Side Population Hematopoietic Stem Cells Promote Wound Healing in Diabetic Mice

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Abstract

Background: Chronic wounds cause significant morbidity and mortality. Early evidence suggests that stem cells play a role in normal wound healing. Various impaired wound healing states might be due to a deficiency in stem cell repertoire. We seek to demonstrate that a new subset of lymphoid progenitor murine hematopoietic stem cells will accelerate wound healing in diabetic mice.

Methods: Bone marrow cells were harvested from C57Bl6/J femurs and separated based on their ability to efflux the vital dye Hoechst 33342, the presence of CD7 and CD34 markers into side (SP) and main populations (MP) using a fluorescent activated cell sorter. SP or MP cells and control solution were applied once topically to $1cm^2$ full-thickness dorsal excisional wounds in lepr db/db and wild type mice on the day after wounding (n=12 in each group). Wound closure was followed by computer planimetry. Wounds were harvested after 7 and 25 days for histological analysis.

Results: Topical SP treatment had a significant effect on wound closure in diabetic animals. The earliest difference can already be seen on POD #7 when SP cell treated animals had a higher percentage wound closure (35% ± 7.2%) than either animals treated with MP cells (16% ± 4.9%) or a vehicle control using saline (14% ± 6.7%), $p<0.05$. When SP cells were given to wild type mice that

already have a normal stem cell repertoire, there was a trend towards better wound closure but no significant differences were found.

Conclusion: SP-treated wounds heal more quickly than MP or control treated wounds in diabetic mice. This finding suggests that one stem cell subpopulation, but not the majority of bone marrow stem cells, harbors the potential for improving healing. Further studies are needed to investigate the mechanism of SP cell mediated healing and to explore its potential as a therapeutic agent.

Introduction

The hallmark of chronic wounds, such as diabetic lower extremity ulcers, is characterized by a dampened immunologic as well as angiogenic response [1]. The idea that stem cell applications can improve wound healing is heralded by the efficacy of topical granulocyte/macrophage colony stimulating factor, a growth stimulant for bone marrow derived progenitor cells, in improving wound closure [2, 3]. Clinically, we have also observed that patients with leukemia whose bone marrow is replaced with non-functioning cells have markedly impaired wound healing. Studies of human endothelial progenitor cells in diabetes also demonstrate a decrease in number as well as function [4, 5]. Together, this suggests that chronic wound states that are associated with diabetes may cause an impairment in stem cell function and subsequently, wound healing.

Recently, uses of bone marrow derived stem cells as well as peripheral blood derived endothelial progenitor cells have both been shown to accelerate healing in diabetic mice [6, 7]. Most bone marrow derived cells, however, suffer from poor long-term engraftment. This is not the case in a side population (SP) of stem cells that is Sca-1^{+}, c-kit^{+}, $Lin^{neg/low}$ and $CD34^{-}$ and purified based on its ability to efflux Hoechst dye [8, 9]. This population of bone marrow derived cells has been shown to exhibit true stem cell characteristics. Long term reconstitution of all hematopoietic lineages has been demonstrated [8]. Their pluripotency have been demonstrated in their ability to regenerate myocardium after ischemia/reperfusion

injury as well as to reverse dysfunctional skeletal muscle in mice deficient in the *mdx* dystrophin gene [10, 11].

Here, we study the effect of this SP cell population on wound closure in an impaired healing model of excisional wounds in genetically diabetic mice.

Materials and Methods

Animals

Male C57/Bl6 wild type mice and lep/r db/db homozygous diabetic mice were purchased from Jackson Labs (Bar Harbor, ME) and used for all experiments.

Animals in this study were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School and those prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [Department of Health, Education and Human Services, Publication no. 85-23 (National Institute of Health), revised 1985].

