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Clock Gene *Per2* Modulates Epidermal Tissue Repair *In Vivo*

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Abstract

Wound healing can be influenced by genes that control the circadian cycle, including *Per2* and *BMAL1*, which coordinate the functions of several organs, including the skin. The aim of the study was to evaluate the role of *PER2* during experimental skin wound healing. Two groups (control and *Per2*-KO), consisting of fourteen male mice each, were anesthetized by inhalation, and two 6mm wounds were created on their dorsal skin using a punch biopsy. A silicone ring was sutured around the wound perimeter to restrict contraction. The wound healing process was clinically measured daily (closure index) until complete wound repair. On Day 6, histomorphometric analysis was performed using the length and thickness of the epithelial migration tongue, in addition to counting vessels underlying the lesion by immunofluorescence assay and maturation of collagen fibers through Picro-Sirius staining. BrdU incorporation and quantification were performed using the subcutaneous injection technique 2 hours before euthanasia and through immunohistochemical analysis of the proliferative index. Additionally, the qualitative analysis of myofibroblasts and periostin distribution in connective tissue was performed by immunofluorescence. Statistically significant differences were observed in the healing time between the experimental groups (means: 15.5 days for control mice and 13.5 days for *Per2*-KO; $p=0.001$). The accelerated healing observed in the *Per2*-KO group ($p<0.05$) was accompanied by statistical differences in wound diameter and length of the migrating epithelial tongue ($p=0.01$) compared to the control group. Regarding BrdU immunoreactivity, higher expression was observed in the intact epithelium of *Per2*-KO animals ($p=0.01$), and this difference compared to control was also present, to a lesser extent, at the wound site ($p=0.03$). Immunofluorescence in the connective tissue underlying the wound showed a higher angiogenic potential in the *Per2*-KO group in the intact tissue area and the wound region ($p<0.01$), where increased expression of myofibroblasts was also observed. Qualitative analysis revealed the distribution of periostin protein and collagen fibers in the connective tissue underlying the wound, with greater organization and maturation during the analyzed period. Our research showed that the absence of the *Per2* gene positively impacts the healing time of the skin *in vivo*. This acceleration

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depends on the increase of epithelial proliferative and angiogenic capacity of cells carrying the *Per2* deletion.

Keywords

Wound healing; *PER2* ; Skin; Circadian Clocks; Tissues

INTRODUCTION

The circadian rhythm is a well-conserved mechanism that connects the human body to the environment. It is governed by the internal biological clock, which determines the cyclic physiological processes and behavior of the organism (Zhang, 2022). The biological clock is driven by specific genes known as circadian genes (Marri, 2023). Circadian genes are present in practically all cells of the human body (Welsh, 2004; Dibner, 2010). They not only play an essential role in modulating the biological rhythm of the organism's food supply, but also fulfill a multifunctional biological role by regulating other genes, including its self-regulation. As a result, circadian genes participate in tissue development, cellular turnover, homeostasis, cytokine synthesis, and other biological processes. On the other hand, disrupted expression of clock genes is linked to some diseases, such as endocrine disorders, cardiovascular diseases, and tumors (Dudek, 2023).

The molecular basis of circadian rhythm involves the participation of specific genes, such as Period Circadian Regulator 2 (*PER2*), in association with other clock genes like Clock Circadian Regulator (*CLOCK)*, Basic Helix-Loop-Helix ARNT Like 1 (*BMAL1, also known as ARNTL)*, and Cryptochrome Circadian Regulator 1 (*CRY1*). These genes trigger actions closely related to transcription and translation (Chiou, 2020). Interestingly, evidence has demonstrated that alterations in circadian genes can affect wound healing. This was demonstrated by studies involving the deprivation of melatonin, which was rescued by its topical application, resulting in positive effects (Ozler, 2010). It is important to note that clock genes are expressed in the skin, and their alteration in their expression may lead to various skin-related changes, including transepidermal water loss, keratinocyte proliferation, and altered blood flow. (Tanioka, 2008; Lyons, 2019; Geyfman, 2009). In our studies, we found that genetic depletion of *BMAL1* resulted in delayed skin healing in mice (Silveira, 2020).

Recent studies have demonstrated that *PER2* plays a pivotal role in several contexts and paradigms, such as inflammatory conditions in the intestine and eyes, cholesterol metabolism, Parkinson's disease, infertility, and early senescence (Davis, 2022; Chen, 2002; Liu, 2022; Xinag, 2023; Schroor, 2023; Wei., 2023). However, the specific molecular mechanisms by which anomalous expression of *PER2* interferes with tissue repair have remained unclear. In this study, we investigated the role of *PER2* in tissue repair *in vivo* by depleting *PER2* and analyzing the skin's response to injury using a well-established skin injury model (Galiano, 2004; Silveira, 2020).

MATERIAL AND METHODS

Experimental mice

Twenty-eight mice (14 control and 14 *Per2*-KO), each 8 weeks old, were observed daily by the investigators and animal care staff. Veterinary care was provided by the facility's veterinarian. The *Per2*-KO mice (B6.129-Per2tm1Drw/J; Jackson Laboratory) and the control mice (C57BL/6J - Jackson Laboratory) were housed under 12-hour light/dark cycles. They received standard rodent chow and water ad libitum in compliance with the AAALAC guidelines. This study was approved by the Institutional Animal Care & Use Committee (IACUC) under protocol number 0007834. It was carried out in compliance with the 'Guide for the Care and Use of Laboratory Animals of the University of Michigan. For the second phase of experiments, skin healing on Day 6, groups with six animals were used.

