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Smad4-dependent desmoglein-4 expression contributes to hair follicle integrity

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ABSTRACT

We have previously shown that keratinocyte-specific deletion of Smad4, a TGFβ/Activin/BMP signaling mediator, results in a progressive alopecia. To further assess the molecular mechanisms of Smad4 loss-mediated alopecia, we examined expression levels of key molecules associated with hair follicle differentiation in Smad4-deleted skin. Among them, Desmoglein 4 (Dsg4) was down-regulated in Smad4-deleted skin prior to the onset of hair follicle abnormalities with gradual depletion coinciding with hair follicle degeneration. Chromatin immunoprecipitation (ChIP) assay showed that Smad4, together with the BMP mediators Smad1 and Smad5, but not the TGFβ/Activin mediators Smad2 or Smad3, bound to the Smad Binding Element (SBE) of the Dsg4 promoter. A Dsg4 reporter assay revealed that Smad4 was required for the maximal transactivation of Dsg4 in cooperation with Smad1 and Smad5. Mutating the SBE of the Dsg4 promoter abrogated Smad4 transactivation of Dsg4. Furthermore, BMP ligands, but not ligands of TGFβ and Activin, induced endogenous Dsg4 expression. Our data demonstrate that in the presence of Smad4, BMP signaling participated in transcriptional regulation of Dsg4. Thus, Smad4 loss-associated Dsg4 depletion contributed, at least in part, to hair follicles degeneration in Smad4 deficient skin.

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Introduction

Epidermal development in mice begins in embryos around E9 with a single layer of the epithelium, and continues with stratification and epidermal barrier formation before birth (Fuchs, 2007). Thereafter, the epidermis undergoes constant self renewal throughout the life of the animal. Epidermal appendages include hair follicles and sebaceous glands. Hair follicle development begins around E14.5 in mice and continues with hair follicle differentiation and production of the hair shaft 1 week after birth (Millar, 2002). Hair follicle cells differentiate into the layers of the outer root sheath (ORS), the inner root sheath (IRS), and the hair shaft. Along with hair follicle morphogenesis, sebaceous glands develop from cells residing in the "bulge" area at the upper portion of the hair follicle. Postnatal hair follicles undergo regenerative cycles of growth (anagen), regression (catagen) and rest (telogen). Following telogen, a new hair shaft is generated adjacent to the previous one through the re-initiation of anagen (for review see Alonso and Fuchs, 2006). Key transcription factors shown to be involved in hair follicle formation and differentiation include Gata-3, Msx-2, FoxN1 among others. Loss of these molecules results in failure of hair shaft formation (Johns et al., 2005; Kaufman et al., 2003; Satokata et al., 2000). Another important molecule in making a normal differentiated hair shaft is Desmoglein-4 (Dsg4). Among the different Dsg isoforms, Dsg4 is the only isoform which is highly expressed in the hair cortex (Green and Simpson, 2007). Loss of function mutations in Dsg4 in humans, rats and mice result in balding due to aberrant hair shaft production (hypotrichosis) (Bazzi et al., 2005).

The process of skin morphogenesis, differentiation and renewal are tightly controlled by multiple signal transduction pathways. Among them, signaling from TGFβ/Activin/BMP, which requires Smad transcription factors as mediators, plays an important role (Li et al., 2003). In the TGFβ family, TGFβ2 has been shown to be required for hair follicle development (Foitzik et al., 1999), whereas TGFβ1 is required for the hair follicle to enter into the catagen phase (Foitzik et al., 2000). Activins have also been shown to play crucial roles in hair follicle development, as knocking out the activin ligand, expressing the activin antagonist, Follistatin, or expressing a dominant negative

Abbreviations: TGFβ, transforming growth factor β; BMP, bone morphogenetic protein; Dsg, desmoglein; SBE, smad binding element; ChIP, chromatin immunoprecipitation; ALK, activin like kinase; K5, keratin-5; TSS, transcription start site; E, embryonic day; P, postnatal day; ORS, outer root sheath; IRS, inner root sheath; DP, dermal papillae; WT, wildtype; KO, knockout; luc, luciferase.

