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Wnt/β-catenin signaling in dermal condensates is required for hair follicle formation

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Abstract

Broad dermal Wnt signaling is required for patterned induction of hair follicle placodes and subsequent Wnt signaling in placode stem cells is essential for induction of dermal condensates, cell clusters of precursors for the hair follicle dermal papilla (DP). Progression of hair follicle formation then requires coordinated signal exchange between dermal condensates and placode stem cells. However, it remains unknown whether continued Wnt signaling in DP precursor cells plays a role in this process, largely due to the long-standing inability to specifically target dermal condensates for gene ablation. Here we use the $Tbx18^{Cre}$ knockin mouse line to ablate the Wnt-responsive transcription factor β-catenin specifically in these cells at E14.5 during the first wave of guard hair follicle formation. In the absence of β-catenin, canonical Wnt signaling is effectively abolished in these cells. $Sox2^{+}$ dermal condensates initiate normally, however by E16.5 guard hair follicle numbers are strongly reduced and by E18.5 most whiskers and guard hair follicles are absent, suggesting that active Wnt signaling in dermal condensates is important for hair follicle formation to proceed after induction. To explore the molecular mechanisms by which Wnt signaling in dermal condensates regulates hair follicle formation, we analyze genome-wide the gene expression changes in embryonic β-catenin null DP precursor cells. We find altered expression of several signaling pathway genes, including Fgfs and Activin, both previously implicated in hair follicle formation. In summary, these data reveal a functional role of Wnt signaling in DP precursors for embryonic hair follicle formation and identify Fgf and Activin signaling as potential effectors of Wnt signaling-regulated events.

Keywords

Wnt signaling; Dermal papilla cells; Stem cell niche; Hair follicle morphogenesis; Hair follicle stem cells

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INTRODUCTION

During embryonic development stem cells give rise to a multitude of complex organs and tissues. To accomplish this, stem cells undergo multiple fate decisions that strike a balance between self-renewal and differentiation into all cell lineages that make up each tissue (Fuchs and Chen, 2013; Li and Clevers, 2010). These cell fate choices are thought to be highly regulated by the microenvironment, or stem cell niche (Moore and Lemischka, 2006; Voog and Jones, 2010; Xie and Li, 2007). Niche influences can include neuronal and humoral inputs, structural aspects and extracellular matrix composition, and typically also involve cell-cell communication and paracrine signal exchange of niche cells with neighboring stem cells (Jones and Wagers, 2008; Scadden, 2006). Stem cell niches have been described in several tissues (Jahoda and Christiano, 2011; Simons and Clevers, 2011; Wang and Wagers, 2011). In skin, dermal papilla (DP) cells are thought to instruct matrix progenitors during hair growth and bulge stem cells during adult hair regeneration in the hair cycle (reviewed in Lee and Tumbar, 2012; Sennett and Rendl, 2012), but the precise molecular mechanisms of DP niche function remain elusive. Likewise, during embryonic hair follicle formation the precursors of DP cells in dermal condensates (Grisanti et al., 2013a) are thought to instruct epidermal placode cells that contain the future hair follicle stem cells (Lee and Tumbar, 2012; Sennett and Rendl, 2012)

During embryonic hair follicle induction, unknown dermal signals downstream of broad dermal Wnt/β-catenin signaling activity are thought to induce epidermal stem cells to switch to a hair placode fate (Chen et al., 2012). Nascent epithelial hair placodes signal back to induce dermal condensates that are clustering DP precursor cells. Fgf20 was recently identified as a crucial placode signal (Huh et al., 2013). For hair follicle formation to proceed, continued signal exchange between the two compartments initiates proliferation and downgrowth, with DP precursor cells at the leading edge (Schmidt-Ullrich and Paus, 2005; Schneider et al., 2009) and hair follicle stem cells set aside at the upper portion of developing follicles (Nowak et al., 2008). At the lower tip of new follicles, stem cell progeny engulf DP cells before starting to proliferate and migrate upwards while differentiating into outgrowing visible hair shafts. In mouse back skin, hair development occurs in three consecutive waves giving rise to four hair follicle types (Schlake, 2007; Sennett and Rendl, 2012) (Fig. 1A). The 1st wave starts around embryonic day (E)14.5 forming primary guard hair follicles that represent ~1–3% of the complete hair coat. The 2nd wave that forms awl/auchene hairs starts at E16.5 (~20%). Finally, the 3rd wave at E18.5 generates zigzag hairs, which comprise the majority (80%) of the down undercoat (Schneider et al., 2009; Sennett and Rendl, 2012). Besides Fgf and Wnt/β-catenin signaling (more details below), Shh, Tgf/Bmp and Eda/Edar/NFkB signaling pathways play an important role in the earliest steps of hair follicle morphogenesis (Botchkarev et al., 1999; Headon and Overbeek, 1999; St-Jacques et al., 1998; Woo et al., 2012; Zhang et al., 2009).

