**Summary**

Animal studies demonstrate that the fetus heals cutaneous wounds by reformation of normal tissue architecture without scar formation. We have developed a new model to study human fetal skin wound healing. Grafts of human fetal skin placed onto athymic mice retain the morphologic features of normal development, although they differentiate at an accelerated rate when placed cutaneously compared to subcutaneously. Full-thickness skin grafts from human fetuses at 15 (n=12), 17 (n=11), 18 (n=25), 19 (n=20) and 22 (n=13) weeks gestational age were placed onto athymic (nu/nu) mice in 2 locations: (1) cutaneously onto a fascial bed and thereby exposed to air or (2) subcutaneously in a pocket under the murine panniculus carnosus. Linear incisions were made in each graft 7 days after transplantation. Grafts were harvested at 7, 14 and 21 days post-wounding and analyzed histologically for scar formation.

By hematoxylin & eosin and Mallory's trichrome stains, complete epidermal and dermal graft wound healing without scar formation was demonstrated in the subcutaneous grafts at each gestational age studied. In contrast, scar was seen at all time points in the cutaneous grafts in both the incisional wound and at the interface of the fetal human skin graft and adult mouse skin, regardless of fetal skin gestational age.

Our results demonstrate that: (1) human fetal skin can heal without a scar in a subcutaneous adult environment; (2) scarless repair does not depend upon either perfusion by fetal serum or the unique amniotic fluid intrauterine environment; (3) in contrast to subcutaneous grafts, cutaneous grafts of 15, 17, 18, 19 and 22 week gestation fetal skin heal with scar.

**Key words:** fetal wound healing, nude mouse, human fetal skin.

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**Introduction**

Surgery on the human fetus is now being performed for a variety of life-threatening conditions (Harrison et al., 1990a,b). The observation of apparent scarless wound repair in the human fetus has led to documentation of scarless fetal skin wound healing in multiple fetal animal models, including the rabbit (Somasundaram and Prathrap, 1970), rat (Goss, 1977), mouse (Whitby and Ferguson, 1991), sheep (Longaker and Adzick, 1991), and monkey (Sopher, 1975). Discrepancies between some of the animal models with respect to fetal skin wound healing characteristics have been observed, which make extrapolation to the human situation difficult. For example, excisional wounds in sheep fetal skin contract and heal (Longaker et al., 1991a), whereas fetal rabbit excisional wounds do not heal but continue to enlarge as the fetus grows (Krummel et al., 1989). Because no studies of human fetal skin wound healing have been performed due to obvious ethical limitations, we developed a model of wound healing using human fetal skin grafts placed onto athymic nude mice.

Nude mice were utilized as adult recipients because of their lack of cell-mediated xenograft rejection, which enables them to maintain lifetime xenografts from a wide variety of mammals, including man (Kischer et al., 1989). Grafts of human fetal skin transplanted onto nude mice retain all the morphological and ultrastructural features associated with normal fetal skin development (Lane et al., 1989). In this study, human fetal skin was transplanted into a postnatal environment where the amniotic fluid effect was removed and the fetal skin was perfused by adult serum. The transplanted fetal skin was then wounded and its wound healing characteristics were studied in the adult environment.

**Materials and methods**

**Animals**

Female adult athymic (nu/nu) nude mice (Charles River Laboratories, Wilmington, MA) at 6-7 weeks of age were housed in groups of 4-6 in sterile cages at the University of
California, San Francisco animal care facility and fed food and water ad libitum.

Skin
Human fetal skin samples from the head and neck were obtained from therapeutic abortion material after obtaining signed consent for the use of this tissue for research purposes under an approved University of California, San Francisco Committee on Human Research protocol. Human fetal tissue collection conformed to the current recommendations of the National Institutes of Health. Gestational age was determined by fetal foot length. Postnatal human skin from the head (42 and 66 years old) and abdomen (3 years old) and adult (6-7 weeks of age) nude mouse skin were used as controls. Skin was placed in cooled serum-free Roswell Park Memorial Institute-1640 media (RPMI-1640; Gibco, Grand Island, NY) with 25 mM Hepes, 0.3 g/1 L-glutamine, 2.0 g/1 NaHCO3, and 1% penicillin/streptomycin. Skin was stored at 5°C for 1-18 hours until transplanted.

Transplantation
Skin samples were washed two times in sequential phosphate buffer solutions (University of California, San Francisco Cell Culture Facility), trimmed of subcutaneous fat, cut into 0.5 cm x 0.5 cm squares and transplanted. All procedures were performed in a laminar flow hood under aseptic conditions with general anesthesia.

