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Cutaneous wound reepithelialization is compromised in mice lacking functional Slug (Snai2)

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Abstract

Background—Keratinocytes at wound margins undergo partial epithelial to mesenchymal transition (EMT). Based on previous *in vitro* and *ex vivo* findings, Slug (Snai2), a transcriptional regulator of EMT in development, may play an important role in this process.

Objectives—This study was designed to validate an *in vivo* role for Slug in wound healing.

Methods—Excisional wounds in Slug null and wild type mice were examined histologically at 6, 24, 48, and 72 h after wounding; reepithelialization was measured and immunohistochemistry for keratins 8, 10, 14, and 6 and E-cadherin was performed. In 20 Slug null and 20 wild type mice exposed three times weekly to two minimal erythral doses of UVR, the development of non-healing cutaneous ulcers was documented. Ulcers were examined histologically and by immunohistochemistry.

Results—The reepithelialization component of excisional wound healing was reduced 1.7-fold and expression of the Slug target genes keratin 8 and E-cadherin was increased at wound margins in Slug null compared to wild type mice. In contrast, no differences in expression of keratins 10 or 14 or in markers of proliferation K6 and Ki-67 were observed. Forty per cent of Slug null mice but no wild type mice developed non-healing cutaneous ulcers in response to chronic UVR. Keratinocytes at ulcer margins expressed high levels of keratin 8 and retained E-cadherin expression, thus resembling excisional wounds.

Conclusion—Slug is an important modulator of successful wound repair in adult tissue and may be critical for maintaining epidermal integrity in response to chronic injury.

Keywords

Wound healing; Snail family transcription factors; Cell adhesion; Keratins

1. Introduction

A crucial aspect of wound repair is reepithelialization, a process that begins within hours after skin injury to cover the tissue defect and reestablish barrier function [1–5]. After injury, keratinocytes at wound margins undergo profound changes in morphology and function. They become migratory, displaying changes in cell:cell and cell:matrix adhesion and reorganization of cytoskeleton as they extend over the wound bed [5–7]. Migratory keratinocytes modify the terminal differentiation program, as illustrated by a decrease in expression of the differentiation associated keratins 1 and 10 and *de novo* production of the injury associated keratins 6, 16, and 17 [1,6–10].

The phenotypic plasticity observed at margins of healing wounds during the reepithelialization process resembles certain aspects of the epithelial–mesenchymal transition (EMT) that occurs during embryonic development and tumor metastasis [5,11]. EMT modulates cell phenotype and behavior during embryogenesis [12–16]. The process of EMT is characterized by loss of intercellular adhesion mediated by adherens junctions and desmosomes, loss of polarity, increased secretion of extracellular matrix-degrading proteinases, shift from keratin to vimentin intermediate filaments, and cell motility. Based on the similarity between events occurring during developmental EMT and at wound margins, wound reepithelialization has been termed a “partial and reversible EMT” [5].

Transcription factors of the Snail family are known regulators of EMT in development, and there is evidence that the Snail family transcription factor Slug (Snai2) modulates EMT-like processes in keratinocytes, including tumor progression and wound healing [11,17,18]. We have reported that Slug is upregulated at the margins of healing wounds *in vitro*, *ex vivo*, and *in vivo* [11]. Slug expression at excisional wound margins *in vivo* and *in vitro* is maximal at 72 h after wounding [11]. Expression of exogenous Slug in cultured human keratinocytes causes EMT-like alterations in cell morphology and behavior including increased cell spreading, decreased intercellular adhesion, and markedly accelerated *in vitro* reepithelialization [11]. Keratinocyte outgrowth is impaired in skin or cornea explants derived from Slug null mice, suggesting impaired wound reepithelialization [1,19]. Moreover, Slug expression is required for growth factor-stimulated reepithelialization *ex vivo* [20]. Interestingly, closure of 3–3.5-mm excisional wounds on the dorsum of Slug knockout mice does not appear to be delayed relative to wild type mice; in both genotypes, wounds appear to close completely in four to five days [11]. This is likely due to fact that contraction of the panniculus carnosus rather than reepithelialization accounts for almost all wound closure in the dorsal skin of mice [21].