Stem cell preparation

Bone marrow specimens extracted from the tibias and femurs of C57BL/6J mice 6 to 12 weeks of age were suspended at 10^6 cells/ml in DMEM supplemented with 2% FCS/10mM HEPES (HyClone Inc., Logan, Utah, USA, and Life Technologies Inc., Carlsbad, California, USA, respectively) and stained with 5 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, Missouri, USA) for 90 minutes at 37°C. The cells were then resuspended in cold HBSS containing 2% FCS and 2 μg/ml propidium iodide. Sorting and analysis of SP cells were performed by

fluorescence-assisted cell sorting (MoFlow;Cytomation Inc., Fort Collins, Colorado, USA). An argon laser tuned to 350-nm emission was used to excite the Hoechst dye. Fluorescence emission was collected with a 405/30 band pass (BP) filter (Hoechst blue) and a 670/40 BP filter (Hoechst red). A second 488-nm argon laser was used to excite PE and FITC. The purity of SP cells in the sorted samples was routinely greater than 91%. The remainder of the marrow not categorized as SP is kept as a negative control designated MP or main population.

Procedure

Mice, aged 6-8 weeks, were anesthetized with intra-peritoneal pentobarbital (60mg/kg). On the morning prior to surgery, hair was removed by shaving followed by the application of calcium hydroxide based depilatory cream (Sally Hansen, Framingdale, NY). Normal saline was used to clean their backs and the mice were allowed to recover.

The wound was created with sharp excision of a 1.0cm x 1.0cm full thickness area of skin through the panniculus carnosus muscle on the dorsum of the mice under sterile conditions. The wound was covered using a pre-cut piece of Opsite® (Smith and Nephew, Largo, Fl) following benzoine (PDI, Orangeburg, NY) application to increase adhesiveness.

0.2cc of topical MP, or SP containing 8000 cells or saline control were injected on post-operative day 1 through the Opsite® using a 30-gauge needle (n=12 for each group). A digital picture of the wound was taken every 2-3 days for a period of 3 weeks until greater than 90% wound closure. All the wounds were harvested after complete wound closure for histological analysis.

Pathological processing

Wounds were harvested on post-operative day 7 during mid healing and also on post-operative day 25 after complete healing. Tissue was fixed in 10% formalin and embedded in paraffin. Hematoxylin and Eosin stained sections were used to determine quality of wound healing as determined by the amount of granulation tissue and the quality of the epithelium.

Statistical Analysis

Digital photograph was taken twice weekly. Wound area was determined using computer planimetry (Scion Corporation). Wound healing rates were expressed as means +/- standard error in the text and figures. Group comparisons were made using analysis of variance with the Bonferroni correction for multiple comparisons applied. In all cases, $p<0.05$ is defined as statistical significance.

Results

SP cells have significant effect on wound closure in diabetic mice.

Topical SP treated animals have the most pronounced effect on wound closure with statistically significant improvement in wound healing seen on POD #7, 12 and 15 ($p < 0.05$, Figure 1A). The earliest difference is seen on POD #7 when SP cell treated animals are 35% ± 7.2% closed while MP treated animals (16% ± 4.9%) and saline treated animals (14% ± 6.7%) are still poorly healed, $p<0.05$ (Figure 1B).

The hypothesis of whether diabetic animals have a dysfunctional repertoire of stem cells which is then "rescued" by the introduction of normal stem cells is further explored by giving additional wild type stem cells back to wild type animals already having a full repertoire of normal stem cells. While there was a slight trend towards improved wound healing in the SP-treated animals, there was no statistically significant differences found in any days between the groups (Figure 2).

SP treated wounds show improved epithelialization

Histological examination of healing wounds taken on POD#7 reveal enhanced epithelialization in the SP-treated wounds. Figure 3A and 3B shows the

transition zone between normal and healing skin from SP and MP treated animals respectively. While the SP-treated wound shows a contiguous epithelial layer, the MP-treated wound shows good granulation tissue formation but an incomplete epithelialization.

When wounds are examined at the end of healing, there are no discernable differences in their gross or histological appearances. Therefore, while the application of SP cells is shown to hasten wound closure rate, it does not result in a qualitatively inferior wound.

Discussion

While it has not yet been conclusively shown that adult stem cells exhibit plasticity across lineages, there is increasing evidence that different stem cell populations play important roles in the healing. Indeed, it has been suggested that they are involved in the healing and regeneration of diverse non-hematological tissues such as the heart, liver, bile ducts and skeletal muscle [12-14]. In the study reported here, we found that a population bone marrow derived hematopoietic stem cells has the capacity to improve regeneration of diabetic wounds. Just as importantly, we found that the "main population" (MP), which contains the majority (99%) of bone marrow stem cells, had no effect on wound healing.

