Slug/Snai2 Is a Downstream Mediator of Epidermal Growth Factor Receptor-Stimulated Reepithelialization

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Many peptide growth factors, including EGFR ligands, accelerate wound reepithelialization *in vivo* and *in vitro*. Furthermore, EGFR expression is transiently increased at wound margins, suggesting an active role for this receptor in wound repair. During reepithelialization of cutaneous wounds, keratinocytes display a phenotypic plasticity resembling aspects of epithelial–mesenchymal transformation. The transcription factor Slug/Snai2 is a regulator of epithelial–mesenchymal transformation during development, and we previously reported that Slug expression is elevated in keratinocytes bordering cutaneous wounds *in vivo*, *ex vivo*, and *in vitro*. In this study we provide evidence that Slug expression is necessary for an EGFR-stimulated reepithelialization response. Epidermal growth factor (EGF) induces Slug expression and the response to EGFR activation is more robust than to other receptor tyrosine kinase ligands. EGFR-stimulated reepithelialization is highly dependent on Slug, as demonstrated by the absence of EGF-stimulated outgrowth in explants derived from Slug null mice. *In vitro* reepithelialization stimulated by ectopic Slug expression was not impaired by an inhibitor of EGFR catalytic activity, suggesting that Slug is a downstream mediator of this EGFR-stimulated response. Our findings provide evidence that Slug is an essential component of the pathway leading to EGFR-mediated epithelial outgrowth.

INTRODUCTION

Successful wound healing is a complex process involving cells of the epidermis, dermis, vasculature, and the immune system (Coulombe, 2003; Arnoux *et al.*, 2005). A crucial component of wound repair is reepithelialization, whereby the epidermal defect is sealed and barrier function is reestablished (Coulombe, 2003; Arnoux *et al.*, 2005). During reepithelialization, migrating keratinocytes undergo numerous functional and phenotypic alterations reminiscent of epithelial–mesenchymal transformation, including retraction of intermediate filaments, disruption of desmosomes and hemidesmosomes, alterations in the actin-based cytoskeleton, and loss of cell polarity (Coulombe, 2003; Arnoux *et al.*, 2005). These changes in keratinocyte morphology and behavior after injury are often referred to collectively as ''keratinocyte activation'' (Coulombe

1997, 2003; Freedberg *et al.*, 2001; Arnoux *et al.*, 2005). Keratinocytes become activated in response to changes in the microenvironment upon injury and then become major participants in the repair process through secretion of various cytokines and growth factors that help orchestrate tissue repair (Coulombe 1997, 2003; Freedberg *et al.*, 2001; Arnoux *et al.*, 2005; Myers *et al.*, 2007).

Ligands for the EGFR are present in the wound environment and promote wound repair (Werner and Grose, 2003). EGFR expression is elevated at the leading edge of healing wounds (Stoscheck *et al.*, 1992; Wenczak *et al.*, 1992), and experimental augmentation of EGFR expression improves wound healing *in vivo* (Nanney *et al.*, 2000). Furthermore, keratinocyte migration *in vitro* and epithelial outgrowth *in vivo* is decreased in EGFR null keratinocytes (Repertinger *et al.*, 2004), indicating that the EGFR is important for optimal wound repair. Although regulation of keratinocyte migration and epithelial outgrowth by ligands for the EGFR has been described (Stoll *et al.*, 1997; Hudson and McCawley, 1998; Repertinger *et al.*, 2004; Li *et al.*, 2006; Tokumaru *et al.*, 2000), the key downstream effectors of EGFR required for epithelial outgrowth are not well characterized.

