Transient activation of β-catenin signaling in cutaneous keratinocytes is sufficient to trigger the active growth phase of the hair cycle in mice

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Wnts have key roles in many developmental processes, including hair follicle growth and differentiation. Stabilization of β-catenin is essential in the canonical Wnt signaling pathway. We developed transgenic mice expressing a regulated form of β-catenin in the skin. Chronic activation of β-catenin in resting (telogen) hair follicles resulted in changes consistent with induction of an exaggerated, aberrant growth phase (anagen). Transient activation of β-catenin produced a normal anagen. Our data lend strong support to the notion that a Wnt/β-catenin signal operating on hair follicle precursor cells serves as a crucial proximal signal for the telogen–anagen transition.

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1219
[DasGupta and Fuchs 1999]. Also, the first postnatal anagen does not occur in mice in which β-catenin expression is progressively lost in the skin (Huelsken et al. 2001). Finally, Wnts 10a and 10b are expressed in postnatal hair follicles at anagen onset, but not in resting follicles (Reddy et al. 2001). These data are consistent with the view that a Wnt signal activates β-catenin signaling in the bulge, thereby driving the resting follicle into active growth.

Whereas the data imply a role for β-catenin in the telogen–anagen transition, no studies have examined the effect of inducible and reversible β-catenin signaling in the skin, with the goal of modeling the transient activation resulting from effects of canonical Wnt signaling. Therefore, we sought to develop a system in which we could tightly regulate β-catenin function in the mouse skin. We have shown previously the function of a chimeric β-catenin protein, in which a full-length β-catenin polypeptide with a codon 33 activating mutation (serine-to-tyrosine substitution—S33Y) is fused in-frame to a mutated version of the hormone binding domain of mouse estrogen receptor-α (ER), is rapidly activated by the ligand 4-hydroxytamoxifen (4-OHT) in cultured cells (Kollig et al. 2002). We expressed the S33Yβ-catenin–ER fusion protein in the skin of transgenic mice using the well-characterized bovine keratin 5 (K5) promoter (Ramirez et al. 1994). We explored effects of chronic and transient activation of β-catenin signaling in cutaneous keratinocytes by topical application of 4-OHT. We show here that prolonged activation of β-catenin signaling results in profound proliferation of the ORS and other epithelial components of the hair follicle. Transient signaling results in activation of a normal anagen phase. These data demonstrate β-catenin signaling provides a potent growth stimulus for hair follicle progenitor cells and is sufficient, when transiently activated in epithelial hair follicle precursors, to trigger telogen to anagen transition.