Current evidence suggests that chronic wound states including those associated with radiation and diabetes are related to a deficiency of functional stem cells for wound healing [4, 5]. Indeed, the introduction of normal wild type stem cells either locally or systemically was effective in improving wound closure in genetically diabetic mice. On the contrary, the application of these stem cells to wild type animals that already have a normal complement of functional stem cells showed no statistically significant improvement in healing.

While the efficacy of SP cells in wound healing is quite dramatic, our understanding of their mechanism of action is still rudimentary. One mechanism

is that they act through the elaboration of cytokines. Indeed, bone marrow has long been known to provide mature inflammatory cells which orchestrate a cascade of events during wound healing that are mediated through cytokines such as platelet derived growth factor, transforming growth factor-beta and vascular endothelial growth factor [15]. Alternatively, applied stem cells may actually become engrafted and incorporated into the wound of experimental animals. While this was not done in this study, investigators who took whole bone marrow stem cells from mice that constitutively express green fluorescent protein have shown incorporation of green cells after wounding into dermal blood vessels and sebaceous glands [16].

In this preliminary study, we have shown here that a subpopulation of bone marrow cells improves wound healing, while SP-depleted bone marrow cells showed no improvement among diabetic animals with impaired stem cell function. Further studies are currently underway in understanding the mechanism of these cells including its terminal cellular differentiation pattern. This study is important not only in suggesting a novel modality for the treatment of chronic wounds but also in demonstrating the potential for plasticity of these stem cells in a non-hematopoietic tissue.

Figures

1. A. Wound closure of diabetic animals treated with topical SP (circle), MP (square) or media (triangle) over a 16-day period. Significant differences were found on POD #7, 12 and 15. B. Comparison of the different groups on POD #7, when the earliest difference can be seen among the groups. Topical SP-treated animals demonstrated significant improvement in wound healing over MP-treated or saline-treated animals, $p<0.05$.
2. A. Wound closure of wild-type animals treated with topical SP (circle), MP (square) or media (triangle) over a 16-day period. B. Comparison of the different groups on POD #7. Topical SP-treated animals demonstrated a trend towards better healing but without statistically significance.
3. Histological examination of healing wounds treated either with topical SP cells (A) or MP cells (B). Arrows show the transition zone between normal and healing skin. While the SP treated wound shows a contiguous epithelial layer, the MP treated wound shows good granulation tissue formation but incomplete epithelialization.