To determine if Slug plays a role in effective wound reepithelialization *in vivo*, we compared healing in primary excisional wounds of wild type and Slug null mice, focusing on events 72 h after wounding, the time at which Slug is most highly expressed at wound margins *in vivo* [11]. Keratinocyte migration from wound edges was significantly delayed in Slug null mice. In addition, expression of two known Slug target genes, E-cadherin and keratin 8, differed at wound margins in Slug null mice compared to wild type mice. Moreover, patterns of keratin 8 and E-cadherin expression in non-healing cutaneous ulcers that developed in Slug null mice during chronic exposure to ultraviolet radiation (UVR) resembled those seen in excisional wounds in Slug null mice. These findings indicate that

Slug is a vital component of successful wound reepithelialization in adult tissue and protects against the development of non-healing wounds in response to chronic low grade injury.

2. Materials and methods

2.1. Excisional wound study

Acute wounding studies were carried out as follows: inbred Slug null and wild type mice on a 129 background were shaved and residual hair was removed by treating with the depilatory cream Nair (Nair, Church and Dwight, Princeton, NJ, USA). Two days later, mice were anesthetized with isoflurane and the shaved skin was washed thoroughly with surgical soap (Betadine, Purdue Pharma, Stamford, CT, USA) and rinsed with alcohol. Then 3.5 mm diameter full thickness wounds were introduced into the upper dorsum using a sterile disposable biopsy punch. Wounds were created by tenting the skin before wounding, thus producing 2 identical wounds lying on either side of the dorsal midline. One to two sets of wounds were introduced into the dorsum of each mouse. Mice were sacrificed 6, 24, 48, or 72 h after wounding by carbon dioxide asphyxiation. Young adult mice of both sexes were employed for these studies.

2.2. Measurement of epithelial migration from excisional wound edges

A total of five 72-h wounds, representing 10 wound margins, with wound margins suitable for histological measurement were identified for each genotype. Digital images of wound margins were captured at 200 $\times$ using an Olympus BX40 microscope (Center Valley, PA, USA) and a Nikon Digital Sight camera (Melville, NY, USA). Analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/niimage/>). The length of the epithelial outgrowth which extended under the serofibrinous crust and over the skin defect was measured by tracing the basement membrane. Wound outgrowths in Slug null and wild type mice were compared using the conservative two-way Student *t*-test with an assumption of unequal variance. A *P* value of less than 0.005 was considered significant.

2.3. UVR exposure study

Inbred 129 Slug null mice utilized for this study (Snai2^{lacZ}) have been described previously [22]. Twenty wild type and 20 Slug null mice were included in the chronic UVR exposure study. Thirteen of the mice in each group were females and seven were males. UVR was obtained from filtered Westinghouse FS-40 lamps that emitted wavelengths between 280 and 400 nm with a peak at 313 nm. Based on determinations of skin thickness, 1600 J/m² represented one minimal erythral dose (MED). Beginning at 10–12 weeks of age, the mice were exposed to 3200 J/m² of UVR (two MED) three times a week for 50 weeks. Forty-eight hours prior to the first UVR dose, the mice were shaved and excess hair was removed using Nair, as described above. Shaving was continued as required for hair removal during the course of the study. All mice were sacrificed at week 50 by carbon dioxide asphyxiation. Mice with tumors exceeding 1 cm in diameter were removed early from the study. These and the animal studies described above were conducted in compliance with institutional and guidelines and in accordance with the National Research Council's criteria for humane care in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.4. Histology and immunohistochemistry

Skin samples were spread on thin cardboard, fixed in neutral buffered formalin, embedded in paraffin, and sectioned at 4–5 μ m thickness. Sections were stained with hematoxylin and

eosin or used for immunohistochemistry to detect expression of the Slug target genes keratin 8 and E-cadherin, as well as markers of keratinocyte differentiation (keratins 10 and 14) and proliferation (keratin 6 and Ki-67). At least two excisional wounds from each genotype at each time point were examined by immunohistochemistry for each protein; for chronic wounds in null mice, immunohistochemistry was performed on four wounds that were clearly not associated with skin tumors.