Results and Discussion

Regulation of β-catenin activity in keratinocytes and transgenic mice

The generation and use of chimeric proteins containing polypeptide sequences of interest fused to the hormone-binding domain of the mouse ER protein has been a successful strategy for exploring protein function, because the activity of the ER fusion protein can be tightly regulated by 4-OHT, an estrogen agonist/antagonist (Littlewood et al. 1995). As noted above, we generated previously a construct encoding a constitutively active β-catenin polypeptide (S33Yβ-catenin) fused in-frame to the ER hormone binding domain (S33Yβ-catenin–ER, Kollig et al. 2002). To investigate β-catenin function in mouse skin, the S33Yβ-catenin–ER sequences were positioned downstream of the bovine K5 promoter, which directs high levels of transgene expression to stratified squamous epithelium (Ramirez et al. 1994, Fig. 1A). In vitro studies with the human 1811 keratinocyte cell line were pursued to confirm the regulation of the S33Yβ-catenin–ER fusion protein by 4-OHT in keratinocytes. Specifically, 1811 keratinocytes were transfected with expression constructs encoding the S33Yβ-catenin–ER fusion protein, and we assessed the ability of the fusion protein to activate a β-catenin/TCF-responsive model reporter gene construct in the presence or absence of 4-OHT. One construct used the K5 promoter sequences, whereas the other construct used mouse Moloney leukemia virus long terminal repeat sequences to direct expression (i.e., pBabe). As shown in Figure 1B, the empty K5 expression construct had no demonstrable effects on TCF transcriptional activity, either in the presence or absence of 4-OHT. In contrast, both the pBabe/S33Yβ-catenin–ER construct, or the K5/S33Yβ-catenin–ER construct along with either the TCF-responsive reporter construct TOPFLASH or control FOPFLASH construct. Cells were then treated with either 4-OHT in ethanol, or ethanol alone, and harvested 30 h later to assess luciferase activity. The assays were performed in duplicate; data are reported as the ratio of relative light units for TOPFLASH:FOPFLASH, normalized for transfection efficiency. (C) Expression of the K5/S33Yβ-catenin–ER fusion protein relative to endogenous β-catenin in transgenic mouse lines. Protein was isolated from tail skin of F1 mice derived from three independently derived founders and subjected to Western blot analysis with a mouse monoclonal anti-β-catenin antibody. The blot was reprobed with an anti-β-actin antibody to verify equal loading and transfer. (D) β-catenin protein localization in a clipped region of dorsal skin from K5/S33Yβ-catenin–ER transgenic mice not treated (−4-OHT) or 24 h after treatment with a single topical dose of 4-OHT (+4-OHT).
screened for the transgene by PCR, and three transgenic lines were selected for further studies based on Western blot data showing differing levels of S33Y-β-catenin–ER fusion protein expression in the skin of the mice [Fig. 1C]. F1 mice were generated by backcross of the founder mice onto a C57BL/6J background. The majority of the studies described here used F3 or later generations of mice generated from the L2 line with moderate levels of transgenic expression. Expression of the chimeric S33Y-β-catenin–ER protein in the skin of transgenic mice appeared to be at levels well below those of the endogenous β-catenin protein [Fig. 1C]. Within 24 h after a single topical application of 4-OHT to the skin of K5/S33Y-β-catenin–ER transgenic mice, strong nuclear staining for β-catenin protein was seen in nuclei of cells in both the interfollicular epidermis and the lower follicle (Fig. 1D).

**Chronic activation of β-catenin function in follicular epithelium induces proliferation and alters differentiation**

Preliminary experiments focused on effects of chronic activation of β-catenin function in the skin. Mice derived from the L2 line were subjected to topical treatment with 4-OHT on a shaved region of dorsal skin. As β-catenin signaling has been implicated previously in several aspects of hair growth and differentiation, we took care to treat mice during a resting phase in the hair growth cycle. Mice are known to undergo two sequential waves of hair growth before entering a prolonged telogen phase by seven weeks of age [Muller-Rover et al. 2001], so we selected day 50 for initiation of 4-OHT treatment to ensure control follicles would be in telogen for at least three additional weeks. Both transgenic mice and wild-type littermates were treated daily with 4-OHT for 1, 3, 7, and 14 d. No demonstrable effects were observed in the skin of wild-type mice treated with 4-OHT [Fig. 2] or transgenic mice treated with ethanol alone [data not shown]. In contrast, dramatic changes were seen in the transgenic mice treated with 4-OHT. Histological studies revealed obvious changes following 3 d of treatment, with an increased number of ORS keratinocytes readily apparent [Fig. 2]. Following 7 d of 4-OHT treatment, hair follicles were hyperplastic with features resembling those normally seen in hair follicles in anagen phase. In addition to marked expansion of ORS cells, the hair follicles had grown into deeper levels of the dermis, compartmentalization of epithelial cell types was evident, and melanin was present in the hair bulb, which contained a normal-appearing dermal papilla. Whereas most of the histological features resemble those in anagen phase [Muller-Rover et al. 2001], the overall size of the follicles appeared to be greater, largely attributable to increased numbers of ORS cells [Fig. 2]. By day 14, follicles in the 4-OHT-treated transgenic mice were even more hyperplastic, composed largely of basophilic cells with scant cytoplasm. Prominent hyperkeratosis of the epithelium at the top of the hair follicles was also seen [Fig. 2]. In the transgenic mice subjected to chronic 4-OHT treatment, hair growth was not evident despite histological features suggesting hair growth might be seen. We speculate the hair was lost as a result of the abnormal follicular epithelial proliferation and/or secondary alterations in hair shaft differentiation and assembly induced by chronic activation of β-catenin signaling function. It is worth noting that antagonism of estrogen receptor signaling by the compound ICI-182780 has been shown to promote anagen onset in mice [Oh and Smart 1996]. However, our data are consistent with a subsequent study [Chanda et al. 2000], which demonstrated that topical treatment with 4-OHT has no significant effect on progression to anagen.