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Activin receptor IB, all give rise to abnormal hair follicle development or cycling (Bamberger et al., 2005; Matzuk et al., 1995a,b). In the BMP family, a recent study has revealed that dermal derived BMP2 and BMP4 are cyclically expressed, which regulate stem cell activation during hair regeneration (Plikus et al., 2008). Studies have shown that overexpression of BMP ligands promotes differentiation and that BMP antagonists, such as Noggin, are required to maintain the undifferentiated state of epidermal progenitors (for review see Botchkarev and Sharov, 2004). Consistently, keratinocyte-specific deletion of the type 1A BMP receptor (also known as Activin Like Kinase 3; ALK-3) results in alopecia due to failure of IRS differentiation and hair shaft formation (Andl et al., 2004; Kobielak et al., 2003; Ming Kwan et al., 2004; Yuhki et al., 2004).

It has been shown that TGFβ/activin signals mainly through Smad2 and Smad3, whereas BMP signals mainly through Smad1 and Smad5 (Li et al., 2003). We have previously deleted Smad4, a common Smad that interacts with both TGFβ/activin-specific Smads and BMP-specific Smads, by crossing Smad4 floxed mice with MMTV-Cre mice (Qiao et al., 2006). In addition to directing Cre expression in mammary epithelia, the MMTV promoter targets Cre expression in keratinocytes, around E13.5 (Wagner et al., 2001). These conditional Smad4 knockout mice develop epidermal hyperplasia, progressive hair loss beginning at the first catagen phase on P16, and spontaneous skin tumor formation later in life (Qiao et al., 2006). Similar phenotypes have also been reported in keratinocyte-specific Smad4 knockout mice when using a truncated keratin 5 (K5) targeting vector, which targets Cre expression in keratinocytes around E13.5 (Yang et al., 2005). It remains to be determined whether the loss of hair follicle differentiation markers is the cause or the consequence of hair follicle degeneration in Smad4 null keratinocytes. Further, it remains to be determined whether Smad4 loss affects epidermal differentiation in addition to epidermal hyperproliferation, particularly when it is lost at stages critical for epidermal development. In the present study, we used a K5 promoter to target the CrePR1 transgene that allows inducible Cre expression in keratinocytes including epidermal stem cells as early as E10.5, when a single epithelial layer begins transitioning into a stratified epidermis (Han et al., 2006; Zhou et al., 2002). When we deleted Smad4 in K5.CrePR1/Smad4flox bigenic mice, we found that Smad4 deletion in keratinocytes either in embryos or after birth did not affect epidermal differentiation. However, Smad4 deletion resulted in degeneration of the hair follicles. We then focused on the identification of direct transcriptional targets of Smad4 that are imperative for hair follicle/hair shaft integrity. We found that Dsg4 is a Smad4 target gene and that loss of Dsg4 expression in Smad4 knockout keratinocytes contributed at least in part to hair follicle degeneration.

Materials and methods

Animals

Smad4 homozygous floxed (f/f) mice (Yang et al., 2002) were mated with K5.Cre.PR1 mice (Zhou et al., 2002). Smad4 deletion in keratinocytes was achieved by daily i.p. injection of 100 μg/kg RU486 with 0.5 mg progesterone in pregnant mice bred from the above two lines at the time points specified in the Results section, or through topical application of 20 μg RU486 to neonatal or adult mouse skin (specified in the Results section), once a day for 5 days. Genotypes of these mice were identified by PCR as previously described to detect the wildtype, floxed allele, the CrePR1 transgene, and cre-mediated Smad4 deletion (Qiao et al., 2006).

