Developmental transcription factor slug is required for effective re-epithelialization by adult keratinocytes.

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Involvement of the Developmental Transcription Factor Slug in Re-epithelialization by Adult Keratinocytes

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ABSTRACT

During re-epithelialization of cutaneous wounds, keratinocytes recapitulate several aspects of the embryonic process of epithelial-mesenchymal transition (EMT), including migratory activity and reduced intercellular adhesion. The transcription factor Slug modulates EMT in the embryo and controls desmosome number in adult epithelial cells, therefore we investigated Slug expression and function during cutaneous wound re-epithelialization. Slug expression was elevated in keratinocytes bordering cutaneous wounds in mice in vivo, in keratinocytes migrating from mouse skin explants ex vivo, and in human keratinocytes at wound margins in vitro. Expression of the related transcription factor Snail was not significantly modulated in keratinocytes during re-epithelialization in vitro. Epithelial cell outgrowth from skin explants of Slug knockout mice was severely compromised, indicating a critical role for Slug in epithelial keratinocyte migration. Overexpression of Slug in cultured human keratinocytes caused increased cell spreading and desmosomal disruption, both of which were most pronounced at wound margins. Furthermore, in vitro wound healing was markedly accelerated in keratinocytes that ectopically expressed Slug. Taken together, these findings suggest that Slug plays an important role during wound re-epithelialization in adult skin and indicate that Slug controls some aspects of epithleial cell behavior in adult tissues as well as during embryonic development.

INTRODUCTION

Re-epithelialization of cutaneous wounds involves extensive modulation of keratinocyte adhesion and motility (Krawczyk and Wilgram, 1973; Sciubba et al., 1978; Martin, 1997). Intermediate filaments retract from keratinocyte surfaces, desmosomes and hemidesmosomes associated with these intermediate filaments are disrupted, partial or complete dissolution of the basement membrane occurs, and keratinocytes lose polarity. These changes are accompanied by profound alterations in the actin-based cytoskeleton and occur concomitantly with an increase in migratory activity (Stenn and Depalma, 1988). In the final stages of re-epithelialization, reversion to the differentiated epithelial phenotype occurs, with formation of stable intercellular and cell-substrate contacts (Krawczyk and Wilgram, 1973; Sciubba et al., 1978; Martin, 1997). The events taking place during wound re-epithelialization are reminiscent of the developmental process of epithelial-mesenchymal transition (EMT). EMT is a dramatic phenotypic alteration characterized by transformation of anchored epithelial cells into migratory fibroblast-like individualized cells. EMT involves complete dissociation of intercellular adhesion structures (adherens junctions and desmosomes), cell elongation, and re-organization of the cytoskeleton (Boyer, 1989; Hay, 1995; Hay and Zuk, 1995; Viebahn, 1995).

The Snail family of zinc finger transcription factors, including Snail and Slug, is involved in EMT during development. Slug was first described as a transcription factor expressed in cells undergoing EMT during gastrulation and neural crest emergence in the chicken (Nieto et al., 1994; Ros et al., 1997; Cohen et al., 1998;

Savagner, 2001; Nieto, 2002). These functions appear to be performed by Snail in the mouse embryo (Sefton et al., 1998; Locascio et al., 2002). Both Snail and Slug induce EMT-like changes when over-expressed in epithelial cell lines (Savagner et al., 1997; Cano et al., 2000; Bolos et al., 2003) and both repress E-cadherin at the transcriptional level in vitro (Batlle et al., 2000; Bolos et al., 2003). Re-epithelialization in the adult skin shares some features with EMT such as modulation of the cytokeratin network, marked remodeling of cell-cell adhesion structures, and the emergence of cell motility (Arnoux et al., 2004). However, during re-epithelialization, migrating keratinocytes retain some intercellular junctions and thus remain part of a cohesive cell sheet. Based on these considerations, we investigated the role of the Snail family in re-epithelialization. In this report, we demonstrate a) increased Slug expression in partially dissociated migrating epithelial cells at wound margins, b) a requirement for Slug in keratinocyte outgrowth from skin explants, c) enhanced re-epithelialization in keratinocytes that constitutively express Slug, and d) an association between Slug expression and desmosomal disruption. Taken together, these findings indicate that Slug modulates some EMT-like events occurring during re-epithelialization.

MATERIALS AND METHODS

Wounding studies in mice

Heterozygous and homozygous Slug-lacZ knockin mice produced by Dr. Thomas Gridley (Jackson Laboratory) have been described (Jiang et al., 1998). These mice are maintained on a mixed background of 129 and C57BL/6 mice. The dorsum of heterozygous Slug-lacZ mice and their wild type littermates was shaved 24 hours before wounding. For wounding, mice were anesthetized with isoflurane and the shaved area was cleaned thoroughly with a topical iodine solution and rinsed with 70% ethanol. Two transcutaneous circular wounds 3 mm in diameter were made on the upper back of each mouse, using a sterile biopsy punch; there was no further treatment of the wound site. For sample collection, mice were sacrificed by CO_2 inhalation, and the area of skin containing the wound was removed and cross-sectioned. Half of each tissue sample was fixed in 10% neutral buffered formalin for routine histology and immunohistochemistry and half was embedded in a mixture of polyvinyl alcohol and polyethylene glycol (O.C.T., Thermo Shandon, Pittsburgh, PA), frozen, and stored at –80º C for subsequent preparation of frozen sections to demonstrate β-galactosidase activity. Frozen sections of wound margins 10 µm in thickness were dried at room temperature, then fixed and stained using the Mirus Beta-Gal kit (PanVera, Madison, WI) as recommended, except that substrate concentration was doubled.

Skin explants

Skin explants from heterozygous and homozygous Slug-lacZ mice were grown as described by Mazzalupo et al. (2002). Briefly, mice were killed by CO_2 inhalation,

the backs were shaved then treated with a depilatory (Nair, Church and Dwight, Princeton, NJ) to remove stubble, washed with a topical iodine solution, and rinsed thoroughly with 70% ethanol. Dorsal skin was removed and flattened in a sterile Petri dish. Circular explants were obtained using 3.5-mm disposable skin biopsy punches. Explants were allowed to adhere briefly to the plastic of 24-well culture vessels, 300 µl of culture medium was added, and tissues were incubated overnight at 37° C in a humidified atmosphere containing 5% CO_2 (Mazzalupo et al. 2002). The next day, 1 ml of fresh medium was added to each well. At harvest, the central skin core was removed and the remaining rim of epithelial cells was stained for β-galactosidase activity as above or for desmoplakin or keratin 14, using rabbit polyclonal primary antibodies against desmoplakin (Serotec, Raleigh, NC) diluted 1:1250 or keratin 14 (Covance Research Products, Berkeley, CA) diluted 1:5000 and the appropriate Vector ABC Elite kit (Burlingame, CA) as recommended.

Cell culture

The HaCaT and SCC 12F human keratinocyte cell lines were generously provided by Prof. Dr. Norbert E. Fusenig (German Cancer Research Center, Heidelberg, Germany) and Dr. William A. Toscano, Jr. (University of Minnesota, Minneapolis, MN), respectively. Culture conditions were described previously (McCawley et al., 1998; Airola and Fusenig, 2001). In vitro wounding studies in SCC 12F cells were performed using established techniques (McCawley et al., 1998) and quantified using ImagePro v. 4.1 image analysis software (GTI Microsystems, Tempe, AZ). Cell spreading was quantified as previously described (Savagner et al., 1997). For both cell lines, internuclear distances between a minimum of 100 adjacent cells were measured using Openlab 3 software (Improvision, Lexington, MA).