Hair follicle growth and differentiation were assessed further in the transgenic mice subjected to chronic 4-OHT treatment through studies of BrdU incorporation and analysis of expression markers that distinguish cell compartments in the follicle [Fig. 3]. Parasagittal skin sections were examined from wild-type and transgenic mice treated with 4-OHT for 14 d. Skin sections from wild-type mice in spontaneous anagen were also examined. A dramatic increase in BrdU-labeling was detected throughout the ORS in the transgenic mice treated with 4-OHT for 14 d [Fig. 3a,b]. In contrast, in wild-type hair follicles in anagen, proliferation was largely limited to the hair bulb [data not shown] with occasional BrdU-labeled cells detected in the ORS [Fig. 3c]. Keratin 17 (K17) expression is seen predominantly in ORS cells, and an expansion in the population of K17-expressing cells was seen following 14 d of 4-OHT treatment of the K5/S33Y-β-catenin–ER mouse [Fig. 3d–f]. An increase in keratin 6 (K6) expression, which localizes to the inner layer of the ORS, was also noted in the transgenic mice [Fig. 3g–i]. Interestingly, there was little proliferation of the basal cell layer of the epidermis despite high levels of K5-directed transgene expression in that compartment [Figs. 1D, 3j–l]. This likely reflects the absence of TCF/LEF factors in the basal cell layer [Gat et al. 1998]. The pattern of expression for the IRS marker trichohyalin was also abnormal in the transgenic mice. Whereas trichohyalin expression in normal anagen follicles is detected in the hair bulb and extends up to about the level of the arrector pili muscle, chronic activation of β-catenin signaling led to more diffuse expression of trichohyalin in transgenic mice [Fig. 3m–o]. Despite striking alterations in the upper part of the follicle, the lower portion appeared to differentiate quite normally, as shown by staining for hair keratin [Fig. 3p–r] and dermal papillae.

![Figure 2](image-url) Growth and differentiation of the hair follicle in K5/S33Y-β-catenin–ER mice treated daily with 4-OHT. A region of dorsal hair was clipped on both transgenic and wild-type littermates and the skin area was treated daily with 4-OHT in ethanol. Parasagittal sections of skin were taken at time points following initiation of treatment and stained with hematoxylin and eosin. Hair follicles in wild-type mice remained in the resting phase (telogen) throughout the experiment; growth and differentiation of transgenic hair follicles was dramatically stimulated.
cause the mice exhibited signs of distress and often became moribund. These findings may be attributable to systemic distribution of 4-OHT in chronically treated mice and activation of K5/S33Yβ-catenin transgene signaling in esophagus and forestomach (D. Van Mater and E.R. Fearon, unpubl.). Of note, mice derived from the L3 transgenic line with high levels of S33Yβ-catenin–ER expression died after roughly 7 d of topical 4-OHT treatment because of bleeding in the upper gastrointestinal tract [data not shown].