The similarities among wound repair, tumor invasion, and developmental processes in the embryo are of interest based on the potential for shared regulatory pathways (Wood *et al.*, 2002; Martin and Parkhurst, 2004; Stramer and Martin, 2005). Slug/Snai2 expression is enhanced at the margins of

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healing wounds *in vitro* (Savagner *et al.*, 2005; Ikuta and Kawajiri 2006), *ex vivo*, and *in vivo* (Savagner *et al.*, 2005); and *in vitro* reepithelialization is markedly accelerated in keratinocytes that ectopically express Slug (Savagner *et al.*, 2005). Because Slug expression appears to be regulated by ras- and mitogen-activated protein kinase cascades (Savagner, 2001; Conacci-Sorrell *et al.*, 2003; Schmidt *et al.*, 2005; Hudson *et al.* 2007) and the EGFR and Slug are both elevated at the margins of healing wounds (Stoscheck *et al.*, 1992; Wenczak *et al.*, 1992; Savagner *et al.*, 2005; Ikuta and Kawajiri 2006), we investigated whether Slug is a downstream mediator of EGFR-regulated reepithelialization.

RESULTS AND DISCUSSION

EGFR activation promotes epithelial outgrowth and Slug expression

Epithelial cell migration from murine skin explants is a model for examining the reepithelialization component of wound repair (Mazzalupo *et al.*, 2002; Savagner *et al.*, 2005). Using this model, epithelial outgrowth from explants was limited when maintained in medium containing 10% fetal bovine serum (FBS) but lacking epidermal growth factor (EGF) (Figure 1). Explant outgrowth was markedly increased when 1 nM EGF was included in the culture medium, and the response was lost in the presence of AG1478, an inhibitor of EGFR catalytic activity (Figure 1). These findings indicate that
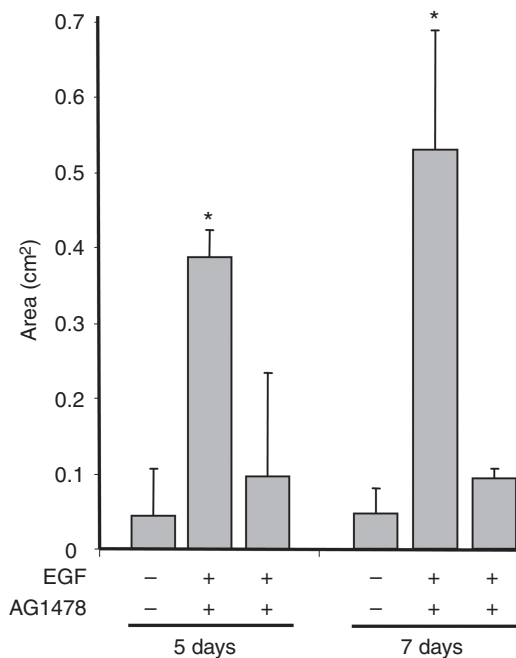
EGFR activation is a potent stimulator of epithelial outgrowth from mouse skin explants.

Because Slug is upregulated at the margins of healing wounds (Savagner *et al.*, 2005) and elevated Slug expression promotes epithelial outgrowth (Savagner *et al.*, 2005; Chandler *et al.*, 2007), we determined if EGF induces Slug expression. EGF stimulated Slug mRNA expression by 4.5-fold *in vitro* (Figure 2a). Keratinocyte growth factor induced Slug expression by nearly 2.5-fold, and modest increases ($<$2-fold) were observed following treatment with insulin-like growth factor-1 or basic fibroblast growth factor. Interestingly, this finding is consistent with results obtained in human skin explants where EGF, insulin-like growth factor-1, and fibroblast growth factor were all identified as important mitogens, but explant outgrowth was substantially greater with EGF (Bhora *et al.*, 1995). Because the EGFR ligand transforming growth factor-α (TGF-α) is reported to be a more robust migration stimulus than EGF (Hudson and McCawley, 1998; Li *et al.*, 2006), we tested both ligands for their ability to induce Slug protein expression. EGF and TGF-α induced Slug within 4 hours and maximal response was observed at 24 hours of treatment (Figure 2b). Induction of Slug was nearly twofold greater with TGF-α than EGF (10 nM, 24 hours treatment, $n=4$).