Canonical Wnt/β-catenin signaling in the hair placode is essential for hair follicle formation (Alonso and Fuchs, 2003). Transgenic mice expressing stabilized β-catenin in the epidermis form excessive hair follicles (Gat et al., 1998), with premature and expanded placode development (Zhang et al., 2008). By contrast, epidermis-specific genetic ablation of β-catenin results in a failure of hair follicle initiation (Huelsken et al., 2001). Similarly, epidermis-specific ectopic expression of Dkk1, a competitive Wnt inhibitor, results in a failure to form hair follicles (Andl et al., 2002). Recent evidence suggests that active Wnt/β-catenin signaling in the dermis plays an important role for hair follicle induction even prior to placode Wnt signaling activity. Several Wnt reporter mice show broad dermal Wnt/β-catenin signaling at E13.5 (Chen et al., 2012; Zhang et al., 2009) that is dependent on epidermal Wnt ligand secretion (Chen et al., 2012). Importantly, this broad dermal Wnt/β-

catenin activity is required for hair placode induction by as of yet unidentified downstream target signals (Chen et al., 2012). After placode induction, dermal Wnt/β-catenin activity becomes more focused in DP precursor cells of dermal condensates at E14.5 (Chen et al., 2012; DasGupta and Fuchs, 1999; Zhang et al., 2009), as nuclear Wnt pathway transcription factors Lef1 and β-catenin are also detected in these cells (DasGupta and Fuchs, 1999; Zhang et al., 2009; Zhou et al., 1995). However, the role of Wnt signaling in directing dermal condensates to interact with placode cells during the progression of hair follicle formation is still unexplored, due to the long-standing lack of DP precursor-specific genetic drivers for gene ablation experiments. Here we utilize our recently characterized $Tbx18^{Cre}$ knockin mouse line (Clavel et al., 2012; Grisanti et al., 2013a; Grisanti et al., 2013b) to express Cre recombinase specifically in embryonic DP precursor cells and ablate the Wnt-responsive transcription factor β-catenin. We report that without β-catenin, whisker and backskin 1^{st} wave guard hair follicles fail to form. Molecular exploration suggests the involvement of Fgf and Activin signaling pathways as potential effectors of Wnt signaling within the DP niche cells. This study reveals that Wnt signaling is required specifically for dermal condensate and early DP niche function during hair follicle morphogenesis, mirroring the known essential role of Wnt signaling within hair follicle stem cells.

MATERIALS AND METHODS

Mice

β-$catenin^{fl/fl}$ mice (Brault et al., 2001) were obtained from Jackson Laboratories (B6.129-$Ctnnb1^{tm2Kem}$/KnwJ). $Tbx18^{Cre}$ and $Sox2^{GFP}$ knock-in and Lef1-RFP transgenic mouse lines have been described previously (Cai et al., 2008; Ellis et al., 2004; Grisanti et al., 2013a; Rendl et al., 2005). BATGAL Wnt reporter (Maretto et al., 2003) and Rosa26 reporter (Soriano, 1999) mouse lines were obtained from Jackson Laboratories (B6.Cg-Tg(BAT-lacZ)3Picc/J).

Cell isolation

To isolate DP precursor cells, β-$catenin^{fl/fl}$/$Sox2^{GFP}$ and β-$catenin^{fl/fl}$/$Tbx18^{Cre}$/$Sox2^{GFP}$ E15.5 embryos were dissected in ice-cold PBS buffer and genotyped. Back skins were microdissected and incubated in dispase (Invitrogen) with 0.2% collagenase (Sigma-Aldrich) and 20U/ul of DNAse for 60 minutes at 37C, then centrifuged at 300g for 10 minutes, followed by trypsinization (0.25% trypsin/0.05mM EDTA, Invitrogen) at 37C for 5 min. Cell purifications were performed on a MoFlo High Speed Sorter (DAKO-Cytomation).