Staining for keratins 6, 10, 14, Ki-67, and E-cadherin was carried out as follows: slides were deparaffinized and re-hydrated. Antigen retrieval was performed with DakoCytomation Target Retrieval Solution (Dako, Carpinteria, CA, USA) and the Biocare Digital Decloaking Chamber (Biocare Medical, Concord, CA, USA) by heating under pressure to 125 °C for 30 s followed by cooling in the chamber to 90 °C and on the bench top for 10 min.

Immunohistochemical staining was carried out manually or using the Dako Autostainer. Slides were rinsed with water, and then treated for 5 min with 3% hydrogen peroxide and with serum-free protein block (Dako, Carpinteria, CA, USA) for 10 min. Slides were incubated for 30 min with primary antibody diluted in DakoCytomation Antibody Diluent with Background Reducing Components (Dako, Carpinteria, CA, USA). Primary antibodies included monoclonal rat anti-mouse anti-Ki-67 (Dako, Carpinteria, CA, USA) diluted 1:200, rabbit anti-mouse keratin 14 (Covance Research Products, Berkeley, CA, USA) diluted 1:10,000, rabbit anti-mouse keratin 10 (Covance Research Products, Berkeley, CA, USA) diluted 1:1000, and biotinylated goat-anti-mouse E-cadherin (R&D Biosystems, Minneapolis, MN, USA) diluted 1:50. Slides were incubated for 30 min with the appropriate secondary antibody (Vector biotinylated goat-anti-rabbit or rabbit-anti-rat antibody; Burlingame, CA, USA) diluted 1:200 in antibody diluent then incubated for 30 min with ABC reagent (Vector R.T.U. Vectastain Elite ABC, Vector Laboratories, Burlingame, CA, USA), treated for 5 min with DakoCytomation Liquid DAB Substrate, counterstained with hematoxylin, dehydrated, and coverslipped. Rinses were performed using DakoCytomation wash buffer (Tris-buffered saline/Tween-20).

Staining for keratin 8 was performed on deparaffinized sections treated for 10 min with 3% H₂O₂ and microwaved in 10 mM citrate buffer for 10 min for antigen retrieval. After a 20-min cooling period, a casein block (Biocare Blocking Reagent, Concord, CA, USA) was applied for 10 min. Slides were treated with rat antibody against keratin 8 (Developmental Studies Hybridoma Bank, Ames, IA, USA) diluted 1:10 for 1–2 h at room temperature, then with biotinylated rabbit-anti-rat IgG (Vector, Burlingame, CA, USA) diluted 1:200 for 30 min. Color development was carried out by incubating slides with SA-HRP for 30 min followed by DAB (BioGenex, San Ramon, CA, USA). Slides were then counterstained with hematoxylin.

3. Results

3.1. Excisional wounds

Little difference was seen between wild type and Slug knockout wound margins at 6, 24, or 48 h after wounding. At 6 h, the epithelium was blunt-ended, with no evidence of keratinocyte migration. By 24 h after wounding, there was very limited outgrowth of epithelium from the wound edge; by 48 h, extensions of epidermis from the wound edge had begun to undermine the wound crust. However, by 72 h after wounding, epithelium had migrated an average of $682 \pm 133 \mu\text{m}$ from the wound edge in wild type mice and $396 \pm 209 \mu\text{m}$ in Slug null mice; the difference between the two genotypes was statistically significant ($P = 0.002$). In general, wound margins in wild type mice consisted of an attenuated sheet of flattened epithelium that undermined the overlying serofibrinous crust and covered the wound margin to the level of the hypodermis (Fig. 1A). In contrast, wound margins in Slug null mice tended to have blunted epithelial extensions that undermined the wound crust to

only a limited extent and did not reach the level of the hypodermis (Fig. 1B). Thus, wound reepithelialization was significantly slowed in the absence of Slug expression.

Our studies and those of others have confirmed a role for Slug in modulating expression of cytokeratins in epithelial cells, with Slug acting as a direct transcriptional repressor of keratin 8 [23,24]. We therefore examined expression of several keratins at wound margin. Keratin 8 was expressed in scattered basal keratinocytes in unwounded Slug null epidermis, but not in basal keratinocytes of wild type epidermis, as shown previously [23]. Keratin 8 expression was not detected at wild type wound margins, (Fig. 2A); however, strong keratin immunoreactivity was seen in basal cells at margins of wounds in Slug null mice (Fig. 2B). Increased keratin 8 expression at wound margins in Slug knockout mice was apparent at 24, 48, and 72 h after wounding, with the strongest expression at 72 h post-wounding.

