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Interleukin 6 and STAT3 regulate p63 isoform expression in keratinocytes during regeneration

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BACKGROUND

Epidermal stem cells express several transcriptional modulators required to maintain their undifferentiated and slow-cycling characteristics. One of these transcriptional modulators is p63, the oldest conserved homolog of the tumor suppressor, p53. p63 activity is vital to skin development, homeostasis, and wound healing. Regardless of genetic background, p63-null mice have no epidermis or epithelial appendages, and severe limb/craniofacial defects. Two different isoforms of p63 have been described and yet, the exact contribution of each isoform (TAp63 and Np63) to skin's functions remains to be determined because of conflicting results from numerous mouse models and human disease states (1). It is fairly well accepted that TAp63, expressed in the uncommitted surface ectoderm, induces proliferation and inhibits terminal differentiation (2) while Np63 isoforms are expressed after the ectoderm has committed to stratification (2).

Our previous work has demonstrated how the IL-6/STAT3 signaling pathway promotes wound-induced hair follicle neogenesis (WIHN) (3). We reasoned that since p63 is required for normal epidermal functions, including hair follicle formation (1), it may also be necessary during WIHN. A link between IL-6/STAT3 and the p63 isoforms TAp63 or Np63 has not been described in skin.

QUESTIONS ADDRESSED

Does p63 play a role in wound-induced hair neogenesis and is p63 expression in keratinocytes modulated by IL-6/Stat3 signaling pathway?

EXPERIMENTAL DESIGN

We examined the role of p63 during WIHN using wild type mice (C57BL/6) and investigated the impact of IL-6/STAT3 signaling on p63 isoform expression using mice and cultured human keratinocytes. All animal protocols were approved by the Johns Hopkins University Animal Care and Use Committee.

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RESULTS

A role for p63 during WIHN has not been described. We hypothesized that WIHN would be decreased in the absence of p63. Following our WIHN assay protocol, 1 cm² full-thickness wounds on the backs of wild type (C57BL/6) 21-day old male and female mice were created as previously described (3). A single intradermal injection of pan-p63 siRNA was introduced into the healing wound, 7 days after wounding (WD7) but before complete re-epithelialization, and was sufficient to inhibit p63 expression by 72% *in vivo* (**Fig. 1A**). This single injection of pan-p63 siRNA reduced the number of regenerated hair follicles ~2.5-fold in wild type mice compared to control injections (n=3; p = 0.07) (**Fig 1B**). These data suggest that p63 is partially necessary for WIHN in mice.

Our prior studies illustrated that IL-6/STAT3 signaling is necessary during WIHN; IL-6 increased WIHN whereas inhibition of Stat3 blocked WIHN (**Fig. 2A**) (3). Therefore, we next investigated whether the IL-6/STAT3 signaling pathway regulates p63 function in human keratinocytes. We hypothesized that IL-6/STAT3 signaling would maintain a basal keratinocyte phenotype, consistent with the described role of p63 in stem cell maintenance, through induction of TAp63 and inhibition of Np63. *In vitro*, TAp63 mRNA expression was significantly increased in normal human epidermal keratinocytes (NHEK) with IL-6 and inhibited by cucurbitacin I, a specific pharmacological JAK/STAT3 inhibitor, in a dose-dependent fashion (**Fig. 2B**). It is plausible that the level of induction from IL-6 alone is modest since cell culture systems already have significant p-STAT3 stimulation. Abundant levels of Np63 were also detected in monolayer keratinocyte cell culture. IL-6 also significantly increased TAp63 protein expression in C57BL/6 mice (**Fig. 2C**). Less consistently, Np63 protein levels decreased with IL-6. Most impressively of all, cucurbitacin I significantly decreased TAp63 and increased Np63 protein expression *in vivo* (**Fig. 2D**).