Two transplant sites were used on the recipients: (1) externally onto a cutaneous site on the dorsal surface where full-thickness native mouse skin had been excised or (2) internally into a subcutaneous pocket where the mouse panniculus carnosus was raised from the underlying fascia in either the left or the right flank (Fig. 1). Each mouse received 2 grafts in bilaterally symmetric locations. The dorsal site was prepared by removing a 0.5 cm x 0.5 cm area of full-thickness skin. The cutaneous graft was trimmed to conform to its prepared bed and anchored with Tegaderm® (3M Medical-Surgical Division, St Paul, MN). A Coverlet® (Beiersdorf, Inc, Norwalk, CT) dressing was placed circumferentially around the mouse trunk and the edges anchored with surgical skin clips with care not to restrict mouse respiratory excursions. The subcutaneous pocket was created by incising a 1 cm length of skin obliquely along the flank and raising a full-thickness flap to excavate a 1 x 1 cm2 cavity. After the graft was placed inside, the incision was closed with surgical clips and healing occurred in an air-tight fashion. Grafts were positioned such that graft dermis apposed mouse fascia.

Wounding
Seven days after transplantation, linear incisions were made through the grafts (Fig. 1). In the case of cutaneous grafts, a 5 mm extension of the incision into murine skin on either side of the graft was made for experimental mouse control wounds. For the subcutaneous grafts, the subcutaneous pockets were reopened and the grafts exposed prior to wounding. The pockets were then reclosed with clips subsequent to wounding. All wounds were marked with India ink to facilitate determining their location during histologic analysis.

Wound harvest
At days 7, 14 and 21 post-wounding, animals were killed (ether anesthesia and bilateral thoracotomy). Wounded fetal grafts, postnatal human grafts and murine skin samples were fixed in 10% buffered formalin and paraffin embedded for subsequent histologic analysis.

Results
Eight fetal donors of six different gestational ages resulted in 81 grafts for analysis. Listing of fetal grafts by gestational age of graft donor and location in the recipient is shown in Table 1. Three human postnatal

<table>
<thead>
<tr>
<th>Gestational age</th>
<th>No. donors</th>
<th>No. cutaneous grafts</th>
<th>No. subcutaneous grafts</th>
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<tbody>
<tr>
<td>15 weeks</td>
<td>1</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>17 weeks</td>
<td>1</td>
<td>5</td>
<td>6</td>
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<tr>
<td>18 weeks</td>
<td>2</td>
<td>14</td>
<td>11</td>
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<tr>
<td>19 weeks</td>
<td>2</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>22 weeks</td>
<td>1</td>
<td>6</td>
<td>7</td>
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Fig. 2. Photomicrographs of 15 week (A) and 19 week (B) human fetal skin cutaneous graft wounds, harvested 14 days after wounding and stained with (A) H & E or (B) Mallory's trichrome. The wounds (arrows) healed with scar and are marked by India ink (arrowheads). Scar formation was present along the bases of the grafts (open arrows). Scale bar equals 100 μm.

donors of 3, 42 and 66 years of age resulted in 24 grafts for analysis.

Cutaneous grafts
Both the fetal and postnatal grafts took readily on their murine fascial bed and were easily distinguished from the surrounding murine skin. Marked contraction of fetal and postnatal human grafts as well as murine skin contraction was evident at each time point. Incisional wounds in the cutaneous fetal grafts healed with scar formation at all gestational ages tested, as demonstrated by hematoxylin and eosin (H & E) and Mallory's trichrome which stains collagen (Fig. 2A, B). Wide scars were evident, composed of thickened, parallel collagen bundles. In addition, scars were present along the bases of the cutaneous grafts. No primitive hair follicles or eccrine glands were present in the wounds. Incisional wounds in the nude mouse skin and postnatal human skin grafts also healed with scar (Fig. 3A, B). Scar was present at the graft-donor interface for both fetal and postnatal human grafts. Melanocytes present in the human basal cell layer marked the murine-human epidermal junction. Near complete healing was present by 7 days, and by 14 days all wounds were healed. At 21 days, scar remained and no loss of graft viability was notable.

Subcutaneous grafts
The fetal human skin grafts remained viable and healed their wounds, unlike the adult nude mouse and human skin grafts which did not remain viable and heal their wounds in the subcutaneous position. The postnatal grafts' high necrosis rate with inability to heal wounds may relate to inadequate neovascularization to support wound repair at 1 week post-transplantation. In contrast to the cutaneous grafts of human fetal skin, the subcutaneous grafts of 15, 17, 18, 19 and 22 week gestational age skin healed without a scar (Fig. 4A-C). The wound collagen deposition was in a normal reticular pattern.