It has been suggested that reduced expression of the keratin 1/keratin 10 pair at wound margins enhances keratinocyte pliability, thus promoting migration [25]. However, we did not observe differences between the two genotypes in expression of either keratin 10 or keratin 14 at wound margins. Keratin 14 was expressed throughout the epidermis at all time points (Fig. 3A and B), and keratin 10 expression was largely absent from the epithelial outgrowth emerging from the wound margin beginning at 48 h after wounding (Fig. 3C and D). Keratin 6 expression is upregulated at wound margins a few hours after skin wounding and remains elevated until the wound is closed [6]. In the present study, we observed similar patterns of keratin 6 expression in the wounds of Slug null and wild type mice, with keratin 6 positivity extending distally from the wound margin for a moderate distance, beginning at 24 h after wounding (Fig. 4A and B). Keratin 6 is often considered a marker of proliferation in mouse epidermis [25], therefore we investigated expression of Ki-67, another marker of cell proliferation, to determine if cellular proliferation was similar in Slug null and wild type mice. In 72-h wounds in both genotypes, a focus of intense proliferation was localized at a slight distance from the wound margin (Fig. 4C and D). This finding is consistent with previous reports of proliferation during cutaneous wound healing [7,26].

A number of studies indicate that Slug modulates, directly or indirectly, expression of a variety of cell adhesion molecules, including components of both adherens junctions and desmosomes [11,14,27,28]. Since altered expression of cell adhesion structures accompanies successful wound healing [5,29], we examined expression of E-cadherin, a component of adherens junctions and a direct target of Slug regulation, at wound margins. No staining for these molecules was seen at the immediate margins of the wounds in wild type mice at 72 h after wounding, while staining was retained at the margins wounds in Slug null mice (Fig. 5A and B). Thus, partial keratinocyte dissociation resulting from reduced adherens junctions probably did not occur in Slug null mice, a finding consistent with the known role of Slug in modulating expression of the E-cadherin gene.

Differences in proliferation do not appear to account for differences in the rate of wound reepithelialization between the two genotypes. Instead, these findings indicate that decreased wound reepithelialization in Slug knockout mice is associated with specifically enhanced expression of keratin 8 and retention of E-cadherin, known targets of Slug regulation, at wound margins. It is likely that these selective alterations in cytoskeleton and cell:cell adhesion retard cell migration from wound margins.

3.2. Non-healing ulcers

An originally independent skin carcinogenesis study [17] utilizing Slug knockout mice and wild type littermates provided unexpected confirmation that impaired wound reepithelialization in Slug knockout mice had important physiologic consequences. Of the 20 Slug null mice chronically exposed to UVR, eight developed non-healing ulcers. One of

these mice developed two separate ulcers. Ulcers were invariably located in the mid-dorsal region, the region of the back expected to receive maximal UVR exposure. Grossly, ulcers appeared as clearly demarcated oval depressed areas with smooth, rounded borders (Fig. 6A). No bleeding or exudation from these ulcers was ever observed. Ulcers were noted as early as 30 weeks of UVR exposure; the average time to development was 42.2 ± 7.0 weeks of UVR exposure. The period from the time of ulcer development to sacrifice averaged 5.2 weeks. During that time, two ulcers did not change in size, two ulcers became smaller, and five ulcers progressively enlarged. At first measurement, ulcers were 30.1 ± 27.1 mm² in area; wound area at time of sacrifice was 77.7 ± 84.1 mm². Although none of the ulcers directly overlay a pre-existing skin tumor, tumors developed at the margins of three of these wounds; all three tumors were spindle cell tumors [17]. None of the 20 wild type mice used for this study developed comparable ulcers, although skin tumors did arise [17].