The molecular switch in p63 isoform expression is critical to epidermal stratification (Koster et al., 2004). In previous work, we show that IL-6 inhibits stratification *in vivo* and epidermal differentiation marker, keratin 1 (KRT1) by 4-fold (**graph inset, Fig. 2E**) (3). In this study, we examined whether or not IL-6's mediated changes in KRT1 expression are dependent on TAp63. In the presence of IL-6, TAp63-specific siRNA sufficiently blocks TAp63 mRNA expression NHEKs (**Supp. Fig. 1**). In NHEKs, TAp63-specific siRNA significantly increased KRT1 mRNA expression compared with non-targeting scrambled siRNA (**Fig. 2E**). Together, these data suggest that TAp63 plays a key role in mediating IL-6's effects in keratinocytes.

CONCLUSIONS

p63 mutations cause ectodermal dysplasia characterized by faulty hair development (1). Increasing TAp63 expression may represent one mechanism whereby IL-6 maintains a basilar keratinocyte phenotype, inhibits keratinocyte differentiation and promotes WIHN (3). TAp63-deficient mice develop skin ulcerations, have significantly delayed wound healing and accelerated aging (4). Additionally, ectopic over-expression of TAp63 decreases terminal differentiation (5). These studies are consistent with our findings.

Moreover, our data suggests a novel function for IL-6/P-STAT3 activation influencing p63 isoform expression in healing wounds and may contribute to WIHN. Unlike Np63, the promoter region of the TAp63 isoform does not contain a putative STAT3 response element; however, multiple NFκB binding sites are present (6). STAT3 and NFκB can interact after IL-6 stimulation and regulate gene transcription at the κB sites within promoters (7-9). It is conceivable that TAp63 expression is regulated by this STAT3/NFκB mechanism. In sum, these findings underscore the important crosstalk between immune and developmental signaling pathways during wound healing and regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Inhibition of p63 attenuates wound-induced hair follicle neogenesis in mice

A-B) Full excision skin wounding to the depth of skeletal muscle was performed in C57BL/6 mice and 7 days after wounding (WD7), 20 μ g of p63 siRNA or scrambled control (Scram) siRNA (in sterile water) was injected into the healing wound.

A) Mean fold change in p63 mRNA 24 hours after injection of pan-p63 or scrambled control siRNA as determined by qRT-PCR and normalized to housekeeping gene, 18S.

B) The number of regenerated hair follicles within scar, as assessed by confocal scanning laser microscopy (CSLM); representative CSLM images are shown. Area of WIHN shown within red box. Original image size is 4mm². n =3, *p < 0.07

Figure 2. IL-6 and STAT3 enhance WIHN with concordant modification of p63 isoform expression in vitro and in vivo

A) Representative CSLM images for control, addition of exogenous IL-6 protein (25ng/mouse) and addition of Stat3 inhibitor, cucurbitacin I (2mg/kg). Area of WIHN shown within red box. Original image size is 4mm². Data originally published in Nelson et al, 2015a and reproduced here for clarity.

B) Mean fold change in TAp63 mRNA after IL-6 (50ng/mL) +/- cucurbitacin I in NHEK for 24 hours as determined by qRT-PCR and normalized to housekeeping gene, RPLP0. (n=4, *p<0.05)

C) *In vivo* TAp63 protein levels after IL-6 (25ng) compared to PBS control in C57BL/6 mice measured by western blot; normalized to β-actin (n=3, *p<0.05).

D) TAp63 and Np63 protein levels after cucurbitacin I (2mg/kg) compared to PBS control in C57BL/6 mice measured by western blot; normalized to β-actin (n=3-4, *p<0.05).

E) Mean fold change in KRT1 mRNA with TAp63-specific or scrambled siRNA (control) in the presence of IL-6 (50ng/mL) in NHEK as determined by qRT-PCR and normalized to housekeeping gene, RPLP0 (n=3, *p<0.05). Graph inset: Data originally published in Nelson et al, 2015a (Fig 4E) and reproduced here for clarity.