Histology and immunostaining

Skin samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Immunofluorescence (IF) or immunohistochemistry (IHC) was performed on OCT-embedded frozen sections or paraffin embedded sections as previously described (Wang et al., 1997). Primary antibodies used for immunostaining included: Adipophilin (1:500) and K14 (1:500) from RDI-Fitzgerald; K1 (1:250), K6 (1:250), Loricrin (1:500), and Filaggrin (1:500) from Covance; AE13 (1:100) from Abcam; Gata3 (1:50) from Santa Cruz; Smad4 (1:200) from Upstate; and E-cadherin (1:200) from BD. The AE15 antibody was a gift from Dr. T.T. Sun. Dsg4 staining was performed using mouse monoclonal anti-Dsg4 (18G8, 1:10) as previously described (Bazzi et al., 2006). Alexa Fluor 488 or 594 Secondary antibodies were purchased from Invitrogen.

Real time PCR

RNA from the dorsal skin was extracted in Trizol and further purified using an RNeasy column with on-column DNase treatment (Qiagen). 50 ng of RNA per reaction was analyzed and then amplified with Brilliant II 1-Step QPCR reagent (Stratagene). Analysis was carried out using MxPro Software V4.0 (Stratagene). Samples were analyzed in triplicate and normalized to an internal VIC-labeled Taqman probe for GAPDH. Other probes were FAM-labeled Taqman Assays as follows: Dsg1a: Mm00809994_s1, Dsg2: Mm00514608_m1, Dsg3: Mm00659652_m1, Dsg4: Mm00812608_m1, Msx2: 00442992_m1, Gata-3: 00484683_m1, Krt31: Mm00657991_gH HoxC13: Mm00802798_m1, FoxN1: Mm00433946_m1, Dlx3: Mm00438428_m1, Gli-1: Mm00494645_m1.

ChIP

Fresh skin was removed at postnatal day 6 (anagen), chopped and disrupted using a Dounce homogenizer, and cross-linked with 4% formaldehyde for 20 min. The cross-linked chromatin was then sheared using the ChIP-IT Express Enzymatic kit (Active Motif) for 10 min. Fifteen μg of sheared chromatin was immunoprecipitated with 1 μg of antibody at 4 °C for overnight, and precipitated DNA was eluted in 50 μl H_2O. Antibodies used for immunoprecipitation included: RNA Pol II, rabbit anti-Smad3, and rabbit anti-Smad4 from Upstate; mouse monoclonal antibodies for Smad1 and Gata-3 were from Santa Cruz; rabbit antibodies for Smad2 and Smad5 from Zymed; and rabbit anti-phospho-Smad1/5 from Cell Signaling. PCR primers encompassing the SBE of the mouse Dsg4 primers are as follows: FWD-5′ ACCCC-CTGAAATAAACTGGAGC and REV-5′ GGTAGGTGCTATGGTGACTAAA-CCC. PCR Primers encompassing a region of −5 kb of the promoter, which does not contain SBE, were used as a negative control: FWD-5′ GCTATCGCTGAAACAAAGGTCACAG and REV-5′ TGATGAGGGACT-CTGGCTAATGC. DNAs precipitated with individual antibodies were used for PCR. Primers for the BMP responsive element in the Msx2 promoter (Brugger et al., 2004), and for the TGFβ responsive Snail1 promoter, were used as controls for binding of Smad1/5 or Smad2/3, respectively.

Luciferase (Luc) assay, site-directed mutagenesis, primary cell culture, growth factor treatments

To avoid interference of high levels of endogenous Smad4 with Luc assay, K5.Smad4−/− keratinocytes were used for Luc assay. Primary mouse epidermal keratinocytes were isolated following suggested media protocol (CellNTec/Chemicon). Primary mouse hair follicle keratinocytes were isolated from neonatal K5.Smad4−/− mice (in C57BL/6 background) as previously described (Han et al., 2006) in CnT-07 media (Chemicon). Briefly, following dermal–epidermal separation of neonatal skins, dermal pieces were finely minced and digested with collagenase for 1 h at 37 °C. These were then centrifuged in a 4% Ficoll gradient before plating. Prior to transfection, cells were placed in CnT-02. Keratinocytes were transfected with full-length individual Smad expression constructs at 50 ng/well each (gifts from XH Feng), 500 ng

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