is considered as 100 % and the wound closure over time is plotted relative to the initial wound area. Representative results are shown in Figure 2 c and d.

4.25 Calculate the percentage (%) of the wound area at each time point using the following equation:

$$\%\ of\ wound\ area\ at\ t(x) = \left(\frac{b \cdot 100}{a}\right)$$

x: time point (day 0, 3, 10 or 14), *a*: wound area at day 0, *b*: wound area at time point *x*

REPRESENTATIVE RESULTS

~~Representative results show primary fibroblasts migration in an *in vitro*~~The scratch assay ~~over time. Cells lacking the β3 protein migrate faster compared with wild type control cells. When wild~~ was performed on a confluent cell monolayer of wild-type and β3-deficient MEFs (Figure 1 c). After performing the "scratch" using a 200 µL pipette tip, cells from both genotypes migrate into the scratch area and close the gap. Images were taken after 6, 10 and 30 hours (Figure 1 a). Cell migration was quantified as percentage (%) of scratch area repopulated by migrating cells 6 hours after performing the scratch. Migrating Cavβ3-deficient MEFs closed the scratch area significantly earlier than MEFs from wild-type mice (Figure 1 a and b). To exclude any effect of cell proliferation, the scratch migration assay was performed in the presence of either 10 % or 1 % FCS. At 10 % FCS both processes are present, the cell proliferation and migration, whereas at 1 % FCS cell proliferation is minimized. Fibroblasts in 10 % (Figure 1 b, left) or 1 % FCS (Figure 1 b, right) showed a similar migration pattern, ruling out the possibility of cell proliferation contribution to the Cavβ3 observed phenotype. Cavβ3-deficient MEFs closed the gap significantly earlier than wild-type MEFs under both conditions. To confirm the Cavβ3-dependent effect observed in β3-deficient fibroblasts, wild-type fibroblasts ~~are~~were transfected with siRNA to ~~downregulate~~down-regulate the Cavβ3 protein~~,~~

~~they~~ (Figure 1 e). As a control for the down-regulation, immunoblots were performed to confirm the efficiency of the siRNA treatment. Two independent *Cacnb3* specific siRNAs (siRNA1 and siRNA2), and a scrambled siRNA (as a control) were used. Fibroblasts treated with the *Cacnb3*-specific siRNAs behave like β3-deficient fibroblasts (Figure 1~~).~~ d), i.e. the migration is increased in the absence of Cavβ3 protein (Figure 1 e).

In vivo, the dorsal skinfold chamber ~~is~~was implanted (Figure ~~2a~~2 a and b) and a defined circular wound ~~is applied. Images of wounds from wild type and β3 deficient mice are taken~~ of 2 mm diameter was generated on the shaved back (Figure 2 b) of wild-type and Cavβ3-deficient mice (8 animals per genotype, 8-12 weeks old and 22-26 g weight). The wound was performed by removing the complete skin with epidermis and dermis. To compare skin wound healing between both genotypes, the wound area in the skin fold chamber was photographed directly after ~~applying the wound~~ wounding (day 0) and then pictures were taken 3, 6, 10 and 14 days post~~-wounding.~~ wounding (Figure 2 c). The sizes of the wounds were measured on these digital images and the wound area at a given day was expressed as percentage (%) of the initial wound area (Figure 2 d). Wound closure is increased in β3-deficient mice compared to wild-type controls. In contrast to the wild-type, the wound in β3-deficient mice was almost completely closed already after 10 days. At day 14 post-wounding, the ~~wound is~~wounds were completely closed in both genotypes~~.~~ (Figure 2 c and d).