In situ hybridization for Slug

The technique used for in situ hybridization was based on that described by Raap et al. (1997) and has been described in detail (Choi et al., 2004). In brief, the pCRII.R.Slug.64-41 plasmid was cleaved with *Eco*RV or *Bam*HI and digoxigenin-labeled riboprobes were produced by in vitro transcription of *Bam*HI-cut template with T7 RNA polymerase or *Eco*RV-cut template with Sp6 RNA polymerase in the presence of digoxigenin-labeled UTP. In vitro transcription was performed as directed by the supplier of the RNA polymerases and digoxigenin-labeled UTP employed (Boehringer Mannheim, Basle, Switzerland). Control A8 cells transfected with the unmodified IRES2-EGFP vector were grown to confluence in chambered slides, then serum-containing medium was replaced with serum-free medium. Approximately 4 hours later, wounds were introduced; additional wounds were introduced 1, 2, and 3 days later. Staining was begun 4 days after the introduction of the initial wound. For some samples, 10 µg /ml mitomycin C was added 1-2 hours before the initial wound to prevent continued cell proliferation. Cell fixation and hybridization with probe were carried out as described by Raap et al. (1997). Briefly, cells were fixed in 4% formaldehyde containing 5% acetic acid and 0.9% NaCl, then dehydrated through a graded series of alcohols and xylene. Cells were permeabilized by incubation in 0.1% pepsin in 0.01 M HCl for 30 min at 37º C. After post-fixation in 0.1% formaldehyde in PBS, cells were again dehydrated, then air-dried. Each riboprobe was added at a concentration of 5 ng/µl to a hybridization mixture containing 4 X SSC, 10% dextran, 1 X Denhardt's, 50% formamide, and 500 µg/ml tRNA, and the mixture was denatured at 80º C for 10 min. Probe solution was applied to cells and covered with a HybriSlip coverslip (Grace Bio Labs, Bend, OR).

Slides were incubated overnight at 37º C in a humidified chamber. The next day, unbound riboprobe was removed by rinsing in 60% formamide-2 X SSC at 40º C and by treatment with 50□g/ml RNAse A in a buffer containing 10 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 1 mM EDTA. Digoxigenin-labeled probe was detected by incubation with anti-digoxigenin Fab (Boehringer Mannheim, Basle, Switzerland) diluted 1:50 followed by components of the TSA Biotin System (NEN Dupont, Boston, MA) as directed by the supplier. Nova Red (Vector Laboratories, Burlingame, CA) was used for visualization, and hematoxylin was used as a counterstain. Slides were dehydrated through graded alcohols and xylene, and coverslips were applied.

Microdissection and determination of Slug, Snail, and E-cadherin mRNA levels

Laser capture microdissection of cells from the first 3 cell layers bordering the wound and from confluent cells at least 10 cells distant from the wound edge was performed with a PixCell IIe (Arcturus, Mountain View, CA). Total RNA was extracted using the Absolutely RNA Nanoprep kit (Stratagene, LaJolla, CA); a DNA digestion step was included. RNA was reverse-transcribed using hexanucleotides and Superscript II reverse transcriptase (Invitrogen, San Diego, CA). Quantitative-PCR analyses were performed on an Applied Biosystems SDS- 7000 (Foster City, CA), using Sybergreen fluorescence and primers for human Slug (5'-CCC TGA AGA TGC ATA TTC GGA C-3'; 5'-CTT CTC CCC CGT GTG AGT TCT A-3'), 36B4 (5'-GTG ATG TGC AGC TGA TCA AGA CT-3'; 5'-GAT GAC CAG CCC AAA GGA GA)-3', human Snail (5' –GCT GCA GGA CTC TAA TCC AGA GTT-3'; GAC AGA GTC CCA GAT GAG CAT TG), and human E-cad (5'-TCA TGA GTG TCC CCC GGT AT-3'; CAG CCGCTT TCA GAT TTT CAT C-3'). Slug,

Snail, and E-cadherin mRNA levels were indexed to levels of the housekeeping gene 36B4, as described by Applied Biosystems.

Overexpression of Slug in keratinocyte cell lines

For transient *Slug* transfection, HaCaT cells were grown to confluence and incubated with FuGENE 6 Transfection Reagent (Roche Diagnostics, Basel, Switzerland) according to the supplier's instructions. Cells were co-transfected with the plasmid pCR3.M.Slug (Savagner et al., 1997) and pCDNA3/LacZ (Invitrogen, Carlsbad, CA), which was used as a marker of transfection. Cells were analyzed 48 hours after transfection. For stable transfection of SCC 12F cells, a 900-base pair *Nsi*I-*Xho*I fragment containing the entire coding sequence of *Slug* cDNA was isolated from the pBL.M.Slug (Savagner et al., 1997) and ligated into the IRES2-EGFP vector (Clontech, Palo Alto, CA) cut with *Pst*I and *Xho*I. The cytomegalovirus promoter drives Slug expression in this construct. The *Slug* gene is located upstream from the gene for enhanced green fluorescence protein. Although a single mRNA sequence encodes both Slug and EGFP, an internal ribosomal entry site assures that 2 separate proteins are produced. Cells were transfected by calcium phosphate using the procedure of Chen and Okayama (1987), and stable colonies were isolated following selection in growth medium containing 300 µg/ml G418 (Gibco, Carlsbad, CA). Detection of Slug mRNA was confirmed using RT-PCR (5'-CAGGGAACTGGACACACATAC-3'; 5'-AATGGGGCTTTCTGAGCCAC-3') and western blot analysis of whole cell lysates using polyclonal goat antibodies to Slug (Santa Cruz Biotechnology, Santa Cruz, CA). GFP expression was evident by western analysis, but was not detectable by immunofluorescence in the stable lines.

Immunofluorescence

SCC 12F cells were stained using mouse anti-desmoplakin (American Research Products, Belmont, MA) or rabbit anti-plakoglobin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies diluted 1:50, biotinylated secondary antibodies diluted 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA), and streptavidin-FITC (Southern Biotechnology Associates, Birmingham, AL). HaCaT cells were stained as described previously (Savagner et al., 1997), using rabbit anti-desmoplakin antibody (Natutec, Frankfurt, Germany) diluted 1:100, mouse anti-E-cadherin (BD Biosciences, San Jose, CA) diluted 1:100, or mouse anti-desmogelin 3 (Zymed, San Francisco, CA) diluted 1:100. DAPI was added to goat secondary antibody (Biosource International, Camarillo, CA) labeled with FITC or rhodamine.

RESULTS

Slug expression at wound margins

In Slug-lacZ mice an in-frame insertion of the β-galactosidase gene into the zinc finger coding region of the Slug gene inactivates the encoded Slug protein. However, the promoter of the Slug gene and the coding region for the Slug nuclear localization signal are preserved. Thus β-galactosidase activity of the fusion protein can be used to monitor Slug expression from its endogenous promoter and the fusion protein is detected in the nucleus. Jiang et al., (1998) showed that tissues that express full-length Slug mRNA also express the fusion protein, as detected by β-galactosidase activity, suggesting that β-galactosidase activity faithfully reflects the presence of Slug protein. However, it is possible that loss of the 3' end of Slug mRNA alters the mRNA stability of the Slug-lacZ transcript or that loss of the carboxy terminus of Slug protein changes its interactions with other proteins, affecting the localization or stability of the fusion protein. Mice possessing a single Slug-lacZ allele are phenotypically normal. Mice homozygous for the Slug-lacZ allele are functional Slug knockout mice; although small in size, these mice are viable and fertile (Jiang et al., 1998).

In intact skin of Slug-lacZ heterozygotes, β-galactosidase activity is confined to hair follicles and adjacent epidermis, where it is localized to nuclei as described elsewhere (Parent et al., 2004). After introduction of excisional wounds in these mice (Fig. 1), β-galactosidase activity in the nuclei of keratinocytes at wound margins became evident within 2 days. At 3 days, β-galactosidase activity was pronounced in keratinocytes constituting the actively migrating front of wound re-epithelialization,

then decreased as the wound resolved to become restricted to islands of basal cells surrounding hair follicles as seen in normal skin (Parent et al., 2004). Epithelial cell migration from murine skin explants mimics the rapid outgrowth of epithelial cells at wound margins and is a useful technique for examining the re-epithelialization component of wound healing (Mazzalupo et al., 2002). β-galactosidase activity was detected in many epidermal cells migrating from skin explants derived from heterozygous Slug-lacZ mice (see below), with maximal β-galactosidase activity at 3-4 days, comparable to the response observed in vivo.