The India ink wound tattoo made histologic wound identification possible as reformation of normal tissue architecture occurred after wounding in the subcu-
Fig. 4. Photomicrographs of 15 and 19 week gestational age human fetal skin subcutaneous graft wounds, harvested 14 days after wounding and stained with H & E or Mallory's trichrome. India ink marks the wound location (arrows) in which no scar is present. Scale bars equal 250 μm. (A) 15 week gestational age skin wound stained with H & E. An epidermal furrow is present at the wound site. (B) 19 week gestational age skin wound stained with Mallory's trichrome. (C) Same 19 week gestational age skin wound section under higher magnification and stained with Mallory's trichrome. India ink is present in the dermis in-between and around hair follicles in the wound. No scar formation is evident. The reticular collagen staining pattern is unchanged from the surrounding unwounded dermis, demonstrating scarless tissue repair can occur in human fetal skin.

taneous grafts at all gestational ages studied. Without the presence of India ink in the dermis or epidermis, one could not identify the wound proper. The India ink mark also demonstrated normal appendages present in-between the wound edges, which are not present in scar.

The Mallory's trichrome stain showed a normal collagen pattern within the wound when compared to the surrounding unwounded dermis in the subcutaneous grafts. This more organized collagen deposition in the subcutaneous fetal graft wounds distinguished them from the collagen pattern seen in the cutaneous graft wounds.

Discussion

With clinical human fetal surgery now being performed (Harrison and Adzick, 1991), the biology of human fetal wound healing becomes more germane not only to the surgeon but also to scientists interested in wound repair and developmental biology. However, human fetal skin wound healing remains relatively unstudied due to the lack of an appropriate model system. We now have a model in which the regulation of scarless human fetal skin repair can be dissected. Not only does scarless skin repair occur, we also have a system in which grafts of identical gestational age skin from 15-22
weeks heal with scar in the cutaneous environment and without scar in the subcutaneous environment. This unique juxtaposition allows for comparison of the mechanisms of skin repair with and without scar formation.

Transplantation of human skin onto nude mice with subsequent wounding is not without precedent. Demarchez and colleagues (1986) have transplanted human adult split-thickness skin grafts onto adult nude mice and later made excisional wounds in the grafts. They demonstrated that the graft reformed human epidermis with normal keratinization and involucrin patterns. By performing immunohistochemistry with antibodies directed against collagens type I and IV, fibronectin, fibrin, laminin, actin, human vimentin, human elastic fibers, or bullous pemphigoid antigen, they showed that grafted human skin preserves its own immunologic markers in the epidermis, basement membrane zone and dermis. After excisional wounds were made, complex human-murine interactions occurred: murine granulation tissue formed in the wound, human epidermal cells re-epithelialized over murine granulation tissue, the basement membrane zone was reconstructed with human components, and human dermal components replaced the murine granulation tissue, thus completing the healing process with scar formation (Demarchez et al., 1987a).

Scar formation in cutaneous fetal grafts may result from the influx of murine granulation tissue with adult murine fibroblasts depositing collagen in an adult pattern. The fetal cutaneous grafts exhibit a layer of scar coursing along the base unlike the fetal subcutaneous grafts (Fig. 2A,B). This subdermal layer of scar in cutaneous grafts may represent murine collagen deposited by fibroblasts in murine granulation tissue since the murine skin is healing by secondary intention with a prolific granulation response. In contrast, subcutaneous graft sites may be less physiologically active than cutaneous graft sites. There is little stimulation of a murine wound healing response at the subcutaneous site since the pocket incision heals by primary intention with little murine granulation tissue formation. Thus, the human fetal cutaneous graft wounds may fill with murine granulation tissue and heal with murine collagen deposition in an adult pattern. This represents one possible scar etiology in the cutaneous fetal grafts since murine granulation tissue was demonstrated in wounds in human adult skin grafts transplanted cutaneously onto nude mice by Demarchez et al., (1987a). This question can be addressed with species-specific immunostaining.

Another possible etiologic factor causing cutaneous fetal grafts to heal with scar is a more rapid rate of graft neovascularization, which would bring earlier wound perfusion by adult serum and inflammatory cells. The profile of growth factors and other inflammatory mediators present in the wound would be in an adult pattern. The earlier presence of adult macrophages and polymorphonuclear leukocytes may modulate the healing in an adult manner, which may preclude scarless repair. An immunohistological study of the revascularization process in human adult skin transplanted onto the nude mouse demonstrated that the graft vessels anastomose to the mouse circulation (Demarchez et al., 1987b). Human endothelial cells are slowly replaced by mouse endothelial cells which produce a murine basement membrane inside the original human graft vessels. This angiogenic process in cutaneous grafts could also regulate a more ‘adult-type’ repair pattern which results in scar.

The role of the environment in fetal skin wound healing can be studied with this model. The scarless healing of human fetal skin transplanted into a subcutaneous pocket demonstrates that perfusion by fetal serum and continuous immersion of the wound in amniotic fluid is not essential for scarless skin repair. This scarless repair process appears to be intrinsic to the fetal skin itself, although the environmental location of the fetal graft is crucial. Scarless repair occurs in a subcutaneous pocket, which is characterized by a sterile, aqueous environment. Conversely, scar formation occurs in the same gestational age skin in the cutaneous environment, which is nonsterile and the skin is subject to desiccation.

