of the exome was affected by CNVs in KNSTRN-mutated tumors versus 1.4% (SD 3.0%) in KNSTRN-wild-type tumors ($P=0.74$). Although this was not statistically significant, our ability to detect differences may have been limited by a small sample size.

In SCC, KNSTRN p.S24F is present in 19% of tumor precursors, suggesting that it arises early in disease progression (Lee *et al.*, 2014). To determine whether KNSTRN mutagenesis is an early event in BCC development as well, we screened 30 early stage BCCs for KNSTRN mutations. We identified a nonsynonymous KNSTRN mutation in only 1/30 (3%) early stage BCCs, suggesting that, unlike in SCC, mutant KNSTRN in BCC appears to be acquired later in disease and is possibly a marker of aggressive behavior (Figure 1c and f). The mutation, pH284D, is absent from dbSNP137 and ESP6500 and is predicted to be deleterious by SIFT; however, it has not previously been reported in the COSMIC database and is not predicted to be damaging by Polyphen.

These findings are the first to implicate KNSTRN in BCC tumorigenesis. Alongside recent data offering a role for KNSTRN in SCC and melanoma, our work supports the classification of KNSTRN as an oncogene and an important contributor to the pathogenesis of malignancies related to UV-exposure. In both SCC and BCC, mutant KNSTRN disrupts sister chromatid cohesion and promotes genomic instability in functional assays. However, unlike in SCC, KNSTRN mutations in BCC appear to occur late in disease progression and are preferentially found in advanced tumors. Further exploration of the role of KNSTRN in skin cancer and genomic stability is warranted.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Estrogen Upregulates Slug to Enhance the Migration of Keratinocytes

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TO THE EDITOR

Declining estrogen levels in postmenopausal women are related to delayed wound healing. It is known that estrogen influences skin re-epithelialization (Ashcroft *et al.*, 1997) and may promote skin wound healing via estrogen receptor-β (ER-β Campbell *et al.*, 2010). However, the cellular and subcellular mechanisms of estrogen's action on keratinocyte migration are still poorly understood. Epithelial–mesenchymal transition (EMT) is an important process in which epithelial cells lose their epithelial characteristics and acquire mesenchymal traits. This significant phenotypic conversion leads to the acquisition of motile behavior. In the skin, EMT has been shown to have a role in skin morphogenesis (Kong *et al.*, 2006), skin cancers (Shimokawa *et al.*, 2013; Wong *et al.*, 2013; Magnoni

Abbreviations: E2, 17β-estradiol; EMT, epithelial–mesenchymal transition; HaCaT, immortalized human skin keratinocyte cell line; mRNA, messenger RNA; PHK, primary human keratinocyte