Elevated Slug expression in migrating keratinocytes at wound margins was confirmed in vitro. In situ hybridization revealed that confluent serum-deprived neoplastic human SCC 12F keratinocytes did not express detectable levels of Slug mRNA; however, enhanced expression of Slug mRNA was evident in these cells at wound margins (Fig. 2). Slug mRNA was detected by 24 h after wounding, remained elevated at 48 and 72 h, and decreased by 96 h. Slug expression was particularly prominent in cells with lamellipodia actively migrating across the gap in the cell layer. Results were similar with or without the addition of mitomycin C to block cell proliferation, suggesting that Slug expression at wound margins was associated with enhanced cell motility rather than with increased cell division. Elevated Slug expression at wound margins was also evident in nontumorigenic human HaCaT cells. In a total of 5 independent studies performed 48 h after wound introduction, quantitative PCR analysis revealed a statistically significant ($p<0.002$ by Student's t-test) mean increase of 1.6-fold in Slug expression in cells isolated from the first 3 layers of cells at the wound margin relative to cells more than 10 cells distal to the wound margin. E-cadherin expression at wound margins was also evaluated by

quantitative PCR analysis, and we found no significant decrease at wound margins. We also investigated the expression of Snail mRNA, a related family member, in cultured human keratinocytes. Snail was expressed at very low levels in confluent or subconfluent HaCaT and SCC 12F cells. Moreover, Snail expression was not modulated at wound margins in HaCaT cells as determined by quantitative PCR (data not shown).

These findings indicate that Slug expression is dynamically regulated in keratinocytes at wound margins in vivo, ex vivo, and in vitro. Slug expression at wound margins was transient in vivo, ex vivo, and in vitro. Moreover, even at times of peak expression, not all cells at wound margins appeared to express the Slug-lacZ fusion protein in vivo or ex vivo. In vitro, elevated endogenous Slug expression was not associated with decreased E-cadherin levels. Furthermore, elevated expression of the related Snail transcription factor was not detected at wound margins. Because Snail has been reported as a transcriptional repressor of E-cadherin (Nieto, 2002), retention of E-cadherin at wound margins was consistent with the observed low levels of Snail expression.

Re-epithelialization is controlled by Slug

Preliminary studies comparing excisional wound healing in Slug knockout and control mice did not reveal a measurable difference in the overall rates of wound closure (data not shown). However, unlike the situation in man, wound contraction is responsible for up to 90% of excisional wound healing in rodents (Hayward and Robson, 1991; Davidson, 1998), and the impact of Slug on early re-epithelialization may have been masked by similar wound contraction kinetics in control and Slug

knockout mice. Because re-epithelialization is the predominant mode of human cutaneous wound repair, we specifically studied this aspect of wound healing. Epidermal cell migration from skin explants was examined using Slug-lacZ homozygous (Slug knockout) and Slug-lacZ heterozygous, same sex littermates. There was rapid and uniform outgrowth of epithelial cells from skin explants of phenotypically normal Slug-lacZ heterozygotes, but outgrowth from Slug knockout skin explants was markedly restricted, with the formation of only a few small islands of epithelium immediately adjacent to the explant (Fig. 3). These findings indicate that Slug plays a critical role in migration of keratinocytes from the wound edge in an ex-vivo assay that mimics in vivo re-epithelialization.

The importance of Slug in keratinocyte migration was confirmed by in vitro studies of Slug-transfected SCC 12F and HaCaT transfected human keratinocytes. Levels of Slug expression in stably transfected SCC 12F cells were similar to those observed after growth factor stimulation of the parental cells, suggesting that persistent Slug expression was within a biologically relevant range (data not shown). Constitutive Slug expression in SCC 12F cells did not alter cell proliferation as assessed by BrdU incorporation (data not shown). Slug-expressing colonies were significantly less compact than control colonies ($p<0.001$ by Student's t-test), as shown by differences in internuclear distances and illustrated in Figure 4. The mean internuclear distance was 69.2+22.2 µm and 66.6+18.5 µm for the 2 Slug-transfected cell lines compared to 46.7 + 10.0 µm and 46.8+10.4 µm for parental and control transfected cells, respectively. The extent of spreading and desmosomal dissolution in transfected SCC 12F cells (Fig. 4) was comparable to that observed in untransfected HaCaT cells at wound margins 48 h after wounding (Fig. 5).

Constitutive Slug expression dramatically enhanced in vitro wound healing in SCC 12F cells (Fig. 6). Cell-free areas of initial in vitro wounds and corresponding fields after 72 h were measured for 2 control cell lines and 2 Slug-transfected cell lines (2 fields in 4 independent cell cultures each). By 72 h after wounding, re-epithelialization by 2 independent Slug-expressing cell lines was nearly complete, with 6.56 ± 1.6% and 10.74 ± 4.2% of the original wound area remaining cell free. Only partial wound closure occurred in parental and vector control cells with 43.03 ± 4.74% and 47.8 ± 4.0% of the wound area remaining. This difference was statistically significant ($p < 0.001$ by Student's t test). Accelerated wound healing was not due to increased proliferation, since the same response was observed when cell proliferation was prevented by mitomycin C (data not shown).

Modulation of desmosome number at wound margins by Slug

Because dynamic regulation of desmosomes is an important aspect of re-epithelialization (Krawczyk and Wilgram, 1973) and Slug modulates desmosomes in rat bladder NBT-II cells (Savagner et al., 1997), we examined the relationship between Slug expression and desmosome number in keratinocytes. During peak explant outgrowth, Slug was expressed in the nuclei of many actively migrating epithelial cells (Fig. 7). Slug-expressing cells were more elongate and had fewer peripheral desmosomal attachments than cells that did not express Slug. These findings show a clear association of Slug expression with desmosomal dissolution in an ex vivo model of epithelial cell outgrowth. We confirmed this relationship in vitro using transiently transfected HaCaT cells expressing both Slug and green fluorescent protein. When a random sample of more than 100 Slug-transfected or

non-transfected cells in the same culture were evaluated, 40 % of transfected cells completely lacked desmosomes, whereas 100% of untransfected cells had desmosomes in all regions of cellular contact (Fig. 8A). This difference was statistically significant ($p < 0.001$ by Chi-square analysis). Figure 8B shows an example of a Slug-transfected cell displaying reduced desmosomal contacts, as detected by desmoplakin staining, and prominent lamellipodia. Similar findings were obtained in SCC12F cells stably transfected with Slug. In colonies of Slug-transfected cells, there was an overall decrease in membrane localization of the desmosomal proteins desmoplakin as detected by immunofluorescence (Fig 4). This decrease was particularly prominent in the actively migrating cells at the edges of expanding colonies and at wound margins. This was very similar to the partial desmosome dissociation observed in untransfected HaCaT cells at the edge of wound margins (Fig. 5). Finally, we performed confocal analysis to compare semi-quantitatively the actual number of desmosome expressed by marginal HaCaT cells versus cells located further away from the edge (Fig. 9). Desmosomes were visualized by immunolocalization of membrane-linked desmoplakin in regions of cell-cell contact. As seen in Figure 9, flattening of cells at the wound margin was accompanied by a decrease in the extent of desmosome-mediated cell-cell contact and the appearance of gaps between the cells; such gaps were not observed in confluent HaCaT cells.

We also looked at cadherin localization during in vitro wound healing in immortalized keratinocytes. The pattern of desmoglein 3 expression reflected that of desmoplakin at wound edges. However, desmoglein 3 expression was more persistent and did not show the characteristic punctate pattern seen for desmoplakin.

Interestingly, there was no decrease in E-cadherin expression at the wound edge and E-cadherin remained associated with the cell membrane (Fig. 10). This observation was consistent with the lack of E-cadherin or Snail mRNA regulation at wound edges that we also observed. These findings suggest that Slug specifically modulates desmosomes rather than adherens junctions at migratory epithelial cell margins.