Tissue histology and immunofluorescence

Embryos or skins were fixed and processed for staining or directly frozen in optimal cutting temperature (OCT, Tissue-Tek) compound. Skins were cryosectioned and stained with hematoxylin and eosin as described previously (Rendl et al., 2008). For detection of β-galactosidase activity, X-Gal staining was performed on whole-mount embryos or cryosections according to standard protocols. For immunofluorescence, 7μm embryo and backskin sections were fixed in 4% PFA and stained as previously described (Rendl et al., 2008). Sections were incubated with antibodies against Gfra1 (goat, Neuromics), Itgb4 (rat, BD Pharmingen), AE15 (mouse, Santa Cruz), AE13 (mouse, Abcam), Lef1 (rabbit, Cell signaling), Ki67 (rabbit, Novocastra), followed by species-specific Rhodamine Red-X conjugated secondary antibodies (Jackson Immunoresearch). Nuclei were counterstained with DAPI. For whole-mount immunofluorescence staining, embryo skins were fixed in 4% PFA and incubated with primary antibodies against Edar (goat, R&D) and Sox2 (rabbit, Stemgent), followed by Rhodamine Red-X anti-goat and Alexa Flour 488 anti-rabbit conjugated secondary antibodies (Jackson Immunoresearch).

Real-Time PCR

Total RNAs from FACS sorted cells were purified using the Absolutely RNA Nanoprep kit (Stratagene), quantified with the NanoDrop spectrophotometer (Thermo Scientific) and reverse transcribed using oligo (dT) primers (Superscript III First-Strand Synthesis System, Invitrogen). Real-time PCR was performed with a LightCycler 480 (Roche) instrument with Lightcycler DNA master SYBR Green I reagents. Differences between samples and controls were calculated based on the $2^{-\Delta\Delta CT}$ method and normalized to Gapdh. Primer sequences are listed in Supplementary Table 1.

Microarray analysis

RNAs were prepared from sorted cells with the Absolutely RNA Nanoprep kit (Stratagene). 3ng of total RNA were labeled using the applause amplification and labeling system (Nugen) and Illumina BeadArrays (MouseWG-6 v2) microarrays were hybridized according to manufacturer's descriptions by the Genomics core facility. Raw data were normalized and expression values were log2 transformed. For clustering analysis, gene lists were filtered based on expression level differences >1.25-fold in both cKO samples compared to WT. Resulting gene lists were analyzed with the Cluster software (Eisen et al., 1998). Hierarchical clustering involved Pearson correlation for population comparisons, and Euclidian distance calculations for gene level clustering. Data were visualized with TreeView software (Eisen et al., 1998). Genes were grouped into functional categories based on Gene Ontology classifications using the PANTHER site (http://www.pantherdb.org) (Thomas et al., 2003).

RESULTS

Active canonical Wnt/β-catenin signaling in placodes and condensates of all hair follicle types

Broad dermal Wnt/β-catenin signaling at embryonic day (E)13.5 is essential for hair placode induction (Chen et al., 2012) and active Wnt/β-catenin signaling in nascent placode cells at E14.5 is required for placode stability and dermal condensate induction (DasGupta and Fuchs, 1999; Huelsken et al., 2001; Zhang et al., 2009). Dermal papilla (DP) precursor cells in these condensates, which are thought to coordinate hair follicle formation with stem cells in the placode, continue to show focused Wnt/β-catenin signaling activity within the dermal compartment, as demonstrated with several Wnt reporter mouse lines (Chen et al., 2012; DasGupta and Fuchs, 1999; Zhang et al., 2009).

To date, it remains unresolved whether Wnt signaling occurs in DP precursor cells and placode cells during the formation of all different hair follicle types that form in three separate waves between E14.5 and birth (Fig. 1A). To analyze in greater detail the dynamics of Wnt activity in all developmental waves we utilized BATGAL reporter mice (Maretto et al., 2003) that express β-galactosidase under the control of Wnt-responsive multimerized Lef/TCF transcription factor binding sites. Analysis of X-Gal stained whole-mount embryos from E14.5–E16.5 showed LacZ expression in a typical hair follicle distribution pattern (Fig. 1B). This suggested that Wnt signaling occurs in both 1st wave guard hair follicles as well as 2nd wave awl/auchene hair follicle types during early hair formation. Histological examination of embryo sections from E14.5 to E16.5 revealed X-Gal labeling in placodes (arrowheads), DP condensates (arrows: 1st wave, open arrowheads: 2nd wave) or both (Fig. 1C–E). Analysis of X-Gal stained sections at E18.5 (Fig. 1F) showed continued β-galactosidase activity in DPs of advanced 1st and 2nd wave guard and awl/auchene hair follicles (arrows, open arrowheads). Placodes (arrowheads) and DP condensates (open arrows) of 3rd wave zigzag hair follicles also demonstrated robust Wnt activity. Quantification of X-Gal stained nascent follicles revealed that >50% of placodes and dermal