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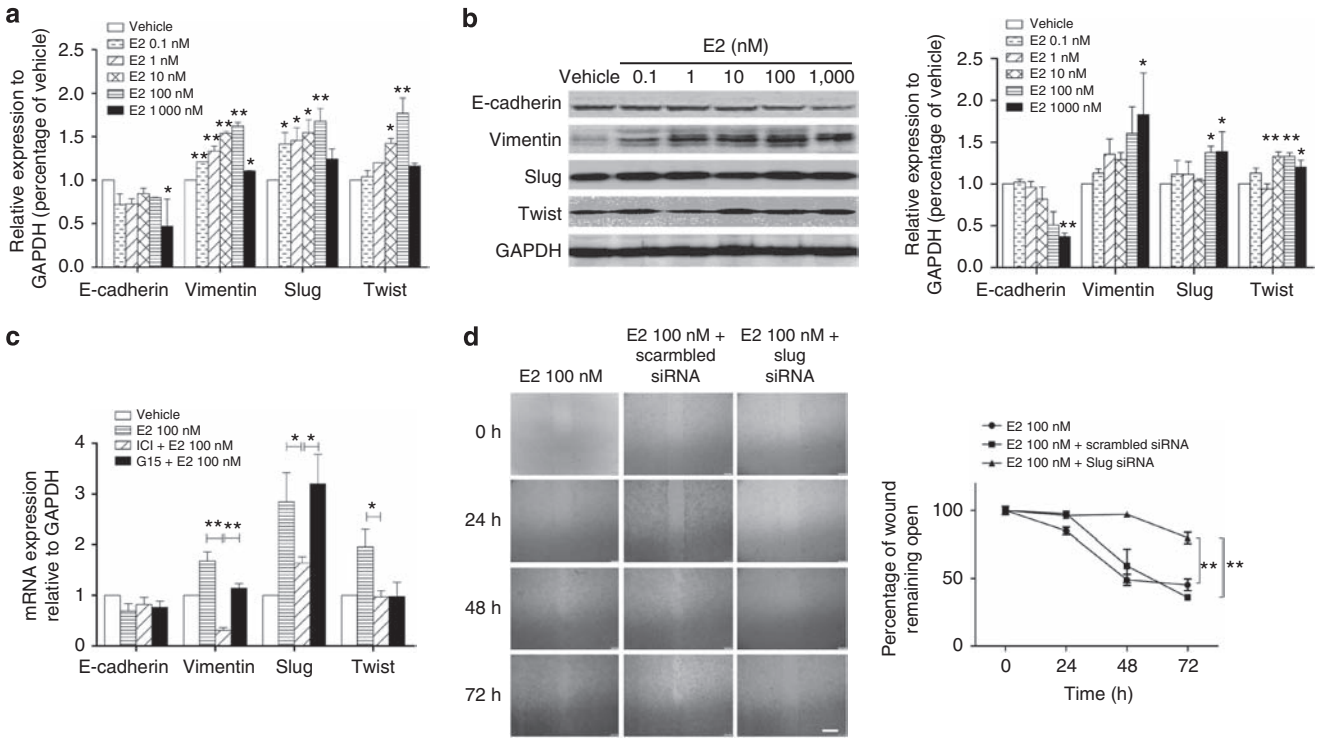

Figure 1. Upregulated Slug, Twist, and vimentin and downregulated E-cadherin in HaCaT cells after treatment with E2. (**a**) Quantitative real-time PCR (qRT-PCR) analysis of E-cadherin, vimentin, Slug, and Twist in HaCaT cells after treatment with 0.1, 1, 10, 100, and 1,000 nM E2 for 48 h. (**b**) Western blot analysis of E-cadherin, vimentin, Slug, and Twist in HaCaT cells. (**c**) Estrogen receptor (ER) mediates E2-induced E-cadherin, vimentin, Slug, and Twist expression in HaCaT cells. Inhibition of ER, but not GPR30, blocked E2-induced Slug, Twist, and vimentin messenger RNA (mRNA) expression. (**d**) Slug knockdown impairs the migration of HaCaT cells. HaCaT cells were transfected with siRNA oligo specific for Slug (Slug siRNA) or a scrambled siRNA oligo (scrambled siRNA) before stimulations with E2, and the migration of cells were assessed using scratch assay and are represented as percentage (%) of wound remaining open (Scale bar = 100 μm). All results are representative or means ± SD of three independent experiments. Differences were determined by one-way analysis of variance (ANOVA) or two-way ANOVA. $*P<0.05$, $**P<0.001$ versus vehicle-treated or E2 100 nM-treated HaCaT cells.

et al., 2014; Qu *et al.*, 2014), wound healing (Savagner *et al.*, 2005; Arnoux *et al.*, 2008; Hudson *et al.*, 2009), and skin fibrosis (Postlethwaite *et al.*, 2004; Takahashi *et al.*, 2013). In this study, we tested the hypothesis that estrogen-induced acceleration of wound healing is mediated by EMT during keratinocyte migration. This study was approved by the institutional review board and written informed consent was obtained from all subjects before they participated in the study. All experimental details are given in Supplementary Materials and Methods online.