DISCUSSION

Cutaneous wound healing requires the rapid and transient activation of a coordinated sequence of events that restores the functional integrity of the skin. The emergence and migration of basal keratinocytes during wound re-epithelialization involves certain morphologic and functional changes that also occur during EMT in development. Because Slug regulates EMT during development and appears to be crucial for migratory behavior during gastrulation and neural crest cell outgrowth in some species (Nieto et al., 1994; Ros et al., 1997; Nieto et al., 2002), we investigated the expression and impact of Slug on the EMT-like events occurring as part of wound re-epithelialization. In the absence of antibodies suitable for immunohistochemistry, we took advantage of heterozygous Slug-lacZ knockin mice to demonstrate the induction of the Slug-β-galactosidase fusion protein at wound margins in vivo and in epithelial outgrowths from skin explants. The ex vivo tissue explant assay we employed was developed to study keratinocyte migration during early wound re-epithelialization in the mouse in the absence of the confounding process of wound contraction. In this system, keratinocytes and other skin cells maintain their normal configuration and relationship to one another. The assay is described as a valuable alternative to in vivo cutaneous wound healing assays (Mazzalupo et al., 2002). We also demonstrated transiently increased expression of Slug mRNA at wound margins in 2 cultured human keratinocyte cell lines. During wound healing in vivo, ex vivo, and in vitro, Slug expression appeared in a sub-population of keratinocytes at wound margins at 1-2 days after wounding, peaked at 3-4 days, and subsequently declined. Thus the timing and localization of Slug expression were compatible with Slug involvement in early wound re-epithelialization. A critical role for Slug in wound re-

epithelialization was confirmed by the demonstration of dramatically reduced epithelial outgrowth from skin explants of Slug knockout mice and accelerated re-epithelialization in keratinocytes that constitutively expressed Slug.

In our studies, acceleration of wound healing in vitro appeared to result from enhanced migration rather than from increased proliferation, since ectopic Slug expression did not increase cell division as measured by BrdU incorporation and the effects of Slug on wound healing were not blocked when proliferation was prevented by mitomycin C in vitro. Moreover, during healing of excisional cutaneous wounds in vivo, keratinocyte proliferation actually takes place a short distance from the wound margin (Krawczyk WS, 1971) rather at the wound margin itself. Thus our findings in vivo and in vitro demonstrate that Slug expression and keratinocyte migration at wound margins can occur in the absence of keratinocyte proliferation. Interestingly, c-kit-positive hematopoietic cells from Slug knockout mice show markedly reduced migratory capabilities in response to stem cell factor (Perez-Losada et al., 2002), suggesting that the role of Slug in modulating cell migration is not restricted to epithelia.

Previous studies have indicated a role for Slug in modulation of desmosomes in tumor-derived cells. Overexpression of Slug in NBT-II rat bladder epithelial cells markedly reduced desmosome number, and Slug expression was required for growth factor-induced desmosomal dissolution in these cells (Savagner et al., 1997). Based on these observations, we investigated the impact of Slug on desmosomes in keratinocytes. We showed that a reduction in desmosome number at wound margins was associated with increased endogenous Slug expression, both in vitro and in skin

explants. In addition, ectopic expression of Slug in keratinocytes in vitro significantly reduced the overall number of desmosome-positive cells. Desmosome reduction in Slug-transfected cells was particularly prominent in migrating cells at wound margins. These findings indicate a functional relationship between Slug expression and desmosome density. The mechanism for Slug-induced desmosomal dissolution is not clear. Some desmosomal cadherins, such as desmocollin 2, include E-box sequences recognized by Snail family transcription factors in their promoter regions, but regulation of these genes by Slug and related proteins has not been examined.

Slug has been reported to repress the E-cadherin promoter in various transformed cell lines by binding to E-boxes (Cheng et al., 2001; Hajra et al., 2002; Blanco et al., 2002; Come et al., 2004; Magnino et al., manuscript submitted). However, E-cadherin expression does not appear to be significantly decreased in migrating immortalized keratinocytes, as shown in the present report both by RNA quantification after microdissection and immunolocalization. The lack of E-cadherin gene regulation in migrating keratinocytes has also been described by other authors (Pedersen et al., 2003). It is notable that co-expression of Slug and E-cadherin has also been described in the bladder carcinoma cell line NBT-II, in normal human mammary epithelial cells, and in invasive ductal carcinomas of the human breast (Come et al., 2004; Magnino et al., manuscript submitted). In these cases, cells remained at least partially cohesive while expressing motile or invasive behavior reminiscent of migrating keratinocytes during wound re-epithelialization.

Snail family members have been implicated in various functions not directly related to EMT, including determination of left-right asymmetry, neural development, apoptosis, cell division, and endoreduplication (Nieto, 2002). Moreover, despite a similar DNA-binding site, differences in function between Snail and Slug are now becoming increasingly evident. In addition, these functions appear to be strongly dependent on species. In the case of cutaneous wound healing in mammals, we found that Slug plays an important role during the transient physiological process of re-epithelialization. More work is needed to define Slug-controlled transcriptional programs and their implications for the adult organism.

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REFERENCES

Airola K, Fusenig NE (2001) Differential stromal regulation of MMP-1 expression in benign and malignant keratinocytes. J Invest Dermatol 116:85-92

Arnoux V, Côme C, Kusewitt DF, Valles H, Cauteret A, Savagner P. (In press) Cutaneous wound healing: a partial and reversible EMT. In Savagner P, ed. Rise and Fall of the Epithelial Phenotype: Concepts of Epithelial-mesenchymal Transition. Georgetown, Landes Bioscience.

Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol 2:84-89.

Blanco MJ, Moreno-Bueno G, Sarrio D, Locascio A, Cano A, Palacios J, Nieto MA (2002) Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. Oncogene 21:3241-3246.

Bolos V, Peinado H, Perez-Moreno MA, Fraga MF, Esteller M, Cano A (2003) The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci 116:499-511.

Boyer B., Tucker GC, Valles AM, Franke WW, Thiery JP (1989) Rearrangements of desmosomal and cytoskeletal proteins during the transition from epithelial to

fibroblastoid organization in cultured rat bladder carcinoma cells. J Cell Biol. 109:1495-1509.

Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA (2000) The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2:76-83.

Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7:2745-2752.

Cheng CW, Wu PE, Yu JC, Huang CS, Yue CT, Wu CW, Shen CY (2001) Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two-hit hypothesis of tumor suppressor gene. Oncogene 20:3814-3823.

Choi C, Hudson LG, Savagner P, Kusewitt DF (In press) An *in situ* hybridization technique to detect low-abundance Slug mRNA in adherent cultured cells. Methods Mol Biol.

Cohen ME, Yin M, Paznekas WA, Schertzer M, Wood S, Jabs EW (1998) Human SLUG gene organization, expression and chromosome map location on 8q. Genomics 51:468-471.

Côme C, Arnoux V, Bibeau F, Savagner P (In press) Roles of the transcription factors snail and slug during mammary morphogenesis and breast carcinoma progression. J Mammary Gland Biol Neoplasia.

Davidson JM (1998) Animal models for wound repair. Arch Dermatol Res 290 Suppl:S1-11.

Hajra KM, Chen DY, Fearon ER (2002) The SLUG zinc-finger protein represses E-cadherin in breast cancer. Cancer Res 62:1613-1618.

Hay ED, Zuk A (1995) Transformations between epithelium and mesenchyme: normal, pathological and experimentally induced. Am J Kidney Dis 26:678-690.

Hay ED (1995) An overview of epithelio-mesenchymal transformation. Acta Anat. (Basel) 154:8-20.

Hayward PG, Robson MC (1991) Animal models of wound contraction. Prog Clin Biol Res 365:301-312.

Jiang R, Lan Y, Norton CR, Sundberg JP, Gridley T (1998) The Slug gene is not essential for mesoderm or neural crest development in mice. Dev Biol 198:277-285.

Krawczyk WS (1971) A pattern of epidermal cell migration during wound healing. J Cell Biol 49:247-263.

Krawczyk WS, Wilgram GF (1973) Hemidesmosome and desmosome morphogenesis during epidermal wound healing. J Ultrastruct Res 45: 93-101.

Locascio A, Manzanares M, Blanco MJ, Nieto MA (2002) Modularity and reshuffling of Snail and Slug expression during vertebrate evolution. Proc Natl Acad Sci U S A. 99:16841-16846.

Martin P. 1997. Wound healing: aiming for perfect skin regeneration. Science 276:75-81.

Mazzalupo S, Wawersik MJ, Coulombe PA (2002) An ex vivo assay to assess the potential of skin keratinocytes for wound epithelialization. J Invest Dermatol 118:866-870.

McCawley LJ, O'Brien P, Hudson LG (1998) Epidermal growth factor (EGF) and scatter factor/hepatocyte growth factor (SF/HGF) mediated keratinocyte migration is coincident with induction of matrix metalloproteinase (MMP)-9. J Cell Phys 176:255-265.