The margins of chronic wounds in Slug null mice were similar to those of the 72-h wounds in these mice, with little or no evidence of active reepithelialization (Fig. 6B). Like the margins of 72-h excisional wounds in Slug null mice, the margins of non-healing ulcers showed prominent keratin 8 staining, strong keratin 14 staining, some loss of keratin 10 staining, and retention of E-cadherin expression (Fig. 6C–F). All skin of Slug null mice chronically exposed to UVR strongly expressed keratin 6, and scattered Ki-67-positive cells were present up to the wound margin (Fig. 6G and H). Diffuse proliferative activity was attributed to the effects of chronic UVR exposure.

4. Discussion

In the present studies we demonstrated delayed wound reepithelialization in excisional wounds and development of non-healing skin ulcers in response to chronic UVR exposure in Slug null mice, in association with altered expression of keratin and E-cadherin, known targets of Slug transcriptional control. In contrast, expression of keratins 6, 10, and 14 did not differ from that seen at wound margins in wild type mice. Further evidence that Slug insufficiency may be related to poor wound healing is provided by our previous analysis of canine non-healing corneal ulcers. These studies revealed that ulcer margins isolated from non-healing canine corneas were deficient in Slug and did not express α -smooth muscle actin, a marker of corneal EMT, or tropomyosin when compared to normally healing cornea [19]. Furthermore, epithelial outgrowth was impaired in both corneal and skin explants prepared from Slug null mice [11,19]. These findings demonstrate that epithelial outgrowth is deficient in Slug null mice and suggest that Slug deficiency is associated with failure to heal.

Keratins 8 and 18 are the first keratins expressed during embryogenesis [30]. In the adult, keratin 8 is expressed in simple epithelia and is not normally expressed in either intact epidermis or at wound margins, although it is often aberrantly expressed in squamous cell carcinomas [31,32]. Slug is a transcriptional repressor of keratin 8 in mammary epithelia [24] and in intact mouse skin [23]. Keratin 8 expression in the skin may reflect dysregulated keratinocyte differentiation. In the present studies, keratin 8 expression was detected in scattered basal keratinocytes of Slug null mice and there was increased expression at the margins of excisional wounds and non-healing ulcers in these mice. The role of keratin 8 in wound healing is somewhat obscure. Keratin 8 is not required for wound repair in the mouse embryo, as shown by normal purse-string wound closure in keratin 8 null mice [33]. However, expression of exogenous keratin 8 in a variety of cell lines results in anchorage-independent growth, shortened doubling times, increased invasive and migratory capabilities [34], and apoptosis resistance *in vitro* [35], as well as enhanced metastasis *in vivo* [36]. It was recently reported that keratin 8 is linked to desmosomes by periplakin [37]. If expression of keratin 8 is suppressed in MCF7 breast cancer cells, there is disruption of

desmosomes and loss of cell sheet integrity resulting in a failure of the sheet to migrate as a unit; on the other hand, loss of keratin 8 expression in vimentin-positive epithelial cell lines results in accelerated keratinocyte migration [38]. Thus, elevated keratin 8 expression appears to be associated with increased migratory and invasive capacities in some contexts; however, elevated keratin 8 expression in Slug null mice does not appear to augment or even to support reepithelialization.

Reversible modulation of cell:cell junctions is required for effective reepithelialization. During reepithelialization, both desmosome and adherens junction density are decreased at wound margins, presumably to promote epithelial cell motility [5]. In these studies we found the expected loss of staining for E-cadherin at wound margins of wild type mice. In contrast, there was a striking retention of both proteins at the margins of both excisional wounds and non-healing ulcers in Slug null mice. Elevated Slug expression has been demonstrated to repress E-cadherin in a number of cell models [39–41], but, to our knowledge, this is the first *in vivo* evidence for a role for Slug in modulating cell adhesion during a normal physiologic process.

Despite reports that Slug expression suppresses keratinocyte proliferation [42], we did not detect Slug-dependent differences in keratinocyte proliferation either in the present *in vivo* studies or in previous or *in vitro* studies [11,43,44]. Expression of neither keratin 6, sometimes used as a marker of epidermal hyperplasia [25], nor the *bona fide* proliferation marker Ki-67 differed substantially in the 72-h wounds of null versus wild type mice. However, both markers were elevated in chronic wounds of UVR-exposed null mice. UVR is known to induce epidermal hyperplasia and keratin 6 expression [45,46]. The difference observed in 72-h versus chronic wounds in Slug null mice is thus likely to be based on the UVR exposure status. Interestingly, our previous studies showed delayed induction of keratin 6 following UVR exposure in Slug null mice [47]. However, in the present study, we did not observe altered kinetics of keratin 6 expression.