condensates were labeled in all three waves, with close to 100% of dermal condensates labeled at E14.5 (Fig. 1G). Placodes and dermal condensates were not always labeled simultaneously, suggesting dynamic regulation of Wnt pathway activation in both compartments. Taken together, the data demonstrate that Wnt signaling occurs at all three waves of hair follicle formation in both the epithelial placode as well as in the dermal niche counterpart.

Ablation of Wnt/β-catenin signaling in dermal condensates impedes formation of 1st wave guard hair follicles

To test whether Wnt/β-catenin signaling in DP precursor cells in dermal condensates plays an important role in hair follicle formation, we sought to completely block canonical Wnt signaling by ablating the Wnt responsive transcription factor β-catenin specifically in these cells, when they are induced at E14.5. For this, we crossed our recently characterized $Tbx18^{Cre}$ knockin line with β-catenin double-floxed mice. $Tbx18^{Cre}$ specifically targets DP precursor cells in nascent hair follicles starting at E14.5 (Clavel et al., 2012; Grisanti et al., 2013a; Grisanti et al., 2013b). After Cre recombination, exons 2–6 of the β-catenin coding sequence are deleted thereby preventing the formation of functional β-catenin protein (Brault et al., 2001). In order to verify inhibition of Wnt/β-catenin signaling, we mated $Tbx18^{Cre}$/β-$catenin^{fl/fl}$ mice with the BATGAL Wnt reporter line, thus generating triple $Tbx18^{Cre}$/β-$catenin^{fl/+}$/BATGAL heterozygous (HET) and $Tbx18^{Cre}$/β-$catenin^{fl/fl}$/BATGAL conditional knockout (cKO) embryos for further analysis. Studies of X-Gal stained sections showed robust Wnt reporter activity in HET 1st wave guard hair condensates (Fig. 2A, arrows), while they confirmed the near complete ablation of Wnt/β-catenin signaling in β-catenin cKO dermis at E14.5 (Fig. 2A, right; the arrow points to a rare condensate with residual Wnt activity). Importantly, Wnt reporter positive placode cells were unaffected (arrowheads). However, in cKO condensates of new 2nd and 3rd wave follicles at E16.5 and E18.5, we observed unabated Wnt/β-catenin signaling (Fig. S1A,B). This was likely due to inefficient early β-catenin ablation, precluding our analysis of the role of Wnt/β-catenin signaling in dermal condensates of these hair types. Sections of back skin from these same crosses after birth confirmed that Wnt/β-catenin signaling was eventually ablated in DPs from all three hair waves (Fig. S1C), resulting in postnatal phenotypes. In summary, these data indicated that we were able to successfully ablate β-catenin and thus abrogate Wnt signaling within dermal condensates of 1st wave guard hair follicles forming at E14.5, but not of follicles in the later waves.

Analysis of hematoxylin/eosin stained sections of β-$catenin^{fl/fl}$ wild-type (WT) control and $Tbx18^{Cre}$/β-$catenin^{fl/fl}$ cKO embryos at E14.5 showed normal 1st wave placodes and condensates (Fig. 2B, arrows). Based on histological comparison, cell adhesion and organization appeared unaffected in cKO condensate cells compared to WT. Interestingly, by E16.5 and E18.5, downgrowth and formation of 1st wave guard hair follicles at these later stages was strongly diminished (H&E in Fig. 2B and BATGAL/DAPI in S1A,B). This suggested that Wnt/β-catenin signaling after the dermal condensate stage is essential for hair follicle formation to proceed. Quantification of total hair follicle numbers at each stage using morphological criteria (Paus et al., 1999) showed a significant reduction of total hair follicles at E16.5 and E18.5 (Fig. 2C), most likely due to the absence of formed 1st wave guard hair follicles. Indeed, morphological analysis of 2nd wave awl/auchene hairs at E16.5 and E18.5 (Fig. 2B, open arrowheads) and 3rd wave zigzag hairs at E18.5 (Fig. 2B, open arrows) revealed that they formed normally in both cKO and control embryos, possibly because of inefficient β-catenin ablation in condensates of these follicle types. Quantification of hair follicle types from each wave at E18.5 confirmed that only 1st wave guard hair follicles were significantly reduced (Fig. 2D).