Nieto MA, Sargent MG, Wilkinson DG, Cooke J (1994) Control of cell behavior during vertebrate development by Slug, a zinc finger gene. Science 264:835-839.

Nieto MA (2002) The snail superfamily of zinc-finger transcription factors. Nat Rev Mol Cell Biol 3:155-166.

Parent AE, Choi C, Caudy K, Gridley T, Kusewitt DF (2004) The developmental transcription factor Slug is widely expressed in tissues of adult mice. J Histochem

Cytochem, in press.

Pedersen TX, Leethanakul C, Patel V, Mitola D, Lund LR, Dano K, Johnsen M, Gutkind JS, Bugge TH (2003) Laser capture microdissection-based in vivo genomic profiling of wound keratinocytes identifies similarities and differences to squamous cell carcinoma. Oncogene 22:3964-3976.

Perez-Losada J, Sanchez-Martin M, Rodriguez-Garcia A, Sanchez ML, Orfao A, Flores T, Sanchez-Garcia I (2002) Zinc-finger transcription factor Slug contributes to the function of the stem cell factor c-kit. Blood 100:1274-1286.

Raap AK, van de Rijke FM, Dirks RW (1997) mRNA in situ hybridization to in vitro cultured cells. Methods Mol Biol 75:367-375.

Ros MA, Sefton M, Nieto MA (1997) Slug, a zinc finger gene previously implicated in the early patterning of the mesoderm and the neural crest, is also involved in chick limb development. Development 124:1821-1829.

Savagner P, Yamada KM, Thiery JP (1997) The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. J Cell Biol 137:1403-1419.

Savagner P (2001) Leaving the neighborhood: molecular mechanisms involved during epithelialmesenchymal transition. Bioessays 23:912-923.

Sciubba JJ, Waterhouse JP, Meyer J (1978) A fine structural comparison of the healing of incisional wounds of mucosa and skin. J Oral Pathol 7:214-227.

Sefton M, Sanchez S, Nieto MA (1998) Conserved and divergent roles for members of the Snail family of transcription factors in the chick and mouse embryo. Development 125:3111-3121.

Stenn KS, Depalma L (1988) Re-epithelialisation. In Clark RAF, Henson RM, eds. The Molecular and Cellular Biology of Wound Repair. New York, Plenum Press, 321-335.

Viebahn C (1995) Epithelio-mesenchymal transformation during formation of the mesoderm in the mammalian embryo. Acta Anat 154:79-97.

FIGURE LEGENDS

Fig. 1. Slug protein expression at wound margins in vivo. Excisional wounds were introduced into the skin of Slug-lacZ heterozygous mice and skin was harvested 24, 48, 72, and 96 h later. The activity of the β-galactosidase-Slug fusion protein was detected in frozen sections of wound margins by histochemistry and sections were counterstained with nuclear fast red. By 72 h after wounding, β-galactosidase activity was strongly expressed in the nuclei of cells at the leading edge of migrating epithelium. Arrowheads show the level of the basement membrane; arrows show wound margins. Bar = 40 μm.

Fig. 2. Slug mRNA expression at wound margins in vitro. Control SCC 12F cells transfected with the unmodified IRES2-EGFP vector were grown to confluence in chambered slides, then serum-containing medium was replaced with serum-free medium. Approximately 48 h later, wounds were introduced; additional wounds were introduced into each cell sheet 1, 2, and 3 days later. Staining was begun 4 days after the introduction of the initial wound. Mitomycin C (10 μg/ml) was added 1-2 h before the initial wound to prevent continued cell proliferation. In situ hybridization with the antisense riboprobe revealed increased Slug mRNA at wound margins (arrowheads) at 24, 48, 72 and 96 h after wounding. Slug expression was not detectable in confluent cells (c). No reactivity was seen at 48 h after wounding when the sense probe was employed (not shown). Bar = 200 μm.

Fig 3. Requirement for Slug for keratinocyte outgrowth from skin explants. Explants from sex matched homozygous and heterozygous Slug-lacZ littermates were cultured

ex vivo for 5 days, then the central skin core was removed, and the remaining rim of migrating keratinocytes was stained for keratin 14 to confirm the epithelial identity of the migrating cells. The solid black area shows the location of the original skin explant (removed for staining); the black arrowhead indicates the expanding edge of epithelial cell outgrowth from the explant; the red arrows show the direction of epithelial cell migration from the explant. Note the uniform thickness of the keratinocyte rim surrounding the explant from the heterozygous Slug-lacZ mouse (upper panel) and the isolated cluster of keratinocytes adjacent to the explant from the homozygous Slug-lacZ mouse (lower panel). Bar = 1 mm.

Fig. 4. Increased cell spreading and desmosome dissociation induced by constitutive Slug expression. Parental SCC 12F cells, control-transfected, and Slug-transfected clones A1 and A18 were cultured under standard conditions for 48 h after plating. Note that colony morphology under phase contrast microscopy was less compact in Slug expressing cells. Further evidence of cell spreading was obtained through nuclear visualization using DAPI staining and fluorescence microscopy. Differences in internuclear distance between Slug-expressing and control cells were statistically significant ($p<0.001$ by Student's t-test). Desmoplakin and plakoglobin localization by immunofluorescence showed a significant decrease in stably Slug-transfected clones A1and A18, especially in the marginal area. Bar = 50 µm.

Fig. 5. Increased cell spreading and desmosome dissociation at wound margins associated with endogenous Slug expression. HaCaT keratinocytes at the wound margin spread and are partially dissociated 48 h after wounding (a-d). DAPI staining of cell nuclei demonstrates compact confluent cells and cell spreading at the wound

edge (c, d). Desmoplakin localization was visualized by immunofluorescence 48 h after wounding in the confluent sheet (e) and at the wound margin (f). The arrowhead indicates a keratinocyte at the wound margin that is connected to its nearest neighbor by only a few desmosomes. Note that the leading edge of this cell is devoid of desmosomes. Bar=50 µm.

Fig. 6. Acceleration of re-epithelialization in vitro by constitutive Slug expression. Confluent SCC 12F cell monolayers of parental, control (vector only), and Slug-transfected stable clones (A1 and A18) were treated with 10 µg/ml mitomycin C for 2 h prior to wound introduction. Cells were washed extensively and maintained in serum-free medium for the duration of the experiment. Defined fields of the wound area were documented by photography immediately after and 72 h following wounding. Arrowheads indicate remaining cell-free areas in the Slug-expressing clones. For each cell line, the cell-free areas of the initial wound and corresponding field after 72 h were measured for 2 fields in 4 independent cell cultures. Cell-free areas for Slug-transfected cells were significantly smaller ($p<0.001$) than for parental or control cells. Bar = 200 µm.

Fig. 7. Association between Slug expression and reduced desmosome number in keratinocyte outgrowths from skin explants. Six-mm skin explants were cultured for 4 days and the migrating epithelial sheet that emerged was stained histochemically for β-galactosidase and immunohistochemically for desmoplakin. Slug-expressing cells were more elongate and expressed less desmoplakin than cells that did not express Slug. Slug-expressing cells were sometimes separated by small clefts