The environment also affects the rate of fetal skin differentiation. Lane et al., (1989) showed that human fetal skin transplanted to a subcutaneous location in nude mice differentiated at a rate comparable to in utero. In contrast, human fetal skin transplanted to the surface of nude mice and exposed to air demonstrated a marked acceleration of development. This more rapid rate of tissue differentiation offers one explanation for why fetal skin of the same initial gestational age may heal without scarring in the subcutaneous location and with scar in the cutaneous location. Further studies are needed to delineate the rate of fetal skin development in this model using immunohistochemical markers for differentiation.

Additional evidence that the stage of fetal tissue differentiation is crucial in scarless repair is that a transition from scarless repair to scar formation has been shown in animal models. Studies in fetal lambs and fetal rats demonstrate a spectrum of fetal healing with a switch to scar and adult-like healing late in gestation when the skin is more differentiated (Longaker et al., 1990a; Ihara et al., 1990). As the fetal skin differentiates, it heals with scar later in gestation, unrelated to the fetal environment. In our model we did not demonstrate a transition from scarless repair to healing with scar formation in the subcutaneous grafts. The latest gestational age tested was 22 weeks, thus a transition to collagen scar deposition presumably exists later than this time point.

To investigate the influence of the fetal environment on adult tissue repair, we transplanted full-thickness adult sheep skin onto the backs of 60 day gestation fetal lambs (term=145 days; Longaker et al., 1990b). Previous work had demonstrated that fetal lambs at less than 77 days gestation do not reject allogeneic skin grafts (Silverstein et al., 1964). The adult graft is thus bathed in amniotic fluid and perfused by fetal blood. Forty days later (100 days gestation), incisional wounds
were made on both the adult skin grafts and adjacent fetal skin, and collagen immunohistochemical analysis was performed 14 days post-wounding. The fetal wounds healed without scarring, while the adult wound collage pattern showed typical scar formation. These findings suggest that the ability of fetal skin to heal without scar formation may be principally a function of the fetal cells and matrix with or without a fetal environmental influence.

Using this model, experiments can be planned to study systematically the expression of specific growth factors and extracellular matrix components in both the subcutaneous and cutaneous grafts. In addition, wounded buried and exposed grafts can be manipulated on the nude mouse through the use of topical dressings and growth factor exposure. Notable differences may provide insights into the mechanisms of scarless repair.

The absence of T cells in the athymic nude mouse does not permit a complete adult cellular inflammatory reaction during graft wound repair. One may be tempted to hypothesize that this lack of T cells may provide an explanation for why human fetal skin exhibits scarless repair in our model. However, scar formation in the native mouse skin, human adult skin grafts and cutaneous human fetal skin grafts illustrates that T-cell-mediated inflammation is not required for scar formation. In fact, incisional wounds in athymic mice heal with a greater tensile strength than do similar wounds in normal murine skin, suggesting that the constitutionally activated macrophage in athymic mice results in stronger wound repair (Barbul et al., 1989a; Sharp and Colston, 1984). With T cell reconstitution, nude mice develop impaired wound healing (Barbul et al., 1989b), which implies that T cells down-regulate wound healing. However, because T cell depletion in normal mice impairs healing (Peterson et al., 1987), T cells may actually have a dual function in wound repair: early up-regulation and later down-regulation (Barbul et al., 1989c). More experimental work is required to delineate the role of T cell function in wound repair.

Transplanting human fetal skin to the nude mouse is not without limitations as a replica of in vivo human fetal skin repair. Whether the fetal skin is placed cutaneously or subcutaneously, it is in an adult environment. Thus, the role of amniotic fluid in this process cannot be studied without reintroducing it into the system. The fetal skin graft is not perfused by fetal serum, nor is it infiltrated with fetal platelets, polymorphonuclear leukocytes and macrophages with their presumably unique milieu of fetal wound growth factors (Longaker et al., 1989, 1991b). Interestingly, the scarless repair of human fetal skin in the adult murine subcutaneous environment demonstrates that these limitations of our model are not requisite for scarless repair, and in fact offer promise that skin can be modulated to heal scarlessly in the adult system.

Fetal wound healing is a complex series of coordinated processes that ultimately ends in scarless repair and reformation of native skin architecture. By understanding its unique mechanisms, modulation of adult wound repair to become more fetal-like may be possible. This may allow development of specific therapies for abnormal postnatal scar-forming conditions: intra-abdominal adhesions, burn contractures, fibrosis and keloid formation. Human fetal skin transplanted to the athymic mouse is a promising new model to study scarless repair.

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References


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