To confirm our observations on embryo sections, we next analyzed E14.5 whole-mount skins stained for the dermal condensate marker SOX2 (Clavel et al., 2012; Driskell et al., 2009; Rendl et al., 2005; Tsai et al., 2009), and also for the placode marker EDAR (Huh et al., 2013; Zhang et al., 2009) (Fig. 2E). In line with our counts on sagittal sections, the numbers of $EDAR^+$ placodes and $SOX2^+$ condensates are comparable between WT and cKO (Fig. 2F), confirming normal placode and condensate induction. Finally, analysis of E18.5 whole-mount skins from $Tbx18^{Cre}$/β-catenin HET and cKO embryos, also crossed with Lef1-RFP to highlight all dermal condensates and DPs (Rendl et al., 2005), confirmed the dramatic reduction of 1st wave guard hair follicles after β-catenin ablation. As shown in Figure 2G, 1st wave guard hair follicles in β-catenin HET skins (arrows) were lacking in cKO samples. Quantification of absolute hair follicle sizes showed clustering of size groups according to hair follicle waves (Fig. 2H, brackets) and hair follicles of the largest guard hair sizes were absent in β-catenin cKO skins (Fig. 2H, top bracket). In summary, while both 1st wave placodes and condensates are induced to form in this model, most incipient hair follicles fail to give rise to downgrowing guard hair follicles in cKO back skin.

Impaired guard and whisker hair follicle formation, but normal lineage and differentiation markers in developed hair follicles after birth

Besides deficiencies in hair development during embryonic formation, DP-specific β-catenin cKO pups are born normally. Body sizes of cKO pups are similar to WT pups at postnatal (P) day P0 (not shown). However, by P10 cKO pups are smaller than WT, and most cKO mice die by P10 with ascites and mobility problems.

Normally, hair shafts from 1st wave guard hair follicles emerge from the skin at P5. As expected, we readily observed guard hairs in WT back skin (Fig. 3A, arrows). In cKO back skin, however, hair shafts were largely missing, which is consistent with our analysis of reduced embryonic hair follicle formation of 1st wave guard hair. Histological analysis of P5 back skin also confirmed fewer hair follicles in cKO compared to WT pups (Fig. 3B). Furthermore, existing hair follicles in cKO back skins were much shorter at this age, and the entire dermis was thinner (Fig. 3B).

Guard hair follicles are most similar to specialized whisker follicles, which form at E12.5 during embryogenesis and are set up in multiple rows with 3 to 7 follicles per row (Brecht et al., 1997). To evaluate whether this hair follicle type is also affected by β-catenin ablation we analyzed E18.5 whisker pads. Histological analysis of cross-sectioned pads, allowing visualization of all whisker follicles at once, showed normal whisker number, size and distribution in WT pups (Fig. 3C). By contrast, in β-catenin cKO sections multiple whisker follicles were missing (Fig. 3C, asterisks), suggesting that Wnt signaling in the DP niche is essential for formation of this hair type as well. This is also evident postnatally, when β-catenin cKO pups lack external whisker shafts (Fig. 3D).

We next determined whether existing hair follicles that did develop in cKO skin had cell lineage formation and biochemical marker expression. LacZ staining of BATGAL cKO pups confirmed that Wnt signaling was missing from hair follicle DPs at P5, while Wnt reporter activity was localized to DPs of maturing follicles in P5 HET pups (Fig. S1C). Immunofluorescence stainings of P5 backskin sections of WT and cKO mice revealed normal Ki67 proliferation marker expression in matrix progenitor cells, which give rise to all differentiated layers of the hair shaft and inner root sheath (Fig. 3E). The transcription factor Lef1 was also present in matrix cells as expected. Additionally, differentiation markers AE15 (Trichohyalin) and AE13 (hair cortex keratins) were detected in inner root sheath and hair shaft, respectively, in a normal pattern (Fig. 3F). Finally, to evaluate whether β-catenin ablation would impact DP maturation in developed hair follicles we examined the expression of DP signature genes GFRA1 and alkaline phosphatase (AP), which also

appeared unchanged (Fig. 3G). Taken together, these data suggest that guard hair and whisker follicles and their shafts fail to develop in the absence of DP Wnt/β-catenin signaling. They further indicate that 2nd and 3rd wave follicles are not compromised in their ability to produce all cell lineages in formed hair follicles, although Wnt/β-catenin activity is ablated at that stage.