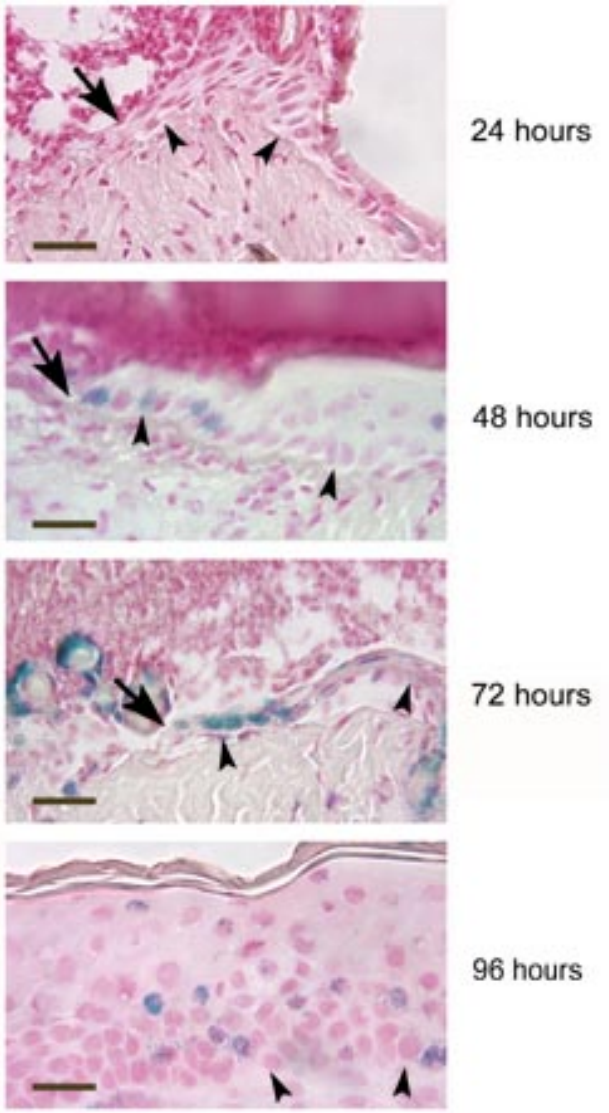
(arrowheads), while cells that did not express Slug were tightly joined by abundant desmosomes (arrow). Bar = 40 µm.

Fig. 8. Reduced desmosomal contacts between HaCaT keratinocytes ectopically expressing Slug. A) Significantly more control cells expressed desmoplakin than did Slug-transfected, GFP-positive cells ($p < 0.001$ by Chi-square analysis) when more than 100 cells of each cell type were examined. B) The same group of HaCaT cells is shown in each of the 3 panels. HaCaT cells transiently transfected with Slug were identified by GFP staining. Slug-transfected cells had prominent lamellipodia (arrowhead). These cells expressed little immunohistochemically detectable desmoplakin (DP) (arrow). The same cells were also stained with DAPI to demonstrate nuclei. Bar = 50 µm.

Fig. 9. Desmosomal quantitation in HaCaT cells. Confocal microscopy images were obtained at 1.5, 3.0, and 4.5 µm above the surface of the culture vessel at the wound margin and in a confluent area. Marginal cells were generally less than 4.5 µm thick. Desmoplakin staining in areas of cell-cell contact was found mostly at heights less than 3.0 µm and revealed gaps between in marginal cells (arrowhead). All images were obtained at the same magnification. The grey dotted line on the bottom right panel indicates the wound edge.

Fig. 10. Expression of desmoplakin, desmoglein 3 and E-cadherin in HaCaT keratinocytes at the wound margin. HaCaT keratinocytes were grown and wounded as described previously. Desmosomal components (desmoplakin, DP; desmoglein 3, DG3) and adherens junctions (E-cadherin, E-cad) were detected by

immunofluorescence. DP and DG3 were observed in the same field by double-labeling. They showed co-localization, but DG3 did not show the punctate pattern observed with DP. There was no significant decrease in intercellular E-cadherin staining at the wound margin compared to the confluent cell sheet.



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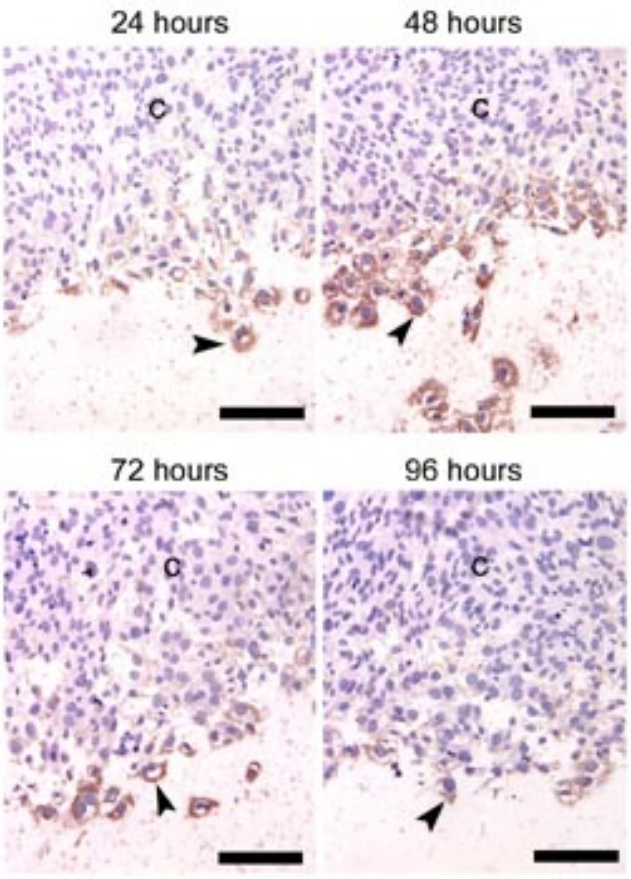

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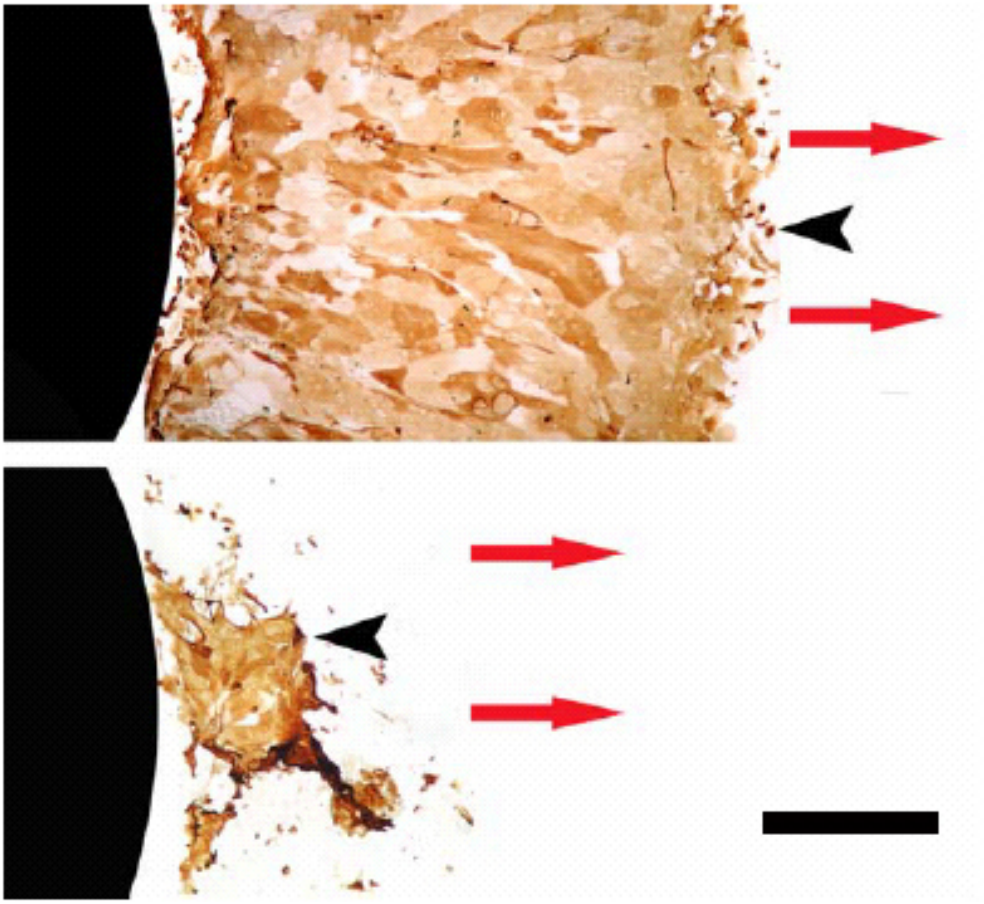