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Figure 1

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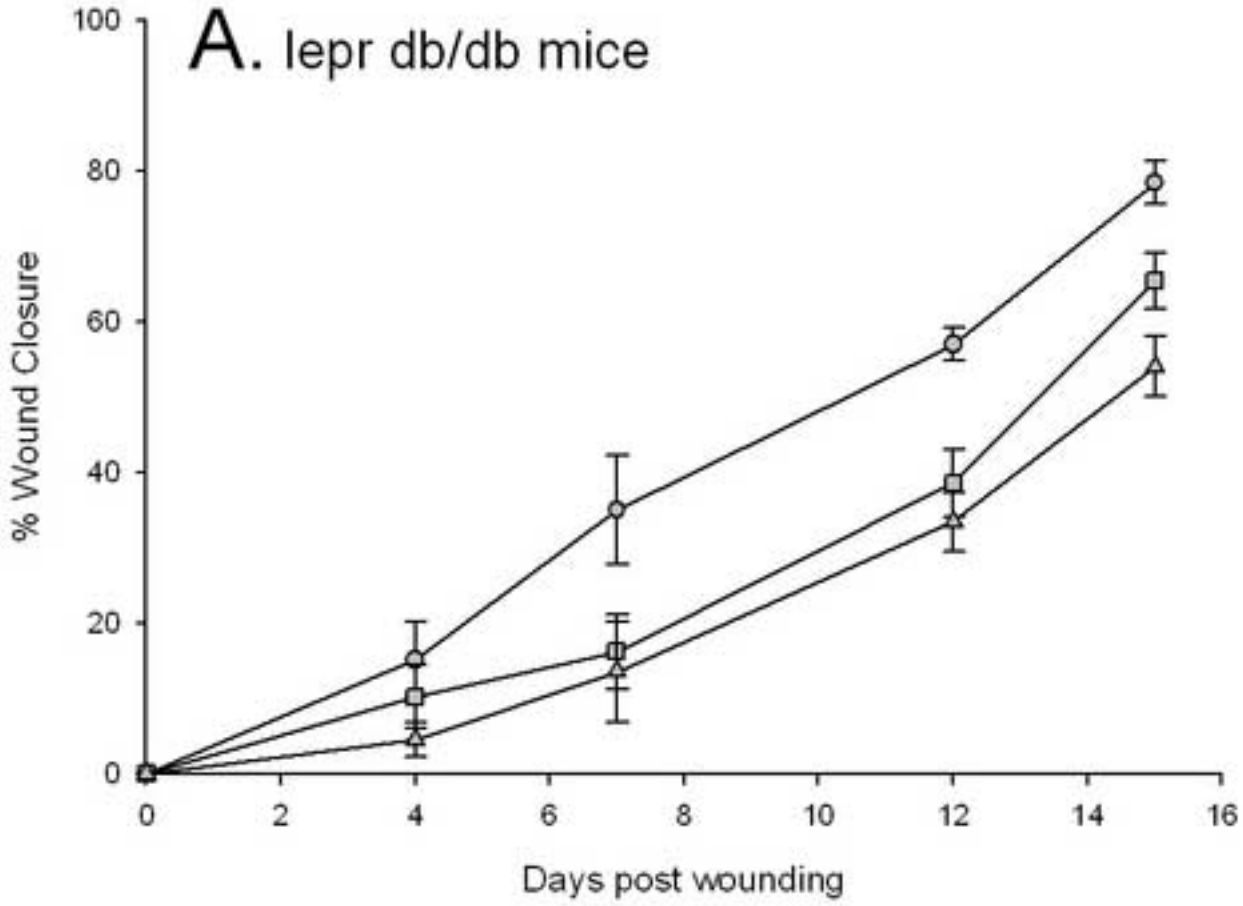