Wound healing assay and clinical measurements

Initially, both control and mutant mice were anesthetized using a mixture of oxygen and isoflurane inhalation (Florane–Baxter Health Care Corporation). Before the procedure, the dorsal area was shaved with an electric clipper and a depilatory cream. Two full-thickness excisional wounds were then made on each side of the mid-dorsal area using sterile 6 mm biopsy punches. To prevent wound contraction, silicone rings (Grace Biolabs, Bend, OR) were placed around the wound (Galiano, 2004). The day of the punch biopsy was designated as day 0 (D0). Wound sites were then monitored and measured daily using a digital caliper, with wound closure serving as the endpoint. The closure index was determined by measuring the diameter and calculating it as a percentage of the wound closure relative to the day-0 wound diameter.

Tissue processing, BrdU incorporation and staining, and immunofluorescence assays

Freshly prepared 5-bromo-29-deoxyuridine (BrdU) was injected intraperitoneally (i.p.) at a concentration of 100 mg/g body weight, 2 hours before the animals were euthanized at ZT12. For immunohistochemical analysis, tissue sections were deparaffinized and rehydrated with xylene, followed by serial dilutions of ethyl alcohol to distilled water. Immunohistochemistry assays were conducted on serial sections post antigen retrieval, utilizing primary antibodies against BrdU (Axyll-Accurate Chemical & Scientific Corporation, Westbury, NY) and biotinylated secondary antibodies (BA-1000, Vector Laboratories). After washing, sections were incubated with an avidin-biotin complex (ABC kit, Vector Laboratories) and developed using the DAB substrate kit (Sigma-Aldrich). The results are represented as the percentage of positive cells per field, with counts performed in both intact skin and wound. Immunofluorescence was performed on tissue sections post antigen-retrieval using antibodies developed against alpha-SMA (Abcam), and periostin (Biovendor). All sections utilized FITC-conjugated secondary antibodies, Alexa Fluor 488 or 568 (Invitrogen, Carlsbad, CA, USA). Nuclear staining was done with Hoechst 33342 (blue stain-Invitrogen, Carlsbad, CA, USA). Images of the independent samples were captured using a 40X objective and a QImaging ExiAqua monochrome digital camera attached to a Nikon Eclipse 80i microscope (Nikon, Melville, NY, USA). Cell counting was performed on the images using ImageJ® software (NIH, Bethesda, MD, USA).

H&E and picrosirius stains and histomorphometry

Tissue samples were collected and stained with Hematoxylin and Eosin (H&E). We excised the wound, including the wound bed and margins, with an additional 5 mm of neighboring skin tissue. We set parameters for histological healing and histomorphometric analysis, such as index closure, wound diameter, epithelial tongue, and epithelial thickness. Wound re-epithelialization commenced from the wound edges and proceeded toward the center of the lesion. Re-epithelialization was defined as the distance traversed by the epithelium, measured in μm. Low-magnification images of the wounds were examined via a blinded sample analysis using a color camera (QImaging micropublisher 5.0, Surrey, BC, Canada) attached to a Nikon Eclipse 80i microscope (Nikon, Melville, NY, USA). Analyses were conducted using Nikon Elements NIS, as described by Silveira et al. (2020). The connective tissue underlying the wound was also analyzed histologically, with some specimens stained with Picrosirius for qualitative analysis of collagen fibers. Thus, we performed Picrosirius red (PSR) staining (KIT/Polysciences, Inc.) according to the manufacturer's protocol. Tissue sections were analyzed under linearly polarized light or brightfield microscopy using a microscope (Olympus, Waltham, MA) equipped with a digital camera with dual charge–coupled device sensors, a rotating polarizer (IX-LWPO) built into the condenser, a fixed analyzer (U-ANT), and a high-luminosity, high-color-rendering LED (Olympus, Waltham, MA). Images determined the organization of collagen fibers in tissues and distinguished between type I (mature fibers, stain yellow for orange) and type III collagen (immature fibers, stain green) in tissue sections.

Pathways Analysis and Visualization of Genomic Data—Pathway analysis for *PER2* molecular interaction was performed using "Pathways Commons" (accessed on October 20, 2023), an integrated resource of publicly available information about biological pathways (Wong et al., 2021; Rodchenkov et al., 2020). Expression of *PER2* in cells of the skin was performed in the Genotype-Tissue Expression portal (GTExPortal), using the data source from single cell snRNA-seq and query group violins by tissue (GTEx Analysis Release V8 - dbGaP Accession phs000424.v8.p2 on October 20, 2023). Lastly, *PER2* expression and genomic alterations were identified on data set from the skin cancer mutational landscape studies using the cBioPortal software (Pickering et al., 2104; Chang & Shain, 2021; Bonilla et al., 2016; Cerami et al., 2012; Gao et al., 2013; Brujin et al., 2023.

Statistical analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical significance between groups was calculated using the Student's t-test, Mann-Whitney, and Wilcoxon test, except for the *in vivo* wound closure readings, which were calculated using the Long-rank/Mantel-Cox test. Differences between groups were considered significant when the p-value was <0.05. Data in graphs are expressed as mean ± SEM. Asterisks denote statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; and NS $p > 0.05$).