EGFR activation also stimulated Slug expression in mouse skin explants. In Slug–lacZ mice, the Slug locus has been inactivated by an in-frame insertion of the β-galactosidase gene into the zinc-finger coding region of the *Slug* gene, thus Slug expression can be monitored by detecting β-galactosidase activity (Jiang *et al.* 1998). EGFR-stimulated Slug expression was also apparent at sites of epithelial outgrowth of explants from Slug–lacZ heterozygotes as determined by expression of a Slug–β-galactosidase fusion protein (Figure 2c) and measurement of Slug mRNA levels in keratinocytes migrating from the explant (data not shown). Slug expression was greatly reduced in explants grown in complete medium containing FBS when EGFR activation was blocked by AG1478 (Figure 2c). These findings indicate that *Slug* is an EGFR responsive gene and suggest that Slug is a candidate downstream mediator of EGFR-dependent reepithelialization.

EGF-dependent epithelial outgrowth is highly dependent on Slug expression

To test whether Slug is required for EGFR-stimulated keratinocyte outgrowth, we compared *ex vivo* reepithelialization in explants derived from wild-type, heterozygous (not shown), or Slug null mice (Figure 3). Time-dependent and EGFR activation-dependent epithelial outgrowth was observed in explants isolated from wild-type and heterozygous mice with some evidence for a partial defect in the heterozygotes at the time of most active outgrowth (5 day outgrowth area: Slug wild type 0.252 ± 0.003 versus Slug null 0.144 ± 0.027 cm^2). Little outgrowth was observed in Slug null explants. Although EGF effectively enhanced epithelial outgrowth in explants isolated from wild-type mice, exogenous EGF did not promote this response in explants derived from Slug null mice (Figure 3). As further evidence that Slug is downstream of EGFR
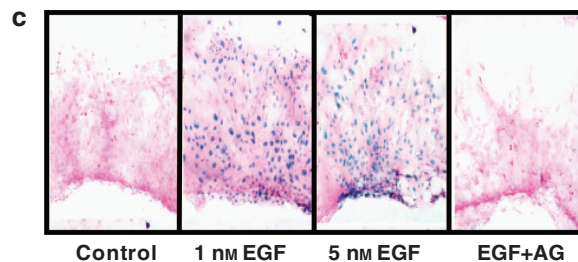

