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Differential use of Erk1/2 and transforming growth factor β pathways by mid- and late-gestational murine fibroblasts

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Abstract

Background: Previously, we demonstrated the rapid closure of mid-gestational excisional murine wounds at 32 hours. In this study, we theorized that mid-gestational wounds would be completely regenerated, whereas late-gestational wounds would heal with scar formation at 48 hours. Furthermore, we theorized that mid- and late-gestational fibroblasts differentially use the transforming growth factor β and mitogen-activated protein kinase pathways.

Methods: Three-millimeter excisional cutaneous wounds were made on murine mid- (embryonic day 15 [E15]) and late-gestational (E18) fetuses and harvested at 48 hours for histology. Percent wound closure was calculated. E15 and E18 fibroblasts were cultured overnight for in vitro scratch wound assay in the presence of the activin receptor–like kinase 4-5-7, Erk1/2, and p38 inhibitors.

Results: E15 wounds healed in a regenerative manner, whereas E18 wounds exhibited scar formation. In vitro scratch closure was similar in the E15 and E18 groups at 8 hours; yet, it increased in E15 compared with E18 groups with activin receptor–like kinase 4-5-7 and Erk1/2 inhibitors. p38 inhibition resulted in reduced scratch closure in both groups.

Conclusion: The scarless mid-gestational excisional wounds compared with the scar-forming late-gestational wounds provides a model to study scar formation. This study also suggests that variable transforming growth factor β and Erk1/2 signaling may influence differences in wound closure between mid- and late-gestational wounds.

Surgical patients often have diseases of abnormal wound healing. These may be characterized as diseases of excess wound healing, as in intestinal adhesions or burn contractures, or deficiencies of wound healing, as in venous stasis ulcers and diabetic pressure ulcers. Despite extensive research, the mechanisms responsible for normal and abnormal wound healing remain poorly defined. As a result, few effective therapies exist, resulting in considerable patient morbidity and mortality.

Transforming growth factor β (TGF-β) has long been associated with conditions of excess scar formation; yet, it has been negligible in fetal scarless wounds.

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To further study the role of TGF-β, we developed a model of excisional wound healing in the fetal mouse and demonstrated that mid-gestational wounds closed rapidly in a grossly scarless fashion compared with late-gestational wounds, which showed minimal signs of closure at 32 hours [1]. In this model, TGF-β1 transcripts were elevated in the mid-gestational wounds compared with normal skin; type 2 TGF-β receptor (TβR-2) transcripts were elevated in both mid- and late-gestational wounds compared with normal skin [1]. This suggests that TGF-β may indeed play a role in both scarless and scar-forming wounds perhaps by differentially using the canonical Smad cell-signaling pathway as well as other signaling mediators such as the mitogen-activated protein (MAP) kinases.

The roles of MAP kinases in the specific aspects of wound healing, including cellular proliferation, migration, and apoptosis, have begun to evolve. MK2, a kinase substrate of p38 MAP kinase, regulates myofibroblast α-smooth muscle actin expression important in postnatal wound contraction [2]. In vitro scratch wound studies of human keratinocytes have demonstrated inhibition of wound closure with Erk inhibitors, and yet, only delayed closure with p38 inhibitors, suggesting a differential role of the MAP kinases in postnatal cellular migration, proliferation, and differentiation [3]. Furthermore, growth factor–stimulated p38 expression can induce epithelial migration, whereas growth factor-stimulated Erk1/2 activation induces cellular proliferation in a model of corneal wound healing [4].

In an effort to further delineate the role of TGF-β and MAP kinase signaling in scarless and scar-forming wound healing, we used an in vitro scratch wounding model and theorized that the mid- and late-gestational fibroblasts would close a scratch wound defect in a differential manner when these pathways are blocked. Furthermore, we sought to further characterize the progression of wound healing in our mouse model of fetal excisional wound healing in which we demonstrated rapid closure of mid-gestational wounds at 32 hours, and yet, only minimal closure of late-gestational wounds. We theorized that mid-gestational murine excisional wounds would heal in a regenerative manner indistinguishable from surrounding normal skin at 48 hours postwounding, whereas late-gestational wounds would contract with significant scar formation.

1. Methods

1.1. Animal model

The animal model was used as previously described in Goldberg et al [1]. Briefly, after institutional review board approval, timed-dated, pregnant, FVB mice (Charles River, Wilmington, ME) at embryonic days 15 and 18 (E15 and E18, representing mid- and late-gestational animals) were sedated and anesthetic levels monitored by respiratory rate and foot pinch response. Laparotomy and hysterotomy were performed to expose the anesthetized fetuses using a Wild Heerbrugg M691 high-powered surgical microscope (Leica Microsystems, Bannockburn, IL). Three-millimeter full-thickness, cutaneous, excisional wounds were created on the dorsum of a maximum of 4 fetuses per doe by grasping and lifting the fetal skin with forceps and using a scissor to transect the raised skin. The base of the wound was marked with India ink before tracing the wound onto parafilm. Amniotic fluid was replaced with 250 μL of normal saline at 37°C. The uterus was closed with a previously placed purse string suture with care taken to avoid compromising the underlying wound. The bicornate uterus was returned to the original position in the doe, and the maternal incisions were closed with a running suture in the fascia and a subcuticular suture in the skin. Operative time ranged from 40 to 70 minutes. Postoperative care consisted of analgesia with a 3-μg subcutaneous injection of buprenorphine and temperature regulation with a homeothermic heating blanket for 90 minutes. At 48 hours after wounding, the does were sedated and anesthetized, and each fetus was killed using decapitation immediately before the wound was measured and tissue harvested. Wounds were retraced onto parafilm and harvested for histologic evaluation. After the fetuses were euthanized and harvested, the does were killed with sodium pentobarbital via intravenous injection.

1.2. Wound measurement

Wound tracings performed at initial wounding and 48 hours (E15 and E18) postwounding were scanned and measured using SigmaScan Pro (Systat, Chicago, IL). Percent wound closure was calculated and defined as the area of the wound at 48 hours divided by the area of the initial wound and then multiplied by 100 and then subtracted from 100.

1.3. Histology

Specimens from E15 and E18 wounded fetuses and adult mice were fixed in 4% paraformaldehyde/phosphate-buffered saline overnight. Tissue samples were embedded in paraffin wax, sectioned at 5 to 7 μm, and mounted on coated glass slides. Sections were also stained with hematoxylin and eosin (H&E) for histology.

1.4. In vitro scratch wound assay

Additional does with E15 and E18 fetuses underwent hysterotomy. Fetal skins were harvested for in vitro studies and digested in dispase and collagenase (1:1). Fibroblasts were grown to confluence overnight in 10% fetal bovine serum (FBS) Dulbecco's Modified Eagles Medium (DMEM). Cells were pretreated for 40 minutes in the presence of an Erk1/2 inhibitor (PD98059, 10 μmol/L), a p38 inhibitor (SB203580, 10 μmol/L), an activin receptor–like kinase