RESULTS

Silencing of the *Per2* gene in vivo leads to accelerated skin wound healing

To test our hypothesis that *Per2* silencing influenced skin wound closure, we tracked and compared daily wound measurements during the wound healing process from both the control and *Per2*-KO groups. Age-matched *Per2*-KO and control/wild-type animals were randomly assigned to the experimental and control groups after genotyping (Figure 1A). In the clinical follow-up of wound closure, it was clearly observed that the lesion area in *Per2*-KO animals gradually got smaller during clinical evaluation and documentation. The most significant differences were identified on and after day 6 (D6) post-wound (Figure 1B–C). Overall, the significantly faster repair of the skin was led by the *Per2* gene ablation (****$p<0.001$). As illustrated in Figure 1D, the comparison graph showed that the *Per2*-KO mouse model achieved complete wound closure by day 12, while the control group reached this stage by day 14. The average wound healing index was 13.5 days for the *Per2*-KO group, compared to 15.5 days for the control group. To better understand which cells might drive this phenotype, we analyzed which cells in the skin expressed *PER2*. Interestingly, most of the expression of *PER2* is found in the basal and suprabasal keratinocytes and vascular cells. (Figure 1E & Suppl. Figure 1A). Notably, *PER2* binds and modulates to other clock genes and molecules associated with cell proliferation and growth, like *ARNTL/BMAL1, CLOCK, TP53*, and *CSNK2B* (Figure 1F).

Histomorphometric parameters and cell migration indicate that wound healing is accelerated in *Per2*-KO mice

To determine the contribution of *PER2* to the regenerative process of the skin, we also analyzed and compared some morphological characteristics of the wound area. For this, we collected tissue at D6 and measured the many parameters, as shown in Figure 2A. The graphic and photomicroscopic images showed a smaller diameter of lesions in the *Per2*-KO group, indicating the presence of smaller wounds in *PER2* mice ($p=0.03$) (Figures 2B–C). Histomorphometric results showed that re-epithelialization tissue has coverage and length in the *Per2*-KO animals compared to controls (Figure 2D); the difference showed evidence of a greater capacity for cell migration during healing. On the other hand, there were no statistical differences in the thickness of the epidermis in the newly formed epithelium, suggesting that *PER2* depletion did not influence epithelial differentiation at this time point (D6) (Figure 2E).

Animals lacking *Per2* exhibit high proliferative and angiogenic activity in the intact skin and the wound bed site.

Based on the histomorphometric analysis, the next step was to test the hypothesis of whether there was a difference in the proliferative activity of the epithelium between the two groups. To accomplish this, we utilized short-pulse bromodeoxyuridine (BrdU) incorporation, enabling us to analyze cell proliferation. We then compared the proliferative index in the intact skin and the epithelial tongue recovering the wounded site in both groups. (Figures 3A–D). BrdU levels had a significant difference in intact skin ($p=0.01$), demonstrating a greater mitotic activity of knockout animals (Figures 3A–B). Likewise, overexpression of BrdU was found in the wound site of *Per2*-KO animals and demonstrated

a higher proliferation than in the control group (p=0.03) (Figures 3C–D). Additionally, we found a significantly greater number of vessels in both the intact skin and the repairing wound site of *Per2*-KO mice, with values of p=0.007 and p=0.01, respectively (Figures 3E–H). This increased presence of vessels was more visible in the wound site, as seen in Figures 3G–H).

The repaired skin of *Per2*-KO mice displays increased myofibroblast presence, enhanced presence of periostin, and collagen fiber maturation

Following the reasoning that the *Per2* gene would act as a modulator of the wound-healing process, we analyzed whether there were changes in the connective tissue in the *Per2*-KO group. Interestingly, as evidenced in Figure 4 (A), a higher density of myofibroblasts in the connective tissue area below the wound was observed in the samples of *Per2*-KO animals - a phenomenon much more discreet in the control animals. Picrosirius red is a specific staining of the extracellular matrix (ECM) components that is especially helpful in studying tissue remodeling, particularly in the case of connective tissue pathologies. It is also one of the most important stains to study collagen networks in different tissues. Thus, picrosirius red stain was used to evaluate whether there were differences in collagen fiber maturation between the two groups. It is important to note that under polarized light, collagen bundles appear in green, red, and yellow fibers, making them easily distinguishable from the black background. This characteristic enables morphometric analysis. Collagen fibers are important for the composition and typical architecture of extracellular matrices of connective tissues, according to López De Padilla et al. (2021). Based on these histological aspects, it was evidenced that in *Per2*-KO animals, a greater organization was present, with evidence of maturation of collagen fibers in the most advanced stages of healing found in D10, compared to the control group (Figure 4B). These differences were not as intense on the D6 in active wounds, when angiogenesis was even more exuberant. Based on the current literature, during wound healing, dynamic alterations on the structure and composition of the ECM are required for effective repair. Periostin, a pro-fibrogenic secreted glycoprotein, is also an important matrix cellular protein that can affect the transition of fibroblasts to myofibroblasts, collagen fibrillogenesis, and ECM synthesis during development, tissue repair and disease (Walker et al., 2016). Interestingly, we found that the expression of periostin displayed a similar expression and distribution in the skin of both experimental groups (Figure 5A). However, the connective tissue under the injury in *Per2*-KO animals showed a marked presence of periostin in parallel alignment (Figure 5B). Indeed, it is important to highlight that in these animals, the collagen fibers are organized parallelly, different from what was observed in the control animals.

DISCUSSION

This study aimed to evaluate the role of *PER2*, an important circadian gene following skin tissue repair *in vivo*. The approach is timely because circadian genes can control the circadian rhythm, affecting human behavior, temperature, sleep, hormone secretion, and metabolism (Ch, 2021). Nevertheless, it has also been described that circadian rhythm is modulated by the external environment, such as the presence of light and time of food intake (Jiang, 2021). Therefore, it is necessary to understand the precise role of circadian genes that

regulate the circadian rhythm individually, especially focusing on tissue repair, as there have been few studies that have addressed this issue so far.

First, our results demonstrated a healing acceleration in the *Per2*-KO group post-skin injury. In the same way, histomorphometric results showed that reepithelialization and all layers of the transitional epithelium had greater length and thickness in the *Per2*-KO group when compared to the control group. Moreover, our results demonstrated high BrdU incorporation and, consequently, proliferation in the *Per2*-KO animals in the intact epithelium, as well as the epithelium recovering the wound.