The present studies confirm a role for Slug in healing of primary wounds. Based on our findings of altered expression of cytokeratin 8 and E-cadherin at wound margins, Slug appears to function primarily in wound reepithelialization rather than in other healing-associated processes. Further, the emergence of non-healing cutaneous wounds in response to chronic UVR exposure suggests that Slug null mice are more susceptible to the effects of chronic low level skin damage than are wild type mice. The findings from this and previous studies suggest that Slug plays an important role in regulating genes essential for normal epidermal homeostasis, for maintaining epidermal integrity upon chronic insult, and for dynamic physiologic processes like wound repair [11,20,23]. It is likely that all of these activities are modulated due to Slug regulation of keratinocyte differentiation, cytoskeleton, adhesion, and motility.

Acknowledgments

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Fig. 1. Histology of 72-h wounds in wild type (A) and Slug null (B) mice (scale bar = 200 μ m). Arrows indicate edge of migrating epithelium. Note the thin and elongated ingrowth of migrating epithelium in the wild type wound which covers the defect to the level of the panniculus carnosus. In contrast, the epithelial ingrowth in the 72-h Slug null wound is short and ends bluntly, covering the defect to a level somewhat above the level of the hypodermis, which overlies the panniculus carnosus. Asterisks indicate the panniculus carnosus.

Fig. 2.

Immunohistochemistry for keratin 8 in 72-h wounds of wild type (A) and Slug null (B) mice (scale bar = 100 μ m). Arrows indicate edge of migrating epithelium. Note the absence of keratin 8 positivity in the wild type wound in contrast to robust staining for keratin 8 in basal keratinocytes at wound margins in Slug null mice.

Fig. 3. Immunohistochemistry for keratins 14 and 10 in 72-h wounds of wild type (A and C) and Slug null (B and D) mice (scale bar = 100 μ m). Upper panels (A and B) show staining for keratin 14 and lower panels (C and D) show staining for keratin 10. Keratin 14 immunoreactivity extends to the margins of all wounds. Note that keratin 10 expression in both wild type and Slug null mice stops fairly abruptly at the initial wound margin (arrows).

Fig. 4.

Immunohistochemistry for keratin 6 and Ki-67 in 72-h wounds of wild type (A and C) and Slug null (B and D) mice (scale bar = 100 μ m). Upper panels (A and B) show staining for keratin 6 and lower panels show staining for Ki-67 (C and D). Keratin 6 immunoreactivity, as shown in panel A, extends from the wound margins for a moderate distance distally in both 72-h wounds; asterisks indicate the extent of distal K6 staining. As shown in Panel B, a focus of prominent Ki-67 expression in the 72-h wounds of both wild type and null mice is located at some distance from the wound margin (arrows).

Fig. 5. Immunohistochemistry for E-cadherin in 72-h wounds of wild type (A) and Slug null (B) mice (scale bar = 50 μ m). Arrows indicate tip of epithelial outgrowth. Note retention of E-cadherin staining at the tip of the epithelial outgrowth in the Slug null mouse.

Fig. 6.

A typical chronic ulcer on the back of a UVR-exposed Slug null mouse (arrow) is shown in the upper left panel (A). Note the smooth rounded edges on the wound and the absence of exudate. The epithelial margin of the chronic wound in the Slug null mouse does not extend into the dermis (B) (scale bar = 100 μ m). There is robust expression of keratin 8 (C) and retention of E-cadherin (D) at the wound margin (scale bar = 50 μ m); keratin 14 is highly expressed in all keratinocytes (E), while keratin 10 expression is not seen at the immediate wound margin (F) (scale bar = 100 μ m). Keratin 6 is expressed uniformly throughout the epidermis (G), and Ki-67 is detected in many basal keratinocytes (H) (scale bar = 100 μ m).