Transient β-catenin activation in telogen follicles induces a normal anagen

Given the proliferative response of hair follicles in transgenic mice chronically treated with 4-OHT and published data suggesting a role for Wnt signaling in initiating anagen, we sought to assess the consequences of transient β-catenin activation in resting (telogen) hair follicles. Transgenic and wild-type mice whose hair follicles were in telogen were treated with a single topical dose of 4-OHT or ethanol alone. The mice were then followed for several weeks. Remarkably, ~15 d after a single 4-OHT treatment, grossly normal hair growth was observed in the clipped and 4-OHT-exposed region of the transgenic mice [Fig. 4A]. In transgenic mice treated with ethanol alone or in wild-type littermates exposed to a single topical dose of 4-OHT or ethanol alone, no hair growth was seen [Fig. 4A].

Histological analyses were pursued on skin specimens obtained at various time points from transgenic and wild-type mice following a single topical dose of 4-OHT. Of note, a single 4-OHT treatment led to a synchronized pattern of normal-appearing follicle growth in the exposed skin, indistinguishable from follicle growth seen in depilation-induced anagen (Muller-Rover et al. 2001; Fig. 4B). The follicles progressed through anagen phase and then entered catagen and telogen at the expected times, between days 19–22. The changes seen in the follicles from transgenic mice exposed to a single 4-OHT treatment mirror the changes seen in follicles during their normal progression through the hair cycle [Muller-Rover et al. 2001]. BrdU-labeling was localized largely to the matrix of the growing follicle, and immunohistochemistry studies with the panel of hair follicle markers described above yielded the expected pattern of expression for normal anagen follicles [data not shown]. The induction of an apparently normal anagen following a single 4-OHT treatment was also seen in the L3 transgenic line [data not shown].

There are several possible explanations for phenotypic differences between the previously reported K14/ΔN87β-catenin mouse [Gat et al. 1998] and our K5/S33Yβ-catenin–ER mouse. Amino acids in the β-catenin N terminus appear important for β-catenin transcriptional regulation [Kolligs et al. 1999], and differing transcription activities may lead to differing functions in vivo. In contrast to the N-terminal deleted β-catenin protein in the Gat et al. [1998] model, our model uses an intact β-catenin protein with a stabilizing point mutation. Yet another variable contributing to differing results seen with the two models might be the possibility that our model allowed a higher degree of β-catenin signaling activity in the keratinocyte. The S33Yβ-catenin–ER protein is inactive unless 4-OHT is present, whereas the K14/ΔN87β-catenin transgenic mouse expresses a con-
The data offer strong support for the notion that a transient Wnt signal provides the crucial initial stimulus for the start of a new hair growth cycle, by activating β-catenin and TCF-regulated gene transcription at the telogen–anagen transition in epithelial hair follicle precursors.

Materials and methods

K5/S33Yβ-catenin–ER expression construct

The generation of the S33Yβ-catenin–ER construct in the phbePuro vector has been described previously [Kolligs et al. 2002]. The K5 expression construct was obtained from José Jorcano (CIEMAT). It consists of 5.2 kb of the bovine K5 upstream region, a rabbit β-globin intron, and two copies of the SV40 polyadenylation sequence [Ramirez et al. 1994]. The S33Yβ-catenin–ER construct was excised from the pBabePuro vector with BamHI and MluI, blunt-ended with Klenow fragment, and cloned into the blunt-ended StuII site of the bovine K5 expression cassette.

Cell culture and reporter assays

The human 1811 keratinocyte cell line was provided by K. Cho (University of Michigan) and propagated in KGM medium (Clonetics). For reporter assays, 3 × 10^5 cells were seeded in 35-mm dishes 12 h before transfection. Cells were transfected with 4 µL FuGENE6 [Boehringer Mannheim], 1 µg of the S33Yβ-catenin–ER construct, 0.5 µg of pTOPFLASH or pFOPFLASH [provided by B. Vogelstein, Johns Hopkins University], and 0.5 µg of pCH110. After 24 h, 500 nM 4-OHT (Sigma, prepared in a stock concentration of 100 µM in 100% ethanol) or ethanol alone was added to the cells. The cells were harvested 30 h after treatment using reporter lysis buffer [Promega]. Luciferase activity was measured with a luminometer and β-galactosidase activities were determined by standard methods to control for transfection efficiency.