Some authors assumed that the regulation of *PER2* gene expression is maintained by some transcription factors, followed by complex biological interactions, all of which have a relevant impact on cellular signaling pathways (Albrech, 2007). It is important to emphasize that *PER2* acts as a tumor suppressor gene (Xiong, 2022; Liu, 2020; Wang, 2020). In fact, *PER2* expression decreased malignant behavior in several cancers of epithelial origin, such as colorectal, oral, and prostate cancers (Xiong et al. 2022; De La Cruz Minyety et al. 2022; Liu et al. 2020). However, some authors also demonstrated reduced *PER2* expression in melanoma and nevus when compared to adjacent non-tumor samples (Lengyel et al. 2022). At the molecular level, *PER2* expression suppresses MMP-1 expression in human keratinocytes *in vitro* (Yeom et al. 2018). In addition, *PER2* ensures mitochondrial homeostasis and activates the GSK3β/β-catenin signaling pathway due to increased proliferative activity (Alexandrou et al. 2018). Taken as a whole, we hypothesize that conversely, the lack of *PER2* and may contribute to the improvement of skin tissue repair.

For this reason, it would be logical to assume that *PER2* expression could inhibit the proliferative activity in cutaneous cells, decreasing its efficiency in promoting tissue repair in wound healing. Following the rationale, Moriya et al. (2007) have demonstrated that the *PER2* gene expression was modulated rhythmically during the day, being correlated negatively with the DNA synthesis by means of bromodeoxyuridine (BrdU) incorporation assay in neural stem cells *in vitro*. On the other hand, other authors have revealed that low *PER2* gene expression was correlated to higher Ki-67 expression in human colorectal alterations (Wang, 2011). Endothelial progenitor cells from *Per2*-KO mice showed increased proliferative activity, migration, and adhesion (Sun, 2014). Mice with *PER2* mutations displayed genes involved in the cell cycle, such as CyclinD1, c-Myc, MDM-2, and CyclinA. Our results are fully in line with the aforementioned studies.

To further understand the role of *PER2* in skin elasticity and repair, we investigated the expression of periostin in this setting. Our results showed that periostin was more expressed in the connective tissue underlying the wound and is best organized into uniform beams and more abundant in the *Per2*-KO group. We can also observe greater fibroplasia in the wound area, showing the beginning of the remodeling phase in this group. The biological significance of periostin expression in somatic cells has been described in collagen-rich tissues, such as the skin, kidney, periodontal ligament, and bone (Wallace, 2019; Cobo, 2016). It has been established that periostin is involved, either in tissue homeostasis or in tissue repair (Yin, 2020; Khurshid, 2020). This is because periostin is highly expressed

in fibroblasts, which are cells responsible for the remodeling of the ECM (Nikoloudaki, 2020; Crawford, 2015). On the other hand, periostin also participates in the regulation of inflammatory conditions in many tissues and systems (Nikoloudaki, 2020).

Some authors have indicated that periostin is one of the most important mediators of skin wound healing, which is up-regulated after skin damage (Elliott, 2012; Ontsuka, 2012). This is because periostin is responsible for the contraction of granulation tissue in skin experimental models and can promote cellular contractility in the dermis (Nikoloudaki, 2020). Additionally, periostin can induce proliferation and differentiation of fibroblasts, keratinocytes, and stem cells in the wound, resulting in faster re-epithelialization and myofibroblast differentiation in the granulation tissue, promoting skin tissue repair (Yin, 2020).

Quantification of vessels in the wound bed and in the intact tissue revealed the presence of angiogenesis in the *Per2*-KO animals, which was more pronounced than in the control group. It has been postulated that mutation in the *PER2* gene is associated with aortic endothelial dysfunction by decreasing NO synthesis followed by increased expression of cyclooxygenase-1 (COX-1) derived mediators (Viswambharan et al. 2007). Conversely, some authors have demonstrated that there was no remarkable difference in angiogenesis of skin between *Per1/Per2* mutants when compared to controls (Zheng, 2019; Nogami, 2022). Further studies are needed to elucidate the issue.