Figure 1. **EGFR activation promotes *ex vivo* reepithelialization.** Explants from wild-type mice were cultured for 5 or 7 days as described in ''Materials and Methods''. Explants were maintained in basal medium without EGF or in basal medium supplemented with EGF (1 nM) with or without 5 μM AG1478. Epithelial outgrowth was measured from digital images. Values represent the mean of 2–4 explants per mouse per treatment group from a total of four individual mice ± standard deviation. *$P<0.05$ EGF treatment compared to untreated control cultures.
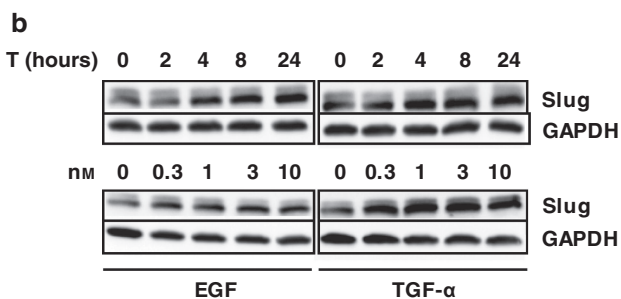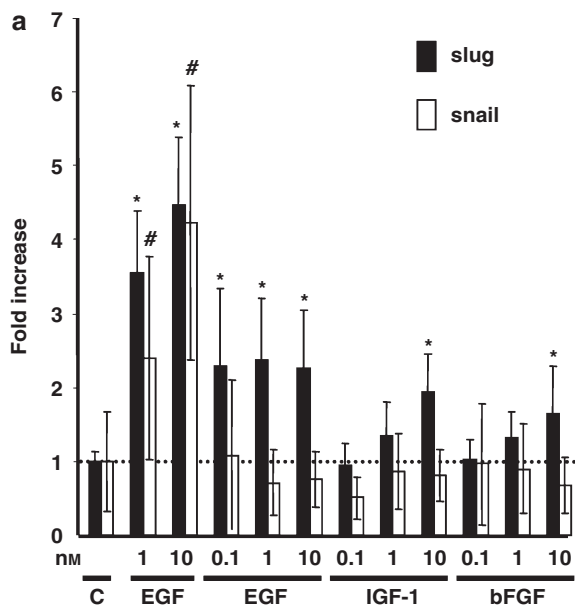
Figure 2. **Induction of Slug and Snail by growth factors.** (**a**) SCC 12F cells were grown to confluence, rinsed with phosphate-buffered saline, and placed in serum-free medium containing 0.1% (w/v) bovine serum albumin for 48 hours before addition of the indicated concentrations of growth factors. RNA was collected after 2 hours and mRNA levels were measured as described in "Materials and Methods". Values were normalized to GAPDH with the baseline level of Slug (filled bars) or Snail (open bars) in untreated cells defined as 1.0 (dotted line) and other values expressed as fold increase. Data shown represent the mean of three independent samples, each analyzed in three separate PCR reactions, ± standard deviation. *$P<0.05$ compared to Slug level in control group; #$P<0.05$ compared to Snail level in control group. (**b**) SCC12F cells were grown as in (**a**), treated with 1 nM EGF or 1 nM TGF-α for the indicated times (upper panels) or with the indicated concentrations of EGF or TGF-α for 24 hours (lower panels). Slug protein was detected by immunoblot analysis. Data shown are representative of three independent experiments. (**c**) Explants from wild-type mice were treated as indicated for 5 days (AG = 5 μM AG1478). Activity of the β-galactosidase–Slug fusion protein (blue stain) was detected by histochemistry.
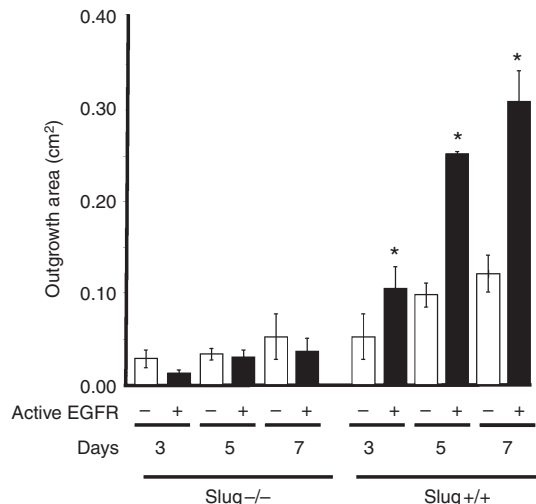

Figure 3. **EGF-dependent epithelial outgrowth is dependent on Slug expression.** Explants from sex- and age-matched wild-type (+/+) or homozygous Slug null (−/−) littermates were cultured in complete medium containing 5 nM EGF (+) or complete medium containing 5 μM AG1478 to inhibit EGF receptor activity (−). Epithelial outgrowth was measured as described in "Materials and Methods" and the legend to Figure 1. *$P<0.05$ comparing activated EGFR versus inactivated EGFR at the corresponding time point.
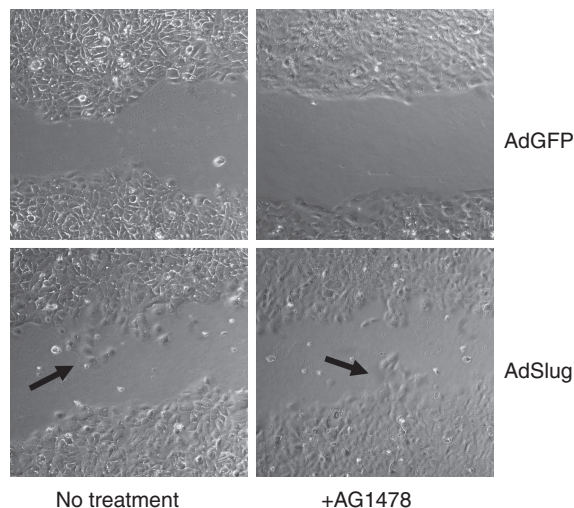