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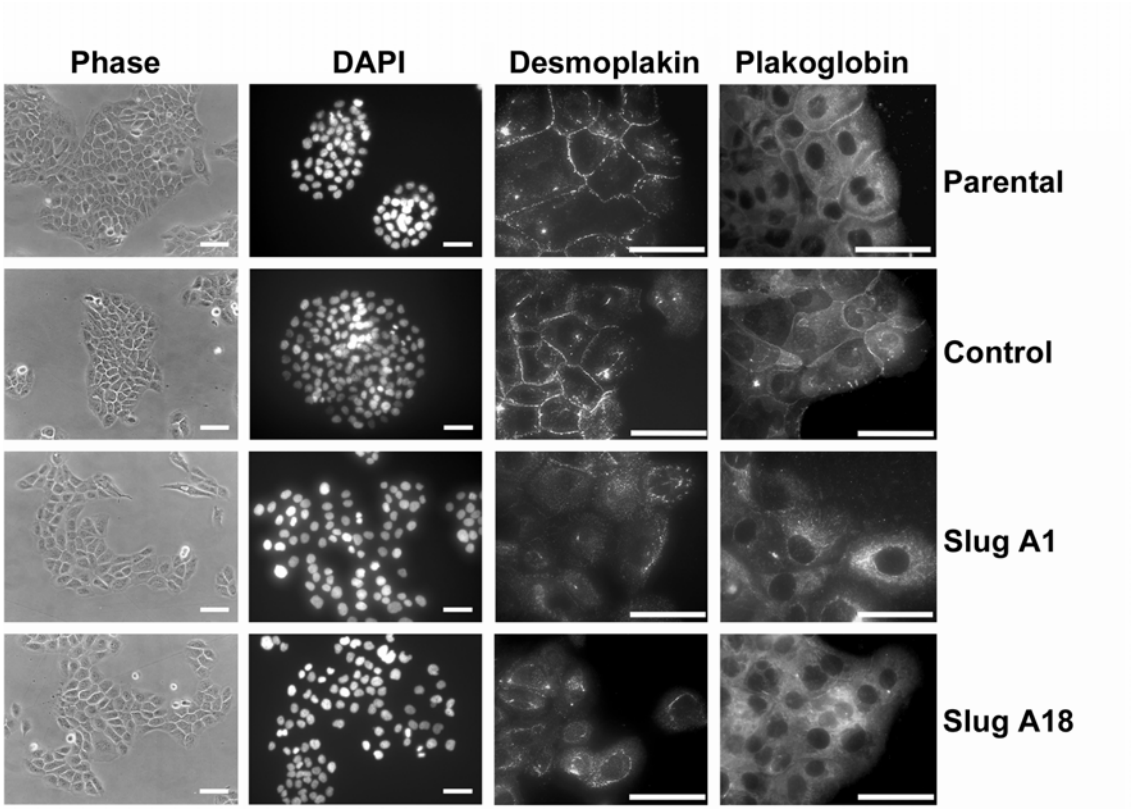

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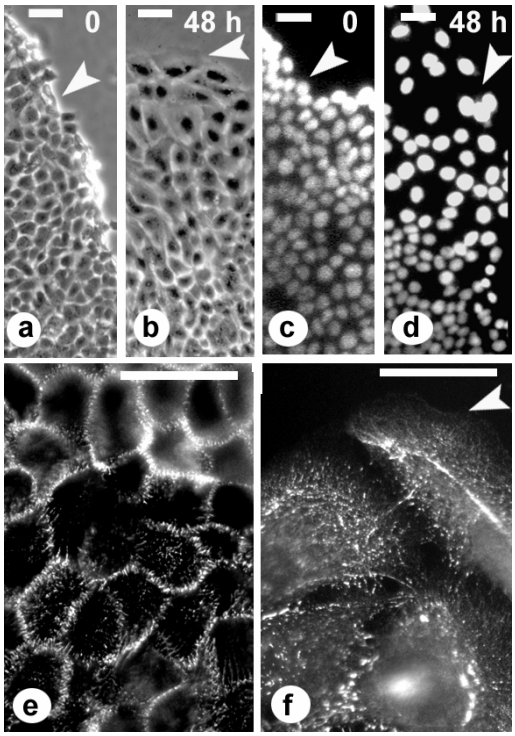

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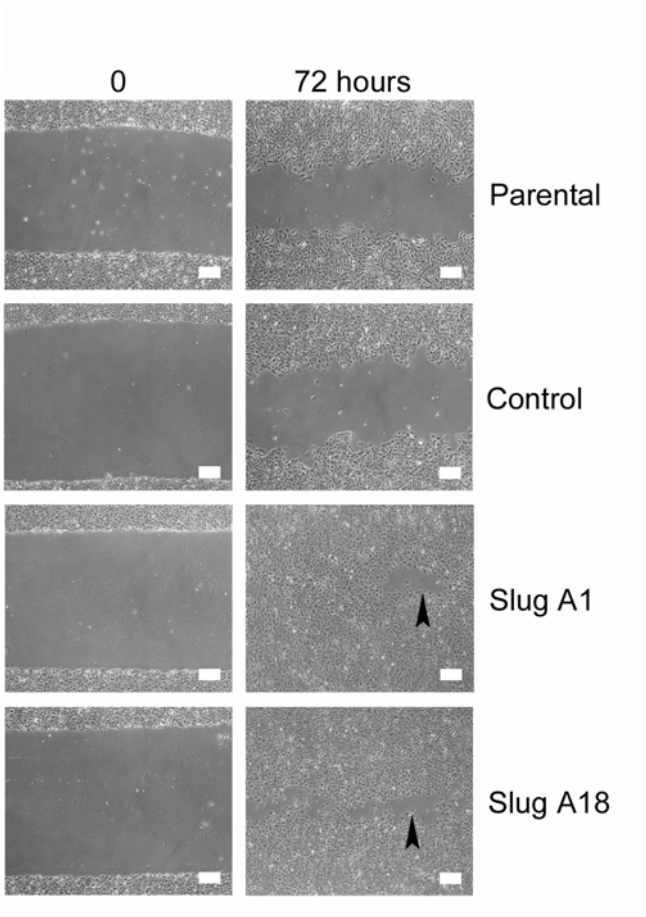

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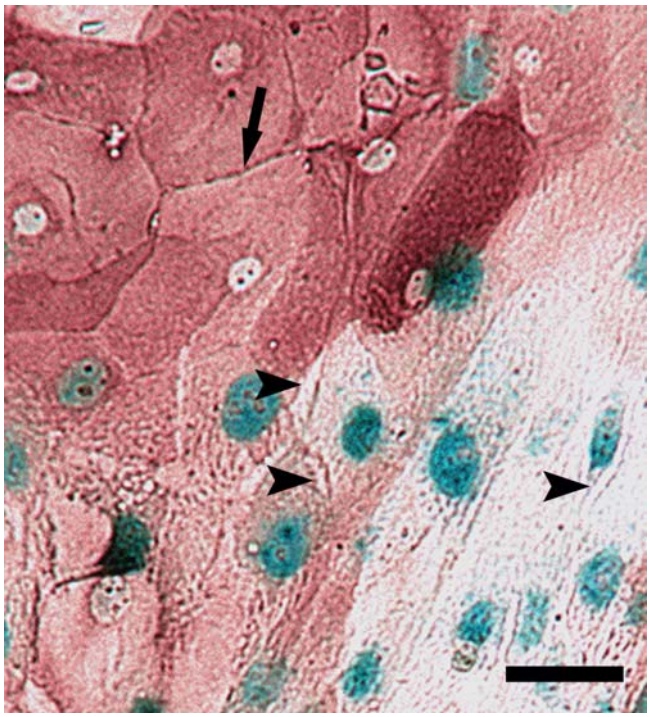

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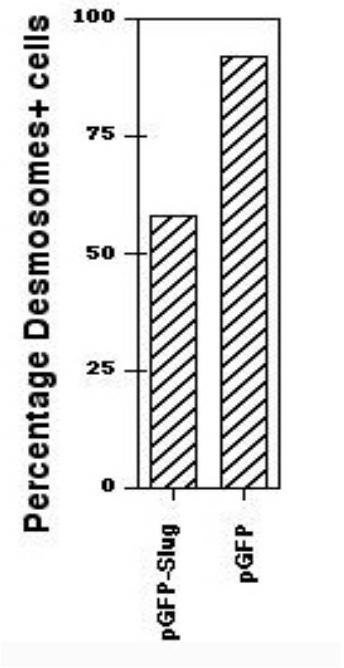