In summary, our results demonstrated that *PER2* downregulation improved skin repair through increasing cell proliferation, angiogenesis, and periostin expression in the cutaneous tissue of mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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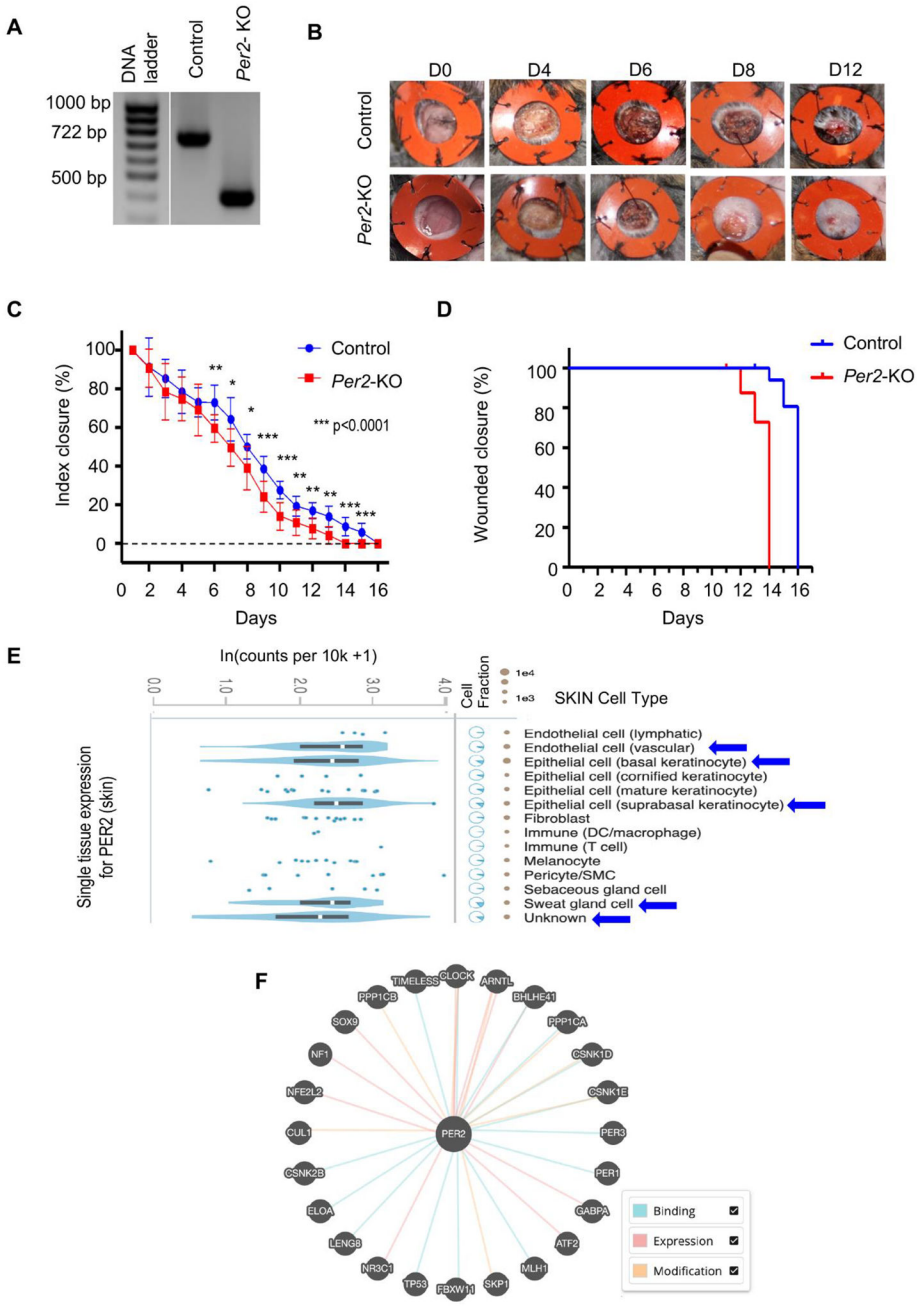

Figure 1. Silencing of the *Per2* gene accelerated the clinical wound healing.

(A) Animal genotyping by PCR. **(B)** Clinical representation of the experimental follow-up showing the 6mm punch wound created on Day 0 and the contain device that was maintained until the wound closed. **(C)** Graphic demonstrating comparison of daily wound area follow-up (Index closure), note that the closing acceleration becomes more evident and constant from Day 6 (6 (T-test/Mann Whitney test). **(D)** Comparative graphic demonstration of wound closure time between Control and *Per2*-KO (Long-rank/Mantel-Cox test). Note that the *Per2*-KO animals started wound healing from day 12, while the Control animals started from day 14. These differences of 2 days were maintained until the last animals of

each group (Means: *Per2*-KO=13.5 days and Control=15.5 days). **(E)** The graph depicts the *PER2* expression in skin cells. Notably, there's pronounced expression of *PER2* in the basal and suprabasal keratinocytes, as well as in endothelial cells (GTEx- single cell snRNA-seq -group violins by tissue & cell type) **(F)** The Diagram displays the *PER2* signaling and molecular interactions (Pathway Commons).