As the expression of ER in human skin remains contentious, the expression of ERs in HaCaT (immortalized human skin keratinocyte cell line) cells and primary human keratinocytes (PHK) was analyzed by immunofluorescence. T47D, a human ductal breast epithelial tumor cell line, was used as a positive control of ERs. Whereas expression of both ER-α and ER-β was observed in T47D cells, HaCaT cells, and PHK expressed only ER-β (Supplementary Figures S1a and c online). The effect of 17β-estradiol (E2) treatment on viability of HaCaT cells was measured using an MTT assay. Although E2 had little effect on cell viability at lower concentrations, there was a statistically significant reduction in cell viability after treatment with 1 μM E2 ($P<0.0001$, Supplementary Figure S1d online).

To investigate the role of EMT in E2-induced keratinocyte migration, we first analyzed whether E2 could modulate the expression of EMT-biomarkers. HaCaT cells were cultured to 80% of confluency and then incubated with 0.1, 1, 10, 100 nM, or 1,000 nM E2 for 48 h. We found that concentrations of E2 ranging from 1 to 100 nM significantly induced the expression of Slug, Twist, and vimentin and significantly repressed the expression of E-cadherin, whereas treatment with 1,000 nM E2 had little or no effect (Figure 1a and b). We next utilized ICI, a potent ER antagonist, and G15, an antagonist of another estrogen-related receptor, GPR30, to evaluate the E2 mechanism of action on HaCaT cells. E2 altered expression of the EMT-biomarkers, Slug, Twist, vimentin, and E-cadherin, was reversed following treatment with ICI; however, G15 could not abrogate the changes in expression of EMT-biomarkers (Figure 1c). These data indicate that estrogen regulates expression of EMT-related molecules through ERs but not GPR30. Next, to determine the role of Slug in the E2-dependent modulation of EMT-related molecules, cells were transfected with Slug siRNA before stimulation with E2. We observed that expression of E-cadherin was increased, whereas that

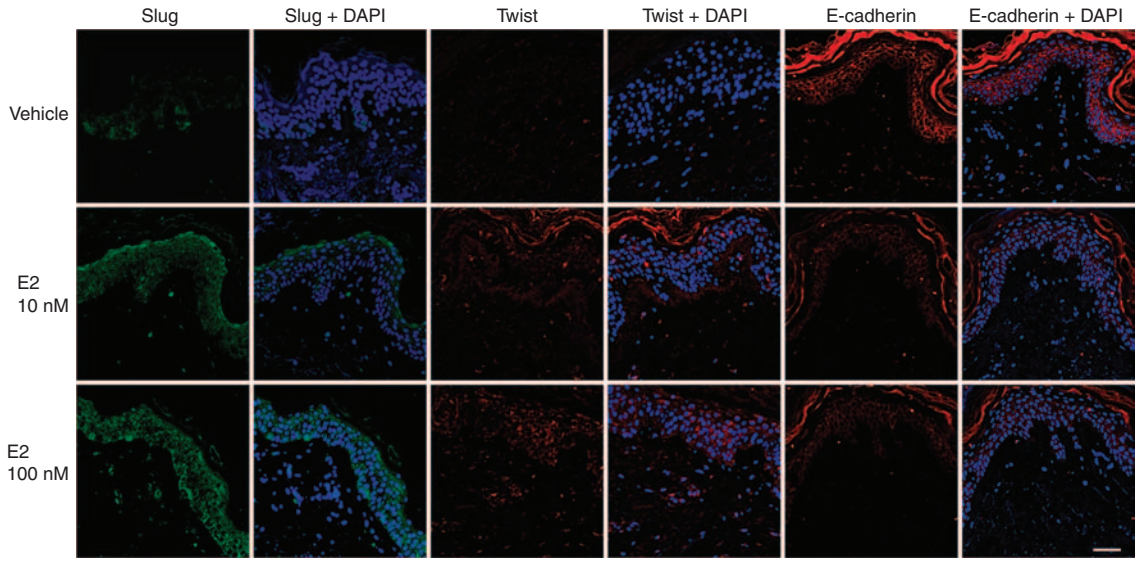

Figure 2. Upregulated Slug and Twist, and downregulated E-cadherin in an *ex vivo* skin explant model after E2 treatment. Immunofluorescence confocal images were obtained for Slug (green), Twist (red), and E-cadherin (red) in vehicle-treated (upper), 10 nM E2-treated (middle), and 100 nM E2-treated (lower) skin. The image is representative data of three independent experiments using nine skin explants obtained from two donors. Nuclei were stained with DAPI (blue). Scale bar = 50 μm.

of vimentin was reduced (Supplementary Figure S2 online), as compared with scrambled siRNA control.