Generation of transgenic mice

Transgenic mice were generated by the University of Michigan Transgenic Animal Model Core. The transgene expression construct was linearized with BsrHII, purified, and injected into the male pronucleus of (C57BL/6 X SJL)F2 mouse eggs, which were then surgically transferred to pseudopregnant foster mice. Offspring were screened for the presence of the transgene by PCR on mouse tail DNA using primers specific for the transgene sequence. Three independent founder lines were generated and transgenic mice were backcrossed to C57BL/6 mice [Jackson Laboratory] for at least three generations. To verify transgene expression, a section of mouse tail was obtained and homogenized in lysis buffer [50 mM Tris at pH 7.5, 120 mM NaCl, 1 mM EDTA, 1% NP40, 10% glycerol, Roche Complete Mini protease inhibitor tablet]. Western blotting was performed on 15 µg total protein using mouse monoclonal anti-β-catenin (BD Transduction Laboratories) and anti-β-actin (Sigma) antibodies at a 1:5000 dilution.

Administration of 4-OHT and preparation of sections for histology

Hair in a roughly 4-cm² region of dorsal skin from the mice was clipped to 0.1 mm, and the clipped area was treated with 0.5 mg of 4-OHT (Sigma) dissolved in 100 µL ethanol once per day for various time courses. One hour before euthanasia, mice were injected with an intraperitoneal injection of 100 µg BSA/g per gram body weight. Skin samples were obtained from transgenic mice and wild-type littermates, fixed overnight in 10% neutral buffered formalin at 4°C, and then transferred to 70% ethanol before being processed and embedded in paraffin. Parasagittal sections (5 µm) of dorsal skin were then stained with hematoxylin and cosin.
Immunohistochemistry
Unstained sections were taken from the paraffin blocks described above. The slides were baked overnight at 60°C and then deparaffinized and rehydrated. Endogenous peroxidase was quenched with 0.3% H2O2 in methanol. Antigen retrieval was then performed in 1 X Antigen Retrieval Citra (BioGenex), according to the manufacturer’s recommendations. Primary antibodies were used at the following dilutions with the Mouse on Mouse (M.O.M.; Vector Laboratories) or Rabbit IgG Vectastain ABC kit (Vector Laboratories): mouse monoclonal anti-BrdU (Zymed; 1:200) and anti-β-catenin (BD Transduction Laboratories; 1:250) and rabbit polyclonal anti-K5 (Covance; 1:1000), K6 (Covance; 1:500), and K17 (provided by Pierre Couble, Johns Hopkins University; 1:1000). The M.O.M. biotinylated anti-mouse IgG or anti-rabbit IgG reagent was then added to the slide, followed by the avidin-biotinylated peroxidase complex. Staining was performed with 3,3′-diaminobenzidine (DAB; Vector Laboratories) as explained in the manufacturer's protocol. Sections were counterstained with hematoxylin and mounted using Cytoseal 60 (Stephens Scientific).

Immunofluorescence
Frozen sections (10 µm) were taken of skin samples and warmed to room temperature. They were then fixed in ice-cold acetone for 10 min and air-dried. Nonspecific binding was prevented by incubating with the blocking reagent included in the Mouse on Mouse kit (M.O.M.; Vector Laboratories), followed by incubation with mouse monoclonal antibodies AE15 (1:50) to detect trichohyalin or AE13 (1:150) to detect hair keratins, both kindly provided by Henry Sun (New York University), and rabbit polyclonal anti-K5 (1:1000). The secondary antibodies [Jackson ImmunoResearch] were FITC-conjugated goat anti-mouse IgG (1:75) and Texas Red-conjugated goat anti-rabbit IgG (1:50).

Staining for dermal papillae
To detect endogenous alkaline phosphatase activity in dermal papillae, frozen sections (10 µm) were treated with the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories) according to the manufacturer's recommendations, followed by hematoxylin counterstaining.

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224 GENES & DEVELOPMENT