FIGURE LEGENDS

Figure1:

***In vitro* scratch migration assay.** (**a**) Representative images of ~~scratch migration~~ cultures from wild-type (WT, left) and Cavβ3 KO (β3 KO, right) primary mouse embryonic fibroblasts (MEFs) immediately, 6, 10 and 30 hours after performing ~~the~~a scratch ~~and after 6, 10 and 30 hours.~~. Images were converted into 8-bit gray scale, and the contrast as well as brightness were adapted to maximally visualize the cell free area. Analysis of the cell free area (% of scratch area repopulated by migrating cells) were performed on the original 24-bit RGB images. (**b**) Bar graphs showing ~~the rate~~percentages (%) of ~~cell migration calculated relative to the initial~~ scratch area repopulated by migrating cells after 6 ~~h~~hours either in the presence of high (10 ~~%~~ %, left) or low (1 ~~%)~~%, right) fetal bovine serum (FCS) in wild~~-~~-type (black) and Cavβ3 KO (red) experiments (**c**) ~~Western blot~~Immunoblot: Protein extracts from wild~~-~~-type brain (50 µg per lane) and fibroblasts (MEFs, 100 µg per lane) using a Cavβ3 specific antibody. The β3 protein (55 kDa) is present in wild~~-~~-type brain~~,~~ (used as a control), and fibroblasts, ~~and~~but is absent in protein extracts of Cavβ3-deficient brain and fibroblasts prepared from Cavβ3-deficient mice. (**d**) Summary of ~~cell migration rate~~percentage of scratch area repopulated by migrating cells after 6 ~~h~~hours in wild~~-~~-type cells treated with either scrambled siRNA (control, black) or two independent *Cacnb3* siRNAs (siRNA1 and siRNA2, red open bars). (**e**) The corresponding ~~Western blot~~immunoblot from the experiment in (d). Data are shown as mean ± SEM, p values were calculated using unpaired two-tailed Student's t test. ~~Figure (~~Panels **a** and **b**~~) are adapted~~ have been modified from [Belkacemi et al., 2018[4]][4].

Figure2:

~~(a) Wild type~~***In vivo* skin wound healing in mice.** (**a**) Titanium frame interior side view showing one half of the titanium chamber and front side view showing the assembled titanium chamber with two symmetrical halves attached with screws and nuts. (**b**) Mouse after shaving the dorsal skin and mounting the dorsal skinfold chamber composed of two symmetric titanium frames (the weight of the titanium frame is ~~arround~~around 2 g) and applying a circular wound (2 mm diameter). (~~**bc**~~) Images ~~indicate~~of the wound ~~area~~ directly after wounding (day 0) and ~~at~~ 3, 6, 10 and 14 days post-wounding. The continuous process of wound closure, with complete epithelialization is shown over 14 days in wild~~-~~-type (WT, top) and Cavβ3 KO (β3 KO, bottom) mice. (~~**cd**~~) At the time points indicated, the wound area was determined using a computer-assisted image analysis program and plotted as percentage of the wound area immediately after injury at day 0 (mean ± SEM of n = 8, β3-KO mice and the corresponding wild~~-~~-type control mice). ~~Wound closure is significantly accelerated in β3-deficient mice and it is almost closed at day 10.~~ P values were calculated using two-way ANOVA and Bonferroni's multiple comparisons test. ~~Figures (**b** and~~Panels **c**~~) are adapted~~ and **d** have been modified from [Belkacemi et al., 2018][4].

DISCUSSION

In this manuscript, we describe an *in vitro* and *in vivo* wound healing assay and correlate the results obtained. For the *in vitro* assay, we ~~use~~used primary mouse ~~embryonic~~ fibroblasts [4, ~~11, 12~~14, 15] ~~but other adherent cell types growing as monolayers can be also used. Plating the same number of viable and healthy cells is of paramount importance in order to obtain accurate and reproducible results. To perform biological and technical replicates is also highly recommended. In case of siRNA treatment, Western blot analysis after each set of experiment is mandatory to make sure that the target protein is efficiently down-regulated. This step of siRNA transfection takes at least 3 to 4 days, but the scratch migration assay can take from 6 hours to 24 hours. After the mechanical scratch of the cell monolayer using the pipette tip, it is essential to rinse the culture twice with phosphate buffered saline to get rid of cell debris and to remove released factors from damaged cells.~~

which play an important role in wound healing and tissue remodeling[11]. Other adherent cell types growing as monolayers (*e.g.* epithelial cells, endothelial cells, keratinocytes) can be used as well. Plating the same number of viable and healthy cells, and applying the scratch at the same degree of confluency are of paramount importance in order to obtain accurate and reproducible results. It is highly recommended to perform biological and technical replicates. In the present method, 6-well plates were used, but 12- or 24-well plates can be also used especially when cells are available only at limited numbers. In case of siRNA treatment, immunoblot analysis after each set of experiments is mandatory to make sure that the target protein is efficiently down-regulated. Transfection reagent and time window should be tested and selected for each cell type before starting with the migration assay. In the case of fibroblasts and the *Cacnb3* gene, it took 3 to 4 days to reach the desired level of down-regulation. In contrast, the scratch migration assay needs shorter times (6 hours to 24 hours). To avoid high variability in the scratch size, it is mandatory that the same investigator applies the scratch for each set of experiments, that equal pressure is administered by the pipette tip and to keep the wound as much as possible vertical to the marked line at the bottom of the plate across the cell monolayer. Application of a mechanical scratch across the cell monolayer leads to the release of different cellular factors (*e.g.* ATP) from damaged cells into the extracellular space. These factors would induce paracrine signaling including Ca^{2+}-signaling in the neighboring cells, which in turn would influence cellular responses[16]. To avoid these effects, culture inserts can be used for plating cells and after removal of these inserts a cell-free gap is created without damaging the neighboring cells[17]. For high-throughput screening, the investigators might consider using instruments available on the market to create reproducible and consistent scratches in 96-well plates. To follow cell migration kinetics continuously over time, users can also consider using high-end commercial systems for automatic image capture. However, automated systems for scratch application and image

capture are not always available because of high costs. A more accessible and cost-effective solution for time-lapse imaging would be for example using the system (ATLIS: an affordable time-lapse imaging and incubation system) described by Hernandez Vera and colleagues [18].

In the absence of any cell proliferation inhibitors, repopulation of the gap in the scratch migration assay is a combination of cell migration and cell proliferation. ~~It is important to suppress~~To monitor only cell migration, cell proliferation ~~in the presence of~~can be suppressed for example by treatment with either Actinomycin C[13,19] or Mitomycin C[14] ~~within the cell culture medium to be able to monitor only cell migration.~~[20]. Adequate concentrations of these compounds must be carefully determined and tested to avoid toxic effects of these compounds, which might affect the viability of the cells and their ability to migrate. As described in the present article, serum starvation or the reduction of the serum concentration in culture medium is another way to reduce the effects of cell proliferation. Serum starvation is used in ~~a number of~~several other cell-based assays ~~in cell biology experiments. Serum starvation~~. It can induce a high number of cellular responses, which might interfere with the obtained results and interpretations[15,21]~~. It has to~~. Serum starvation must be carefully applied and its effect on cell viability should be assessed before starting the experiment. In the present article, ~~we show~~ migration of primary fibroblasts in the presence of either 10 % or 1 % serum~~.~~ is shown (see Figure 1 b). Migration rate, as expected, is slower at low concentrations of serum. However, β3-deficient ~~cells~~fibroblasts migrate faster than ~~its control~~ wild~~-~~type cells at both conditions; low and high serum concentration.

The skin wound healing assay using the dorsal skinfold chamber is ~~a straight forward assay~~relatively straightforward procedure to investigate skin wound closure over time *in vivo*. The implantation of the titanium dorsal skinfold chamber was described for the first time in rats[16,22]. Sorg et al. used this technique in SKH1-hr hairless mice to follow wound healing and formation of new blood vessels[9]. The skinfold chamber model ~~we describe~~described in this article has many advantages over the classical wound healing assays performed on the dorsal skin ~~or~~, on the ear ~~of mice.~~[23] or hind limb[7] of mice. Covering the wound area with a glass coverslip prevents infections and tissue trauma and limits desiccation~~.~~ of the wound. The observation chamber covered with the glass coverslip ~~allows topical application of different reagents~~can be opened at any time during the healing process~~.~~, allowing topical application of different pharmacologically active compounds (for example siRNA for Cavβ3 as solutions or ointments) and the chamber can be closed again. Murine skin ~~contracts directly after wounding, influencing~~ wounds healing process is composed of both contraction and epithelialization ~~and healing process.~~[24]. Using the dorsal skinfold chamber in mice ~~overcomes this problem and~~ minimizes skin contraction~~.~~ and gives the opportunity to study mainly the epithelialization process. It provides also a clear window to observe and monitor the wound closure process. One disadvantage of the titanium chamber is that the mouse has to carry the titanium chamber, which has a weight of around 2 g (7.7 % of the weight of a 26 g mouse), for 14 days. This might cause some discomfort for the mouse, although it seems that mice tolerate well this chamber and they are comfortable and can easily reach food and water. The skin wound healing model presented in this article can study only one wound per mouse. Other published methods[25, 26] suggest to apply two wounds per mouse which would reduce the number of animals needed for a study. It is of great importance to create a circular wound of similar size for all mice to get objective information as well as reliable and reproducible results.