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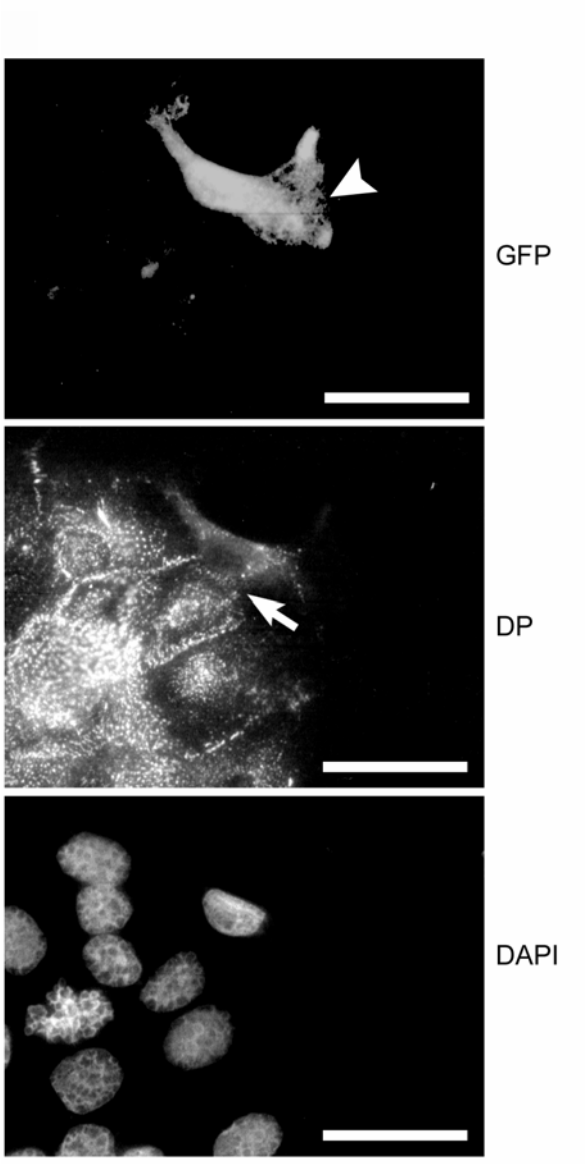

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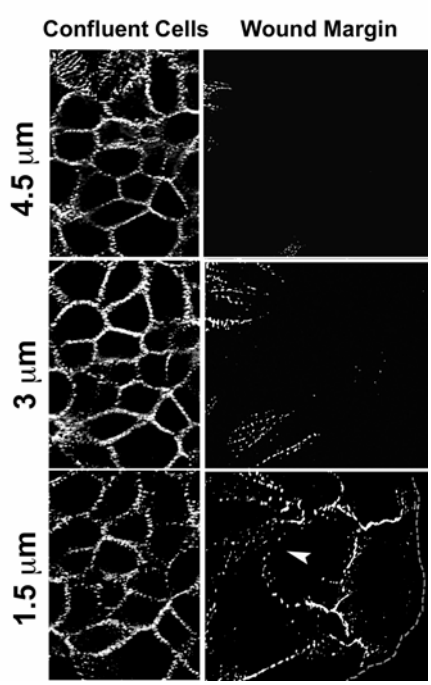

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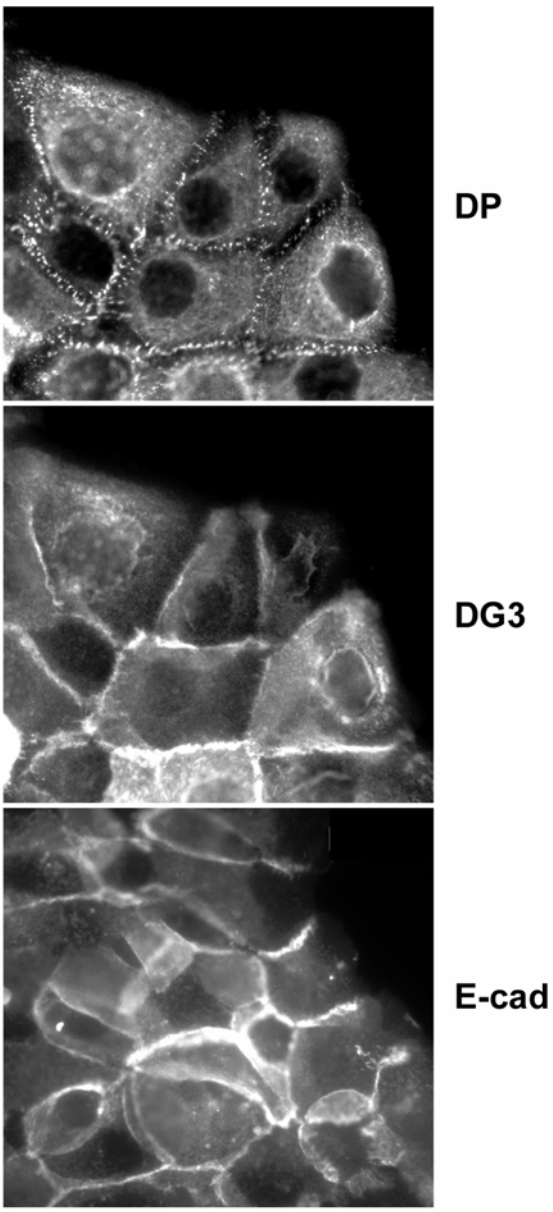

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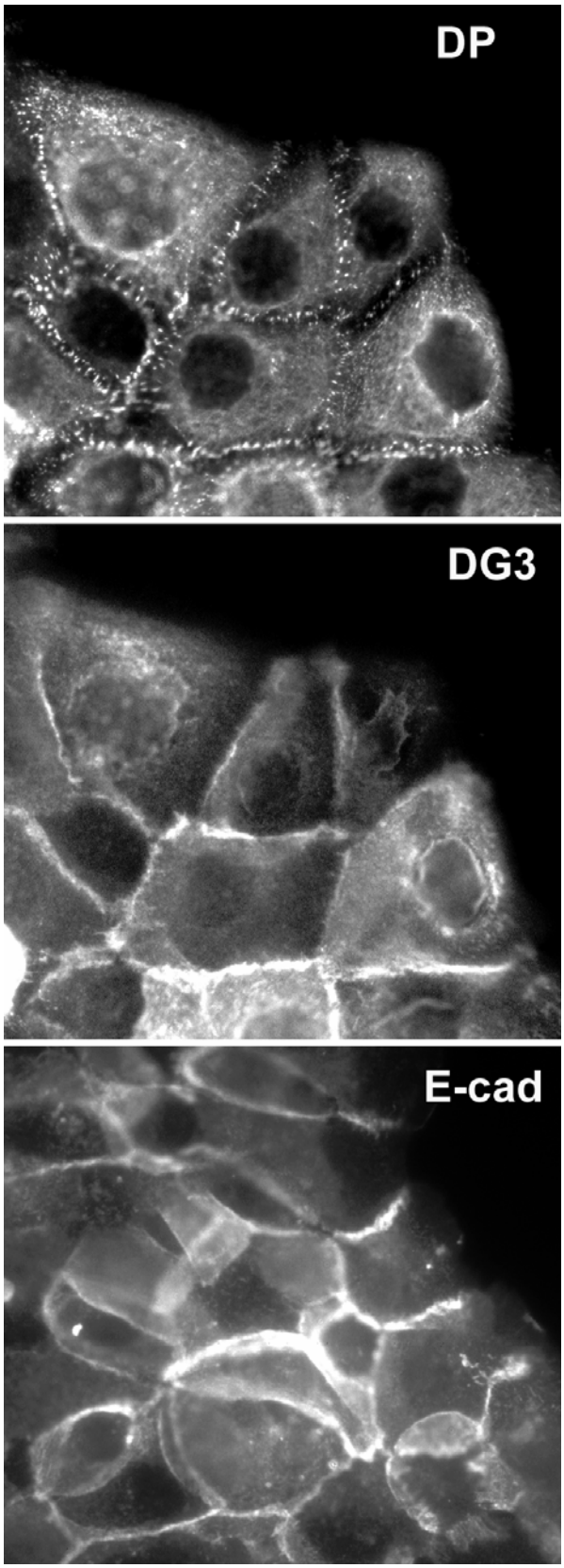

Figure 10.