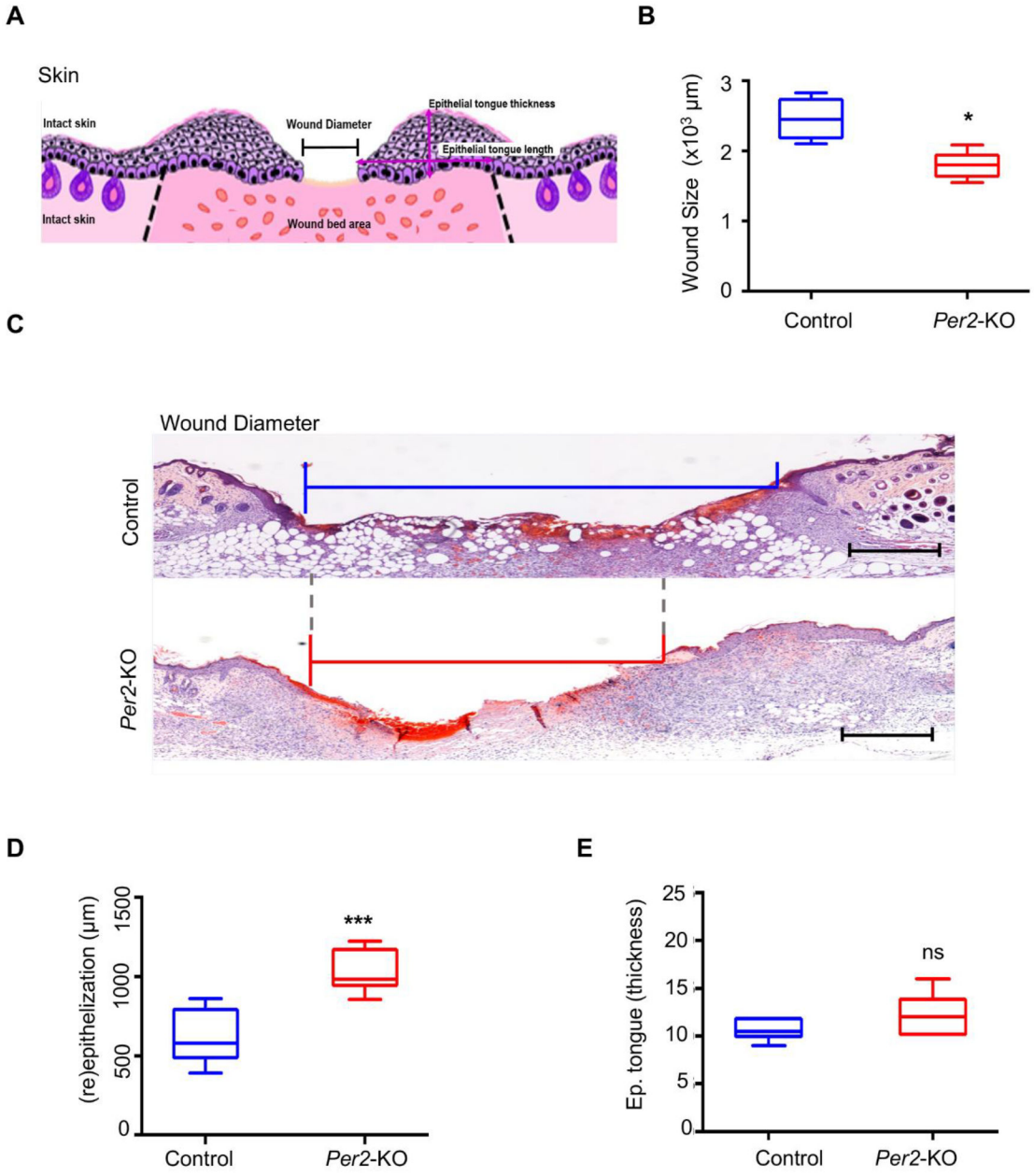

Figure 2. Differences in histomorphometric parameters of cell migration ascertained that the wound healing of *Per2*-KO mice was accelerated.

(A) Schematic figure showing the histomorphometric parameters analyzed; n=6 per group. **(B)** Graphic showing the smaller diameter of lesions on D6 in *Per2*-KO animals (*p=0.03; T-test/Mann Whitney test). **(C)** Photomicrograph representation by H&E staining of wound area collected on Day 6 after injury (scale bar = 100 µm), note the difference of wound diameter that is small in the *Per2*-KO specimen. **(D)** Graphic demonstration showing a greater length of the epithelial tongue in *Per2*-KO, therefore more cell migration in healing (*** p=0.0159; T-test/Mann Whitney test). **(E)** Graph with data of epithelial tongue thickness, despite no evidence of difference between the two groups, we found

the hyperplasia that occurs around the lesion and the time of wound closure reinforces the acceleration of healing in *Per2*-KO animals (T-test/Mann Whitney test).

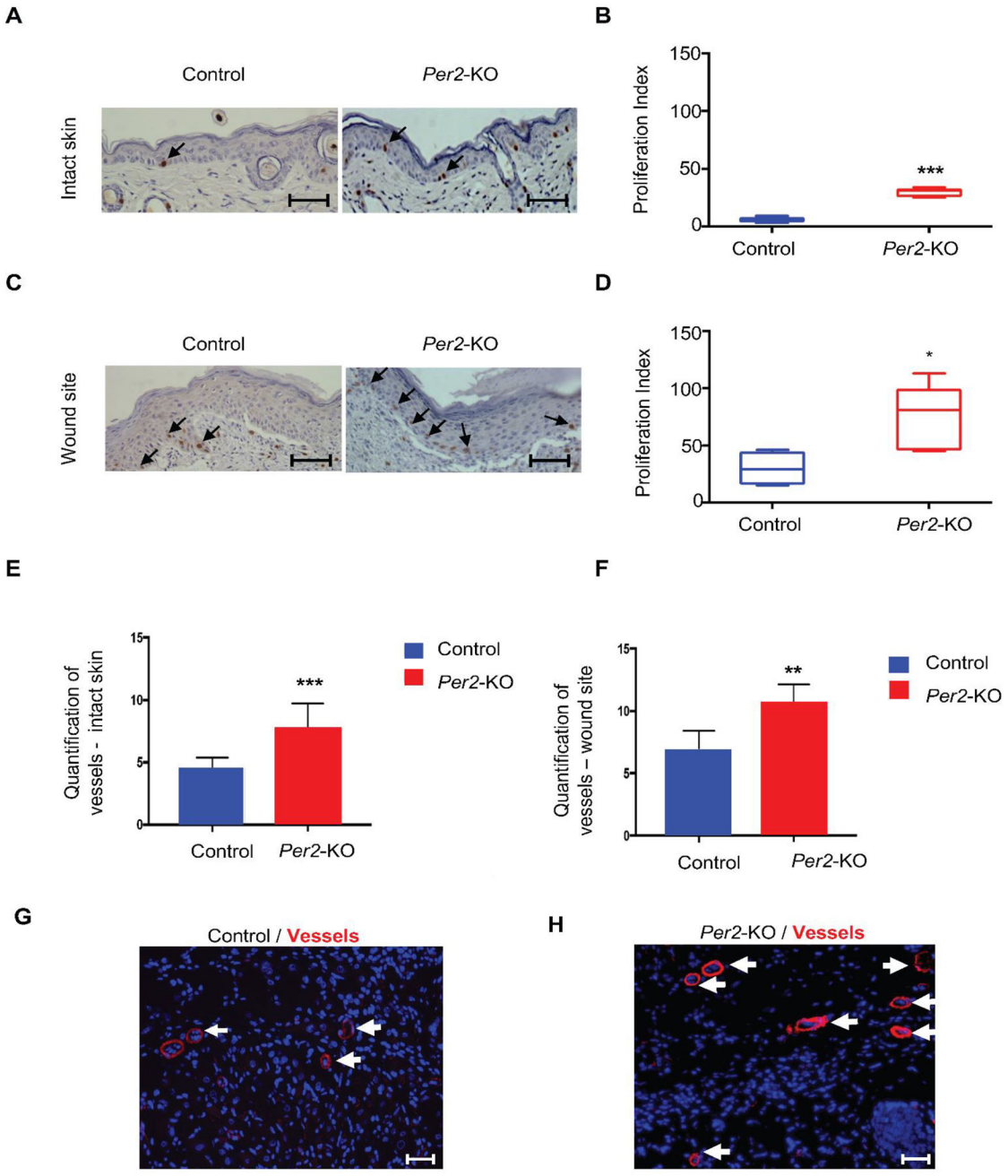

Figure 3. Proliferative activity and angiogenic potential from *Per2*-KO animals are significant in intact skin and wound beds.

(A) Representative photomicrographs immunostained for BrdU in intact skin at D6 show that *Per2*-KO animals have more positive cells stained with anti-BrdU antibody (arrow). **(B)** Graphic showing statistically significant difference in the proliferation index between the Control and *Per2*-KO (***p=0.01; T-test/Mann Whitney test) in intact skin **(C)** Photomicrographs immunostained for BrdU in wound site at D6 demonstrating a greater proliferative activity in *Per2*-KO group when compared to Control (n=6 mice; scale bar, 200 μm), we can see this difference in graphic **(D)** (***p=0.01; T-test/Mann Whitney test).

(E) *Per2*-KO mice showed a greater amount of blood vessels in the region of intact skin (*** p=0.007; Wilcoxon test), and **(F)** showed the count vessels of the area underlying the wound, keeping this difference (n=6 per group). **(G-H)** Representative photomicrographs are immunostained vessels in red (indicated by the white arrows) in both groups, *Per2*-KO and control mice (scale bar, 400 μm).

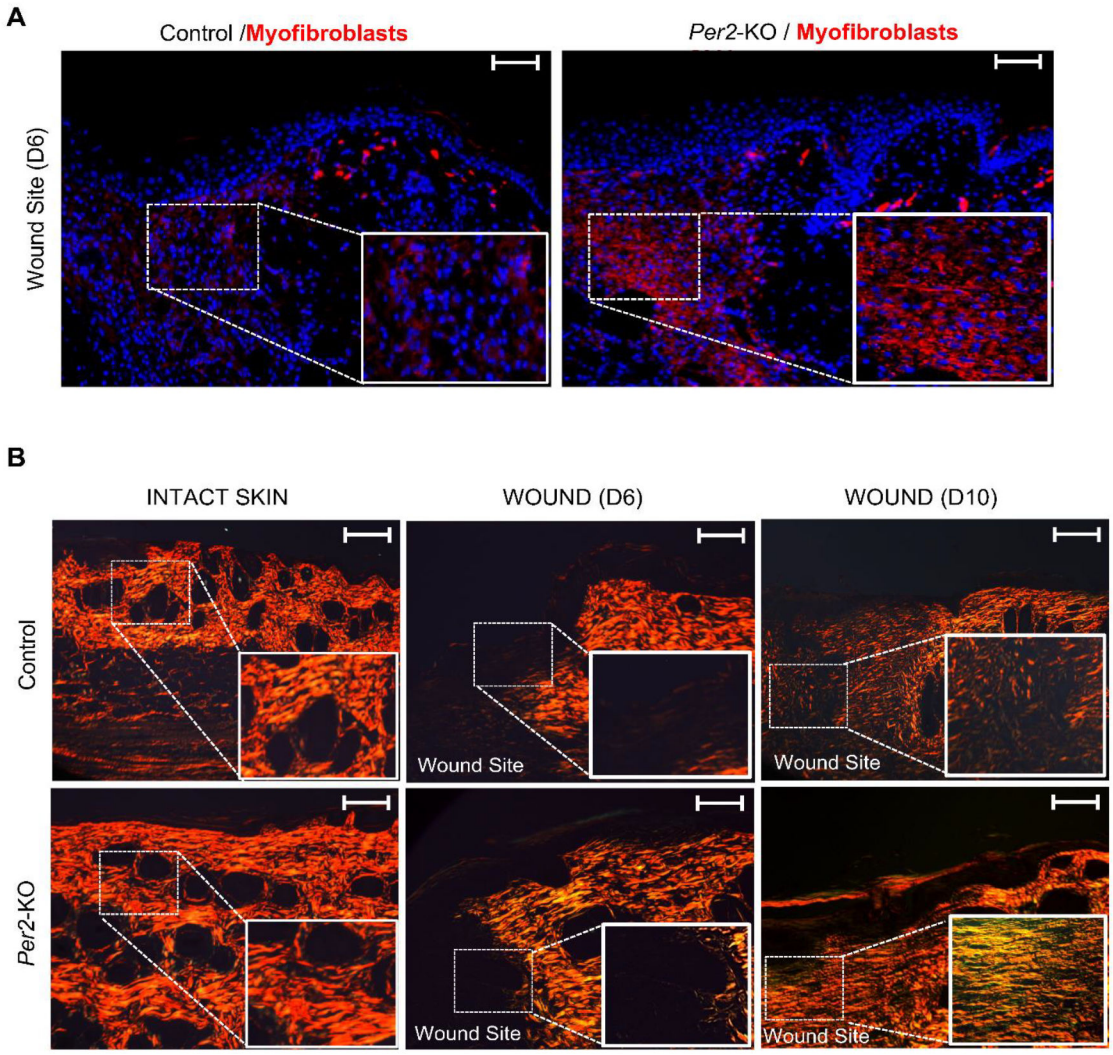

Figure 4. *Per2*-KO mice have an evident presence of myofibroblasts and collagen fibers matures in the wound bed area.

(A) The wound area in the immunofluorescence assays with alpha-SMA displayed that *Per2*-KO animals had an exuberant presence of myofibroblasts, while in the control animals, these findings were discreet. Observe greater fibroplasia in the connective tissue underlying the wound (scale bar, 100 μm). **(B)** Photomicrographs in the special staining of Picrosirius red depicted under polarized light microscopy of the Control animals and *Per2*-KO without wound and D6 and D10 after injury (second and third column of the image) at detail images, which displayed orange collagen fibers (more mature) were evident in the *Per2*-KO animals with a good organization of collagen fibers during the tissue repair process, especially as the healing process advanced (scale bar, 200 μm).

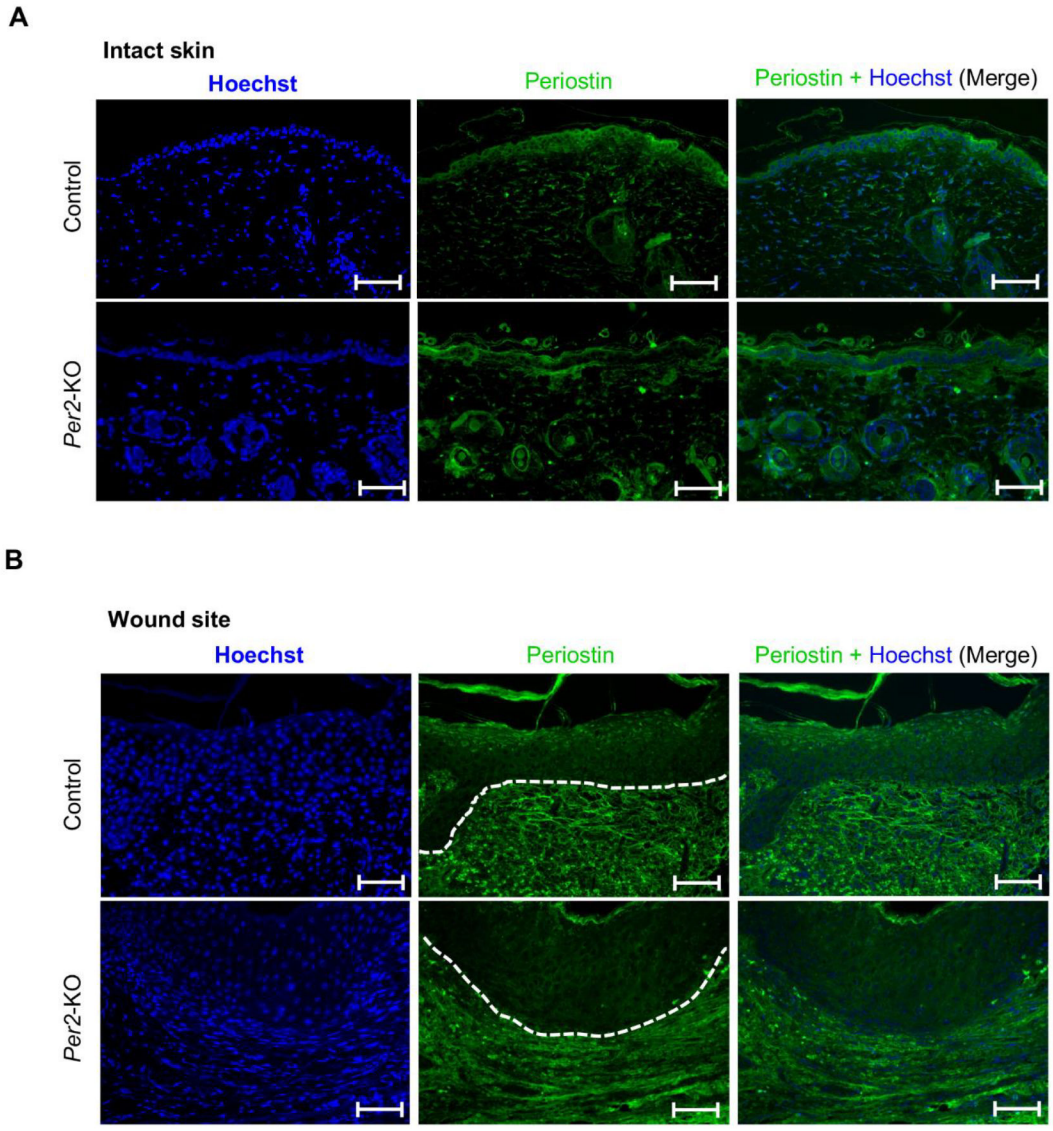

Figure 5. *Per2*-KO animals demonstrated greater organization of periostin in the wound bed area.

(A) Representative images of immunofluorescence staining for periostin by immunofluorescence assay using anti-periostin antibody in intact tissue. A similar distribution of periostin is observed in the intact skin of Control and *Per2*-KO mice (scale bar, 100 μm). **(B)** The same assay in wound site on D6 after injury for Control and *Per2*-KO animals, it is possible to verify the presence of periostin in both groups, but *Per2*-KO samples demonstrated a pattern of organization of periostin and extracellular matrix that was localized in parallel arrays in the granulation tissue underlying the epithelial tongue of the wounds (scale bar, 200 μm).