Figure 4. **Inhibition of EGFR does not disrupt keratinocyte outgrowth in cells expressing exogenous Slug.** SCC 12F cells were infected with either AdSlug or AdGFP as described in "Materials and Methods". An *in vitro* wound was introduced into cultures treated with 5 μM AG1478 or untreated, and outgrowth into the wounded area was monitored by phase contrast microscopy. As previously reported, ectopic Slug expression enhanced *in vitro* reepithelialization and cell spreading (Savagner *et al.*, 2005), but note that the Slug-associated keratinocyte outgrowth (arrows) was evident even when EGFR activity was inhibited by AG1478.

activation, inhibition of EGFR by AG1478 did not disrupt migration of SCC12F cells expressing exogenous Slug (Figure 4). The defect in outgrowth does not appear to be due to changes in cell proliferation. Quantification of Ki-67-positive cells in the intact epidermis did not reveal a

significant difference between wild-type and knockout epidermis (12.04 ± 3.32 versus 16.08 ± 7.07, $P = 0.112$ using Student's *t*-test), nor were significant differences in Ki-67 staining evident at the wound margins of wild-type and Slug null mice (manuscript in preparation).

Interestingly, epithelial outgrowth in Slug null mice was impaired despite EGF stimulation of Snail expression (Figure 2a), illustrating that Slug and Snail may play distinct roles in the epidermis. A recent publication highlights divergent transcriptional programs regulated by Slug and Snail despite their significant structural similarities (Moreno-Bueno *et al.*, 2006).

Several lines of evidence suggest an important role for Slug in normal adult epidermis. Slug is expressed in adult skin predominantly in hair follicles and interfollicular epithelium adjacent to hair follicles (Parent *et al.*, 2004). Slug expression coincides with outgrowth from wound margins (Savagner *et al.*, 2005; Ikuta and Kawajiri, 2006) and ectopic expression of Slug promotes epithelial outgrowth of keratinocytes (Savagner *et al.*, 2005) and corneal epithelium (Chandler *et al.*, 2007). Furthermore, Slug expression is greatly reduced at the margins of nonhealing corneal ulcers (Chandler *et al.*, 2007). Collectively, these findings suggest that Slug expression plays a positive role in wound reepithelialization. Our findings provide evidence that Slug is an essential component of the pathway leading to EGF-stimulated epithelial outgrowth and supports the conclusion that Slug contributes to regenerative processes in adult tissues.

MATERIALS AND METHODS

Cell culture studies

The nontumorigenic human keratinocyte cell line (SCC 12F) was generously provided by Dr William A. Toscano, Jr (University of Minnesota, Minneapolis, MN) and maintained in a medium consisting of 50% Dulbecco's modified Eagle's medium and 50% Hams-F12 medium (DME:F12; Sigma, St Louis, MO) containing the antibiotics penicillin and streptomyocin, 5% glutamine, and 5% FBS (Gibco, Gaithersburg, MD) as previously described (Hudson *et al.*, 2007). To minimize basal expression of Slug and Snail for induction studies, confluent cultures were maintained for 2 days in serum-free medium before growth factor treatment (Hudson *et al.*, 2007). Growth factors were obtained from Sigma and included EGF, TGF-α, keratinocyte growth factor, insulin-like growth factor-1, and basic fibroblast growth factor. For experiments involving ectopic Slug expression, subconfluent SCC 12F cells plated in a 12-well multiwell plate were incubated with AdSlug, AdGFP, or with virus-free medium (Chandler *et al.*, 2007) as follows: adenovirus (AdGFP or AdSlug, 10 μl, viral titer of 10^8 plaque-forming units per ml) was incubated with 2.5 μl Lipofectamine (Invitrogen, Carlsbad, CA) for 15 minutes at room temperature then added to 300 μl serum and antibiotic free DME:F12 medium. Cells were then incubated for 4 hours at 37 °C, the medium replaced with 500 μl DME:F12 containing 5% FBS and further incubated for 48 hours. A preliminary study titrating the virus concentration against the level of transgene expression at 48 hours indicated a transduction efficiency of 65–80% at this concentration (data not shown). After 48 hours, cells were moved to complete medium containing 5% FBS with or without 1 μM AG1478 (Cal Biochem, San Diego, CA). A cell-free

area was introduced into the now confluent cell cultures by scraping the monolayer with a sterile pipette tip and washing extensively to remove cellular debris (Savagner *et al.*, 2005). *In vitro* reepithelialization was monitored by migration of cells into the cleared area after 24 hours.

Skin explants

Skin explants from wild-type, heterozygous, and homozygous Slug-lacZ mice were grown exactly as described by Mazzalupo *et al.* (2002) (Savagner *et al.*, 2005). For specific studies, EGF was eliminated from the medium or supplemented to levels indicated in figure legends. To measure outgrowth area, the central skin core was removed and the remaining rim of epithelial cells was fixed briefly in 10% neutral-buffered formalin, permeabilized for 5 minutes in 70% ethanol and stained for 5 minutes with a 1:10 dilution of Mayer's hematoxylin. Images were captured digitally, adjusted in Adobe Photoshop to enhance contrast, and the area representing outgrowth from the skin plug was quantitated using NIH ImageJ. β-galactosidase staining of explants was performed as previously described (Parent *et al.*, 2004).

RNA isolation and quantitative mRNA measurement

Total RNA from cultured cells and explant outgrowths was isolated using Trizol as directed by the manufacturer (Invitrogen). DNA was removed using the DNA-free kit (Ambion, Austin, TX) as directed, and cDNA was produced from 250 to 500 ng of RNA using SuperScript II reverse transcriptase and oligo(dT) primers as recommended by the enzyme supplier (Invitrogen). For explants, the central tissue cores were removed before RNA isolation. Each explant sample analyzed consisted of a pool of RNA from four explants, representing two samples from each of two mice.

Quantitative PCR was performed using the Brilliant SYBR Green QPCR mix (Stratagene, LaJolla, CA) as directed and 100 nM of each primer. Primer sets included those for human Slug (5′-CCCTGAA GATGCATATTCGGAC-3′; 5′-CTTCTCCCCCGTGTGAGTTCTA-3′), human Snail (5′-CGGAAGCCTAACTACAGCGA-3′; 5′-GGACAGAGT CCCAGATGAGC-3′), human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-GCCGTGGAATTTGCCGT-3′; 5′-GCCATCAAT GACCCCAT-3′), mouse Slug (5′-GATGTGCCCTCAGGTTTGAT-3′; 5′-ACACATTGCCTTGTGTCTGC-3′), mouse Snail (5′-TGTCCAGAG GCTACACCTCA-3′; 5′-CTCACTGCCAGGACTCCTTC-3′), and mouse GAPDH (5′-ACCCAGAAGACTGTGGATGG-3′; 5′-CACATTGGGG GTAGGAACAC-3′). Amplifications were carried out on a Stratagene MX3000P Real-Time PCR System. In total, 45 cycles of 94 °C (30 seconds), 60 °C (30 seconds), and 72 °C (30 seconds) were performed. Electrophoresis of the products revealed single bands of the appropriate size (data not shown). RNA concentrations were calculated using the LinReg PCR program (Ramakers *et al.*, 2003) and normalized to GAPDH values.

Immunoblot analysis of Slug

Slug antibody for western blot assay was a kind gift from Dr Pascale Leroy. A total of 25 μg of protein was loaded on 15% SDS-PAGE gel, transferred to nitrocellulose membrane and blocked in 5% powdered milk in Tris-buffered saline Tween-20 (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween 20). The membrane was incubated with Slug primary antibodies (1:5,000) overnight at 4 °C in 5% powdered milk in Tris-buffered saline Tween-20 and was then

washed extensively with Tris-buffered saline Tween-20, and incubated with 1:8,000 anti-chicken secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized with the ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). Equivalent loading of proteins in each well was confirmed by loading controls using antibodies to GAPDH (1:2,000).

Statistical analysis

Results for different treatment groups were compared by Student's *t*-test and the value for statistical significance was considered to be $P < 0.05$.

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