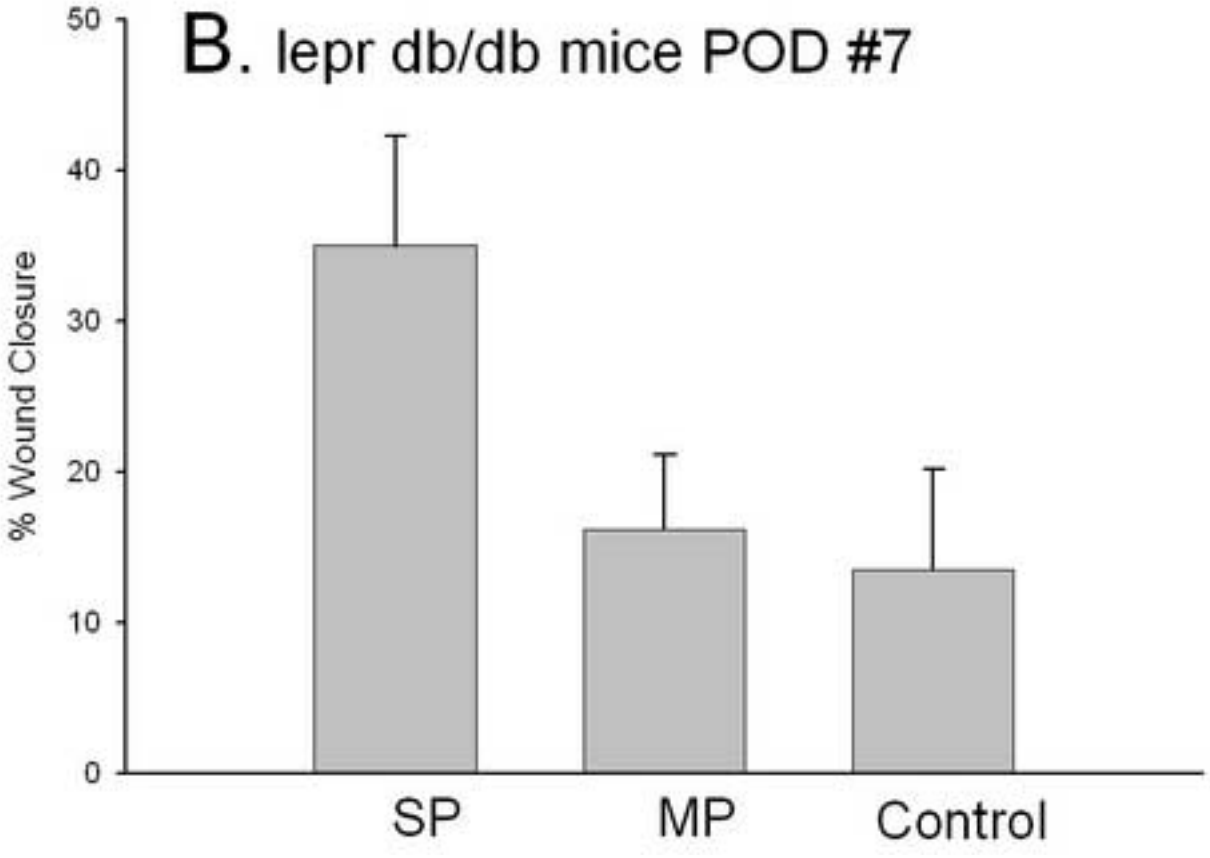

Figure 2

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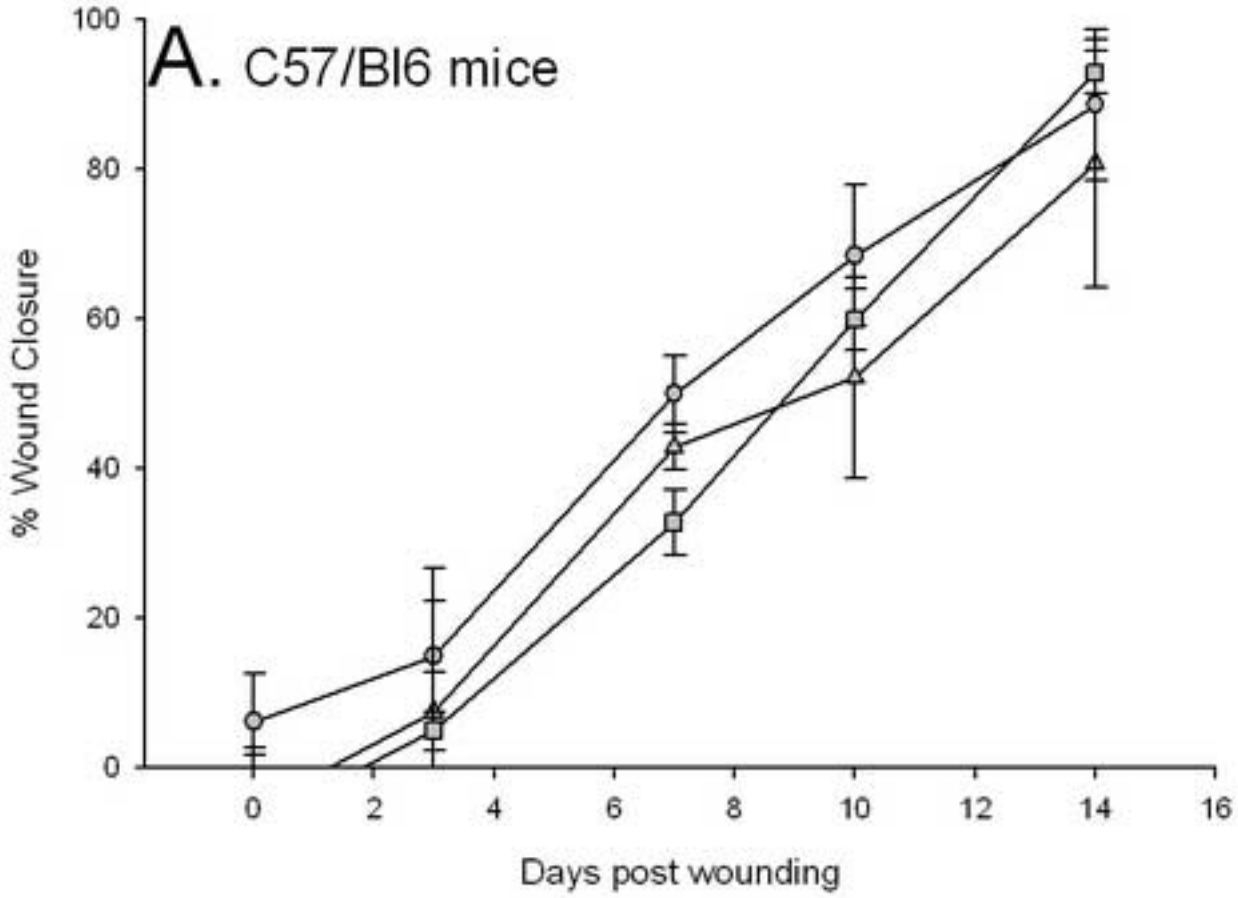