To take images of the wounds over time, mice were fixed on a ~~plexiglas~~mouse restrainer, ~~and then~~ placed on a stage, and the skinfold chamber is positioned under a stereomicroscope. Using the restrainer~~,~~ helps in avoiding anesthesia and minimizing stress ~~which might influence the results. At the end of the wound healing experiment, mice will~~for the mice. Mice can be

sacrificed and tissues from the wounded area can be explanted and collected at different stages of the healing process (either after complete healing or at earlier time points) for histological analysis, RNA collection or protein biochemistry.

In summary, we have shown two techniques~~;~~, an *in vitro* scratch assay using primary fibroblasts and an *in vivo* skin wound healing assay in mice. In both assays, wound healing-/gap closure is ~~accelerated~~increased in the absence of the Cavβ3 subunit of voltage--gated calcium channels. As with wild--type and Cavβ3-deficient mice or cells, both assays might well correlate in the absence or presence of other specific molecules.

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DISCLOSURES

The authors have nothing to disclose.

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***In vitro* and *in vivo* analysis of wound healing by means of the scratch migration assay and the dorsal skinfold chamber**

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1 Primary cell culture and siRNA transfection

1.1 Using an ultrafine permanent marker, mark a 6-well plate at the bottom in order to be able to identify later the same region of interest and follow the closure of the scratch.

1.2 Plate primary fibroblasts, isolated from wild type and β3-deficient mice[4], in a 6-well plate at a density of 5 x 10^5 cells/well in the presence of 2 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FCS).

1.3 Label the 6-well plate with the cell type, genotype and the date.

1.4 Move the 6-well plate into the cell culture incubator and maintain cells at 37 °C and 5 % CO_2 for 24 hours.

1.5 Next day, take cell out of the incubator, aspirate cell culture medium out of the well, discard it and replace it with 2.25 mL fresh culture medium by adding it carefully against the wall of the well.

1.6 In order to transfect fibroblasts with siRNAs, the transfection reagent listed in the materials table was used following the manufacturer´s protocol.

1.7 For each well prepare the following: tube (1) contains 9 µL of the transfection reagent diluted in 150 µL Opti-MEM and tube (2) contains 1.5 µL siRNA (*Cacnb3* siRNA-1, *Cacnb3* siRNA-2 or scrambled siRNA as a negative control) diluted in 150 µL Opti-MEM.

1.8 Add diluted siRNA tube (2) into diluted transfection reagent tube (1) and vortex for 2 s.

1.9 Incubate the mixture for 5 min at 21°C.

1.10 Label wells with either *Cacnb3* siRNA-1, *Cacnb3* siRNA-2 or scrambled siRNA.

1.11 Add 250 µL of the siRNA-transfection reagent mixture dropwise to the cells.

1.12 Place the 6-well culture plate back into the incubator and keep cells at 37 °C and 5 % CO_2 for 72 h.

2 *In vitro* wound healing assay (Scratch migration assay)

2.1 Aspirate culture medium out of the well and discard it.

2.2 Scratch manually the cells using a pipette tip (200 µL) to create a smooth and vertical scratch over the confluent cell monolayer in the middle of the well.

2.3 Rinse each well twice with 2 mL phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH7.4 to remove any remaining cells and debris from the scratched area. Add the 2 mL PBS carefully against the wall of the well to avoid detaching cells from the cell culture well.