To investigate whether estrogen can alter the rate of cell migration, a scratch assay was performed. Confluent monolayers of HaCaT cells were scratched with a sterile P200 pipette tip, and cellular debris was completely removed by washing. Cells were then treated with 0.1, 1, 10, or 100 nM E2, and images of the same field were captured at 0, 24, 48, and 72 h after scratching. As shown in Supplementary Figure S3a online, HaCaT cells incubated with all concentrations of E2 migrated toward the center of the wound to a greater extent than those treated with vehicle. In order to confirm results obtained from the scratch assay, transwell assays were performed. When various concentrations of E2 (0.1, 1, 10, and 100 nM) were added to the lower chambers, HaCaT cells in the upper chambers significantly migrated towards E2, as compared with those treated with vehicle (Supplementary Figure S3b online). To further determine whether Slug is involved in the E2-induced migration of HaCaT cells, scratch assay after Slug siRNA transfection was performed. The migration of HaCaT cells was markedly inhibited by transfection with Slug siRNA, as compared with cells transfected with scrambled siRNA control (Figure 1d). Collectively, these results suggest a critical role for Slug-mediated EMT in the E2-mediated enhancement of keratinocyte migration.

Because HaCaT cells are immortalized keratinocytes that may behave differently from normal human skin, we investigated the effects of E2 on expression of Slug in an *ex vivo* skin explant model. As the endogenous estrogen levels and response to exogenous estrogen are different depending on subjects' age and gender, skin explants were obtained from postmenopausal donors (Supplementary Table 1 online). At 2 days post-treatment with 10 or 100 nM E2, epidermal thickness was significantly increased in E2-treated epidermis compared with that in vehicle-treated epidermis. (Figure 2). Immunofluorescence labeling together with confocal laser microscopy using specific mABs revealed higher cellular expression of Slug and lower expression of E-cadherin in the 10 nM E2-teated (Figure 2, middle image) and 100 nM E2-treated (Figure 2, lower image) epidermis compared with vehicle-treated skin control (Figure 2, upper image). In addition, Slug was expressed mostly in the lower epidermis of vehicle-treated skin, however, it was expressed in the entire epidermis of 10 nM E2- and 100 nM E2-treated skin. Also, at 2 days post-treatment with 10 or 100 nM E2, epidermal thickness was significantly increased in E2-treated epidermis compared with that in vehicle-treated epidermis (Supplementary Figure S4 online). This suggests that E2 can modulate EMT in normal epidermis.

The Snail family of transcription factors is best known for its ability to trigger EMT through repression of E-cadherin (Batlle *et al.*, 2000; Cano *et al.*, 2000; Bolos *et al.*, 2003), which is important for the strength of cellular adhesion in a tissue and cellular motility. Savagner *et al.* (2005) analyzed the expression of Slug during wound healing using *in situ* hybridization and reported a crucial role for Slug in keratinocyte migration *in vitro*. Hudson *et al.* (2009) also showed that re-epithelialization of excisional wound healing was significantly impaired in Slug-deficient mice. In addition, EGF, the most potent effector of keratinocyte migration, induced activation of ERK5 and upregulated Slug (Arnoux *et al.*, 2008).

The estrogen-induced molecular changes demonstrated in this study are consistent with changes seen during EMT. Estrogen also accelerated keratinocyte migration, and this effect was lost in Slug knockdown cells. Our data indicate that estrogen might

promote re-epithelialization in wound healing via an EMT process. This highlights the possibility that further exploring the role of estrogen-induced EMT in the skin may yield novel insights into critical processes such as aging, fibrosis, and cancer metastasis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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