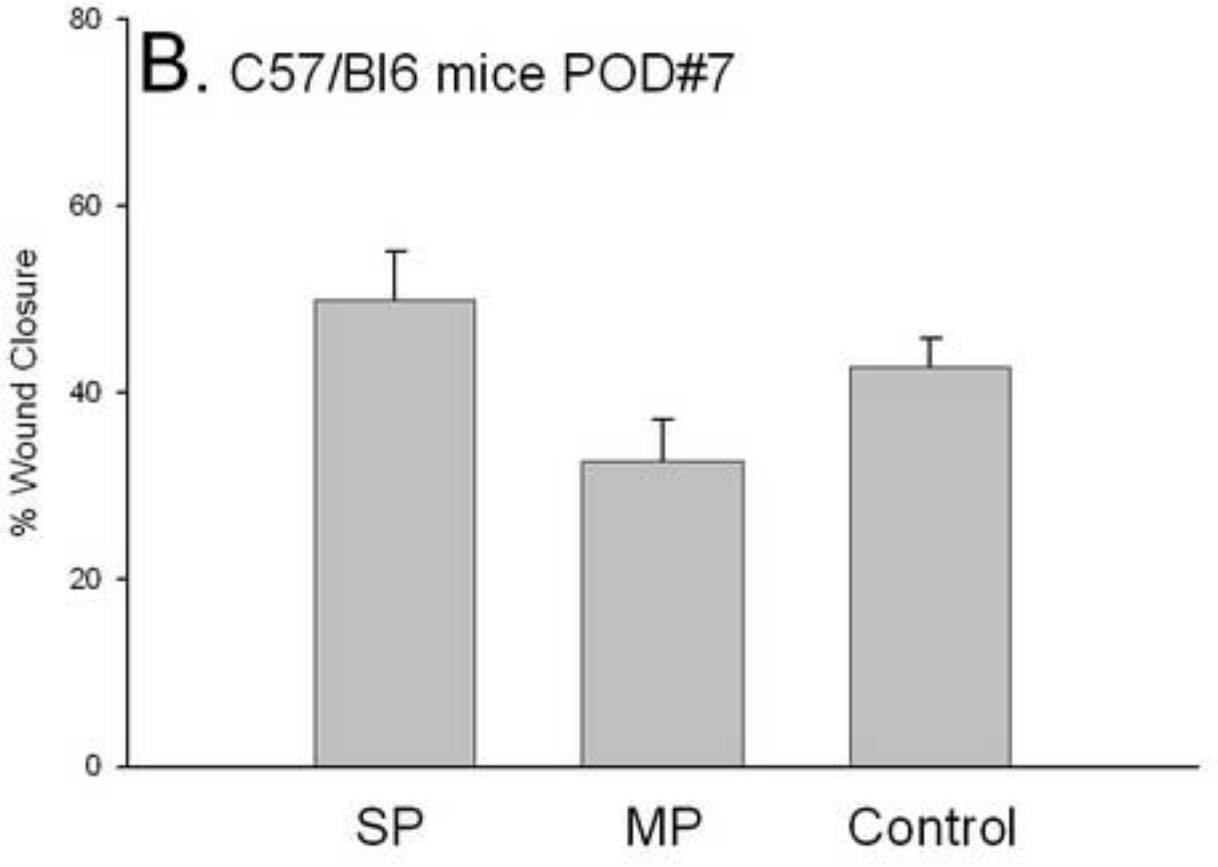

Figure 3

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