2.4 Add 2 mL cell culture medium containing either 10 % serum or 1 % serum carefully to each well.

2.5 Move the plate to the microscope stage and capture bright field images of the cell-free area (two pictures per well) immediately after scratching (t=0h) at a 10X magnification using a light microscope. To image the same region of interest, use the line, which was prepared in step (1.3), and take one image above this line and one image below this line. Save images as TIFF or JPEG.

2.6 Because the microscope stage does not maintain cell growth condition, move the plate back to the cell culture incubator and keep the cells at 37 °C and 5 % CO_2.

2.7 Using ImageJ[13], quantify the initial cell-free area (100 %) and the remaining area after 6, 10 and 30 hours. The % of scratch area repopulated by migrating cells is then calculated relative to the initial scratch area.

3 *In vivo* skin wound healing assay

3.1 Anesthetize a wild-type or Cavβ3-deficient mouse (22-26 g body weight) by intraperitoneal (i.p.) injection 0.1 mL saline/10 g body weight containing a mixture of ketamine (75 mg/kg body weight) and xylazine (25 mg/kg body weight).

3.2 To avoid dryness or damage of the eye, apply ophthalmic ointment to both eyes and repeat application if necessary over time.

3.3 Carefully shave the mouse dorsum, using a shaving machine followed by application of a depilation cream to the shaved area to remove any remaining hair. Take care not to injure the mouse skin.

3.4 The depilation cream needs around 10 min to remove completely the hair.

3.5 Prepare the titanium chamber by taking one part of the symmetrical titanium chamber frame, and fix on one side the connecting screws with nuts. These nuts will serve as spacer to keep a distance of 400-500 µm between the two symmetrical parts of the chamber and to avoid compression of blood vessels on the skin.

3.6 Disinfect the hair-free area of the mouse with 70 % (vol/vol) ethanol. Take a fold of the back skin of the mouse under a light source and position the middle line of the double layer of skin where the titanium chamber will be implanted. After that, fix the skinfold with a polypropylene sutures cranially and caudally and hang the other side of the suture on a metal rack. Adjust the height of the rack to allow the mouse to sit comfortably.

3.7 Implant the titanium chamber into the fold of the back skin of the mouse in a way to sandwich the folded dorsal skin layer between the two symmetrical halves of the titanium frame. Attach by polypropylene sutures the first half of the titanium frame on its superior edge to the back of the dorsal skinfold.

3.8 Before moving to the next step, check reflexes of the mouse to make sure that the depth of anesthesia is maintained.

3.9 At the base of the skinfold, pass the two connecting screws, attached to the first half of the titanium chamber, to penetrate the skinfold from back to the front side. Make small incisions on the skin (using fine scissors) to help smooth penetration of the connecting screws.

3.10 Detach the mouse from the rack and place it on a lateral position. Put the second complementary half of the titanium chamber on top of the 3 connecting screws (see Figure 2 a) and apply slight pressure with fingers in order to pass these screws through the second half of the titanium frame. Then, fix both symmetrical parts with stainless steel nuts. Pay careful attention to the tightness of the screws at this step, because if it is too loose; it might detach. In contrast, if it is too tight; it will squeeze the skinfold, reduce blood flow and can lead to tissue impairment and necrosis.

3.11 In the center of the skin bordered by the observation window of the skinfold chamber, mark the wound area by a standardized biopsy punch (2 mm in diameter), in order to ensure reproducible wound sizes.

3.12 By using fine forceps and scissors, create a circular wound within the marked area by removing the complete skin with epidermis and dermis. The final wound area will be around 3.5-4.5 mm^2, see Figure 2 b.

3.13 Clean the wound with 0.5 mL sterile saline (0.9 % NaCl, 37 °C).

3.14 Cover the wound with a glass coverslip and fix this glass coverslip with a snap ring using the snap ring plier on the titanium chamber.

3.15 Immediately after finishing the surgical procedure, place the mouse on a homemade acrylic glass platform and take pictures (day 0) under a stereomicroscope equipped with a camera. Take pictures under illumination, with different magnifications (X16, X25 and X40) and record all pictures by a DVD system for subsequent off-line analysis.

3.16 Keep the mouse at a warm area during recovery from anesthesia for at least 2 hours. Thereafter, transfer mice in individual cages back to the animal facility (12 hours light/dark cycle) and make sure that mice have access to food and water.

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