Hair shafts are shorter during later postnatal hair growth

Our embryonic analysis of hair follicle waves, together with the absence of external guard hair shafts at P5 suggested that guard hairs failed to form, while initial 2nd wave awl/auchene and 3rd wave zigzag hair formation was not affected, likely due to inefficient β-catenin ablation. The lack of external guard hair shafts became even more obvious in β-catenin cKO pups by P10, when hair shafts of 2nd and 3rd wave follicles also appeared in both cKO and WT pups. Guard hair shafts greatly overshadowed all other hair types in WT back skin, yet were largely absent in knockout skin (Fig. 4A). Additionally, the overall hair coat appeared to be shorter in the cKO compared to WT. Histological analysis in hematoxylin and eosin stainings again showed fewer follicles, shorter hair follicles and thinner skin in cKO back skins (Fig. 4B).

To ascertain and precisely determine which hair follicle types were affected by inactivation of Wnt/β-catenin signaling in the DP niche, we next plucked hair shafts and phenotyped them by a combination of three distinguishing key features: (i) number of medulla cells, (ii) kinks and bends of hair shafts and (iii) shaft length (Schlake, 2007). 1st wave guard hairs contain two columns of medulla cells and do not have any kinks or bends (Fig. 4C,D). They are the longest and are considered to have tactile function, much like whiskers (Fig. 4C). 2nd wave awl hairs are shorter than guard hairs and contain 3–4 columns of medulla cells (Fig. 4C,D). Auchene hairs are also 2nd wave hair follicles that look like straight awl hairs, except that their hair shafts have one bend (Fig. 4C,D). Zigzag hairs have only one column of medulla cells and typically two kinks in the hair shaft (Fig. 4C,D).

Using these classifications we quantified >2,000 plucked hair shafts from WT and cKO back skins (Fig. 4E). In WT samples, we identified ~3% guard hairs out of all follicles, while in cKO guard hair numbers were reduced by 82%. 2nd wave auchene hairs were undetectable and awl hair numbers were relatively increased in cKO, although this difference did not reach statistical significance. Intriguingly, the absolute increase of awl shafts is similar to the number of missing auchene hairs, suggesting that auchene hairs may have turned into awl-like shafts, as previously reported (Chi et al., 2013). Zigzag hair follicle numbers were comparable between WT and cKO confirming the analysis in embryonic skins. Within awl hair shafts the number of pigmented medulla columns was reduced to 1–2 columns (Fig. 4D), which is in line with a recent report in which β-catenin was postnatally ablated in the DP niche between P4–P7 (Enshell-Seijffers et al., 2010). Moreover, all hair follicles that did form in cKO skins were reduced in length to ~60% (Fig. 4C), as reported previously after postnatal β-catenin ablation in the DP (Enshell-Seijffers et al., 2010). These data suggest that our early ablation of β-catenin in DP cells of the 2nd and 3rd hair follicle waves recapitulates the later hair growth phenotypes in formed follicles seen during postnatal inhibition of Wnt signaling in the DP niche.

Ablation of Wnt/β-catenin signaling alters DP precursor gene expression, including signaling molecules Fgf10 and Inhba

To investigate how Wnt/β-catenin signaling in the dermal condensate regulates hair follicle formation, we next isolated WT and β-catenin cKO condensate cells from embryonic skins and interrogated gene expression changes at the genomic level by microarrays. To isolate pure condensate cells we crossed $Sox2^{GFP}$ reporter knock-in mice (Ellis et al., 2004) with

