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Time Course of Angiogenesis in the Wound Tissue in *per2*-mutant Mice

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Abstract

The multifunctional nuclear protein NONO (non-POU domain-containing octamer-binding protein) is a binding partner of circadian period (PER) proteins, and its defect resulted in defective wound repair. We have previously shown the difference in circadian rhythms of angiogenic factors in the skin and wound tissues in *per2* mutant B6.Cg-Per2tm1Brd Tyrc-Brd/J mice compared with control B6(Cg)-Tyrc-2J/J mice. Therefore, we hypothesized that the mutant mice would show a difference in the recovery of vasculature from the skin injury. In this study, we analyzed the time sequence of the vascular development in the skin wound tissues in *per2* mutant and control mice, and examined their developmental patterns. However, our results show no statistically significant difference in the percentages of the vascular areas in the wound tissues during the course of recovery from the wound infliction. Our result indicates that the loss of the circadian rhythm in the mutant mice has no significant effect on the vascular areas in the recovery from the skin wound.

Introduction

The programmed activation of senescence in wound myofibroblasts has been considered an integral part of skin injury repair response [1]. In support of this view, non-POU domain containing octamer binding (NONO/p54nrb) was implicated in timing the replicative senescence of wound myofibroblasts [2]. The defect of NONO resulted in defective wound repair [2]. NONO is a binding partner of a circadian period protein *Per2*, and the NONO-PER protein complex was shown to couple the clock and the cell cycle by activating the rhythmic transcription of the cell cycle checkpoint gene Ink4a [2]. We have previously shown the difference in circadian rhythms of angiogenic factors in the skin and wound tissues in *per2* mutant B6.Cg-Per2tm1Brd Tyrc-Brd/J mice compared with control B6(Cg)-Tyrc-2J/J mice[3].

In this study, we analyzed the time sequence of the vascular development in the skin wound tissues in *per2*-mutant and control mice, and examined their developmental patterns.

Materials and methods

Animals

Six-week-old male B6.Cg-Per2tm1Brd Tyrc-Brd/J mice (*per2* mutant mice) and B6(Cg)-Tyrc-2J/J mice (control mice) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA), and maintained in our institution[4]. The mutant mice harbors a deletion mutation in the PAS domain (a dimerization domain found in Per, Arnt and Sim) [4]. The mice were exposed to luminescent light during the daytime (8 A.M. through 8 P.M.), and kept in the dark at night. The approximate intensity of the light in the daytime was 600 lux.

Evaluation of vascular areas in the skin wound healing tissues

The dorsal skin on each mouse was shaved, and the several incisions were performed on each mice during the daytime under isoflurane inhalation, using sterile scissors. After 7, 10, 14, 21, 28, and 35 days, the wound tissues were collected, fixed in 4% paraformaldehyde for 3 hr, embedded in paraffin, and 3 micrometer paraffin sections were prepared. The sections were incubated with the rat monoclonal anti-mouse CD31 (PECAM-1) antibody, Clone SZ31 (Dianova #DIA-310, Japan) diluted 10-fold by the BOND RX automated immunostainer (Leica, Germany) using BOND polymer refine detection system [5]. The percentages of the vascular areas in the wound healing tissue areas were calculated using cellSense software (Olympus, Tokyo, Japan) under the microscope.

The figure was drawn using Prism ver.7 (GraphPad Software, USA). Statistical analysis was performed by ANOVA and Sidak's multiple comparison test using Prism.

Results and Discussion

Our results show that histologically, CD31-positive endothelial cells of the vasculature were detected in the wound areas throughout the observed periods (Figure 1). Seven days after the wound infliction, the vasculature ran vertically to the surface in the wound tissue (Figure 1 A and D). The vasculature gradually progressed to the normal form (Figure 1 C and F). The results of the vascular area assessment in the wound tissues of *per2* mutant mice are shown in Figure 2. There was no statistically significant difference between mutant and control mice in the percentages of the vascular areas in the wound tissues 7

through 35 days after the skin incision. We have previously shown the difference in circadian rhythms of angiogenic factors in the skin and wound tissues in *per2* mutant B6.Cg-Per2tm1Brd Tyrc-Brd/J mice compared with control B6(Cg)-Tyrc-2J/J mice [3]. Therefore, we hypothesized that the mutant mice would show a difference in the recovery of vasculature from the skin injury. However, our results show that the loss of the circadian rhythm in the mutant mice had no significant effect on the vascular areas in the wound tissues during the course of recovery. Kowaslka et al showed the wound healing defects in *per1/per2* double mutant mice, in which they focused on the proliferation defects of keratinocytes and fibroblasts [2]. On the other hand, our data focused on the vascular recovery in the wound regions. Our results may indicate the difference in the responses between vascular cells and fibroblasts in wound healing process. Another possibility is that there could be some delay in wound healing and the angiogenesis lasts longer in *per2* mutant mice.

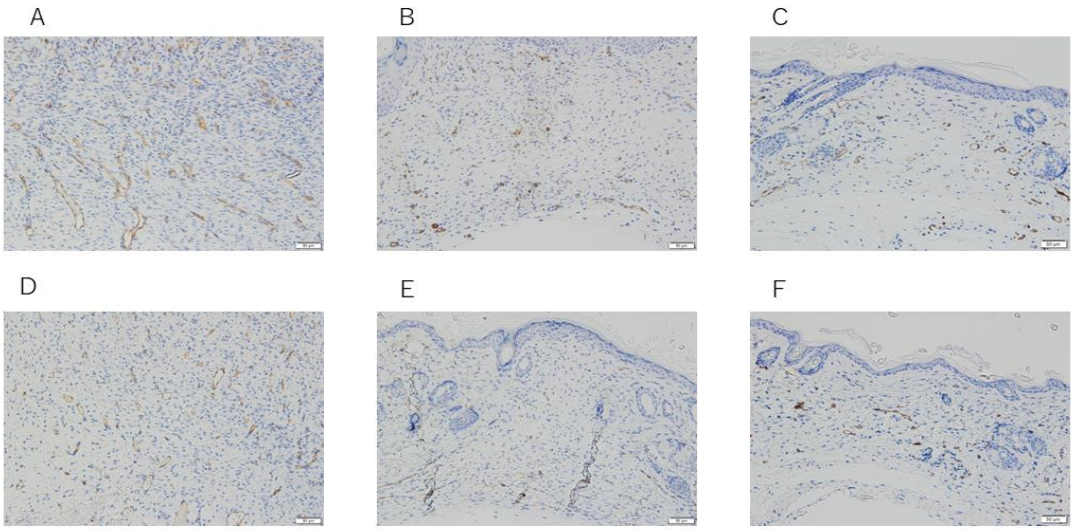

Figure 1: Histology of the skin wound tissues after the skin incision. Anti-CD31 immunohistochemistry for vascular endothelial cells stained with DAB (brown). Right bottom scales show 50 micrometers; Seven (A and D), 14 (B and E), 28 (C and F) days after the skin incision; A, B, and C: *per2* mutant mice. D, E, and F: control mice.

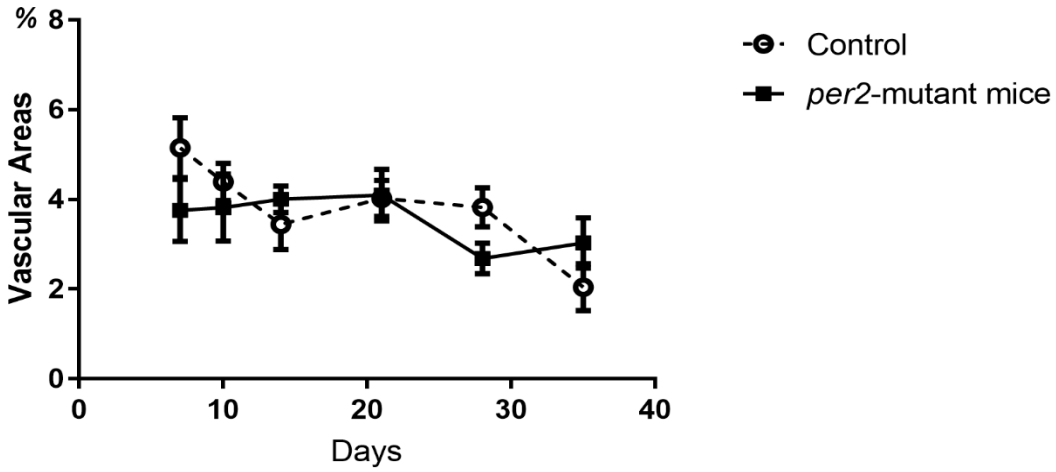

Figure 2: The percentages of the vascular areas in the wound tissues of *per2* mutant and control mice during the time course of the incision wound recovery; N= 9, 10, 9, 6, 7, 3 for control mice 7, 10, 14, 21, 28, 35 days after incision respectively; N= 9, 10, 9, 5, 4, 3 for *per2* mutant mice 7, 10, 14, 21, 28, 35 days after incision respectively; There is no statistically significant difference between mutant and control mice.

There is also a possibility that our negative result may come from the use of *per2* mutant mice instead of *per1/per2* double mutant mice.

Our result warrants the further study of using *per1/per2* double mutant mice or the analysis of the difference in fibroblast proliferation in *per2* mutant mice.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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