our $Tbx18^{Cre}$/β-$catenin^{fl/fl}$ lines to generate β-$catenin^{fl/fl}$/$Sox2^{GFP}$ WT and $Tbx18^{Cre}$/β-$catenin^{fl/fl}$/$Sox2^{GFP}$ cKO embryos. Sox2 is a DP marker (Rendl et al., 2005) that is specifically expressed in embryonic DP precursor cells of 1st and 2nd wave follicles (Clavel et al., 2012; Driskell et al., 2009; Tsai et al., 2009). In $Sox2^{GFP}$ reporter mice GFP is detectable in dermal condensates where Sox2 is expressed (Fig. 5A). Using E15.5 embryos, we isolated 1st wave condensate cells that are β-catenin deficient. Single cell preparations from E15.5 reporter-positive back skins were subjected to fluorescence activated cell sorting (FACS) and condensate cells were isolated as the 1.5–2% GFP^{hi} population (Fig. 5B). To analyze the enrichment of condensate cells we also isolated negative cells as a control population and compared by real-time PCR the expression of several known embryonic condensate signature genes (Fig. 5C). As expected, Sox2 gene expression was nearly 100% enriched. Confirming this enrichment, Tbx18 and the DP marker Enpp2 (Grisanti et al., 2013a; Grisanti et al., 2013b; Rendl et al., 2005) were also preferentially expressed in condensate isolates (Fig. 5C).

We next isolated WT and cKO condensate cells and determined a 70% knockout efficiency based on β-catenin mRNA levels (Fig. 5D). We then proceeded with transcriptional profiling of two independent biological replicates using Illumina BeadArrays (MouseWG-6 v2). Comparative analysis between WT and cKO samples revealed 185 genes significantly up- or downregulated more than 1.25-fold. We grouped the list of regulated genes into functional categories based on Gene Ontology classifications and determined the overall enrichment of categories compared to their representation in the transcriptome. Significantly enriched categories included ectoderm and mesoderm development, cell motion, signal transduction, and cell communication and cell-cell signaling, suggesting that regulated genes are highly involved in developmental processes (Fig. 5E). Hierarchical clustering of gene expression changes arranged the genes into co-regulated groups revealing that the majority (over 80%) of altered genes were downregulated, suggesting that Wnt/β-catenin signaling mostly acts as an activator in this context (Fig. 5F).

Real-time PCR further validated the downregulation of several genes in β-catenin cKO condensate cells (Fig. 5G). Among these genes, Axin2 and Nkd2 were >2x downregulated, which is expected since both genes have been previously shown to be affected by Wnt/β-catenin pathway regulation. Axin2 is a direct transcriptional target of Wnt/β-catenin signaling (Jho et al., 2002), and its downregulation in sorted cKO cells underscores the loss of functional pathway activation. Nkd2 encodes an inducible antagonist of Wnt signaling that is upregulated to counterbalance pathway activation (Zeng et al., 2000), and reduced levels in cKO condensate cells suggest functional operation of a negative feedback loop. The fact that we did not observe stronger regulation of many genes likely reflects the timing of β-catenin ablation, since residual mRNA was still detectable (Fig. 5D). Similar Axin2 and Nkd2 regulation was also reported previously after β-catenin ablation in postnatal DP niches (Enshell-Seijffers et al., 2010). Several other downregulated genes directly interact with β-catenin and the Wnt pathway. For example, Cdk5r1, also called p35/cdk5, binds and phosphorylates β-catenin affecting protein-protein interaction (Kesavapany et al., 2001). Enc1 encodes an actin binding protein that is a transcriptional target of Wnt signaling in colorectal carcinomas (Fujita et al., 2001). Downregulation of Prdm1/Blimp1 suggests secondary regulation of its respective target genes. Interestingly, Prdm1/Blimp1 ablation in vivo results in lack of sensory whisker formation (Robertson et al., 2007).

Finally, downregulation of Fgf10 and Inhba identifies Fgf and Activin signaling pathways as putative downstream targets of Wnt signaling in the DP niche. Fgf signaling plays a crucial role in hair follicle development, underscored by studies showing that ablation of Fgf10 results in failed whisker formation (Ohuchi et al., 2003) and deletion of epithelial Fgfr2 leads to missing hair follicles in the back skin (De Moerlooze et al., 2000; Petiot et al.,

2003). Decreased Inhba expression in β-catenin depleted condensate cells should lead to reduced Activin/TGFβ signaling and Activins have previously been implicated in hair follicle morphogenesis as well (Matzuk et al., 1995a; McDowall et al., 2008).

DISCUSSION

After initial hair follicle induction in embryonic skin, the progression of hair follicle formation relies on dynamic interactions between placode cells and the specialized DP precursor cells in dermal condensates. Although multiple signaling pathways have been implicated for several years (reviewed in Lee and Tumbar, 2012; Schneider et al., 2009; Sennett and Rendl, 2012), the precise mechanisms behind this crosstalk remain unclear. Numerous studies firmly established an essential role of canonical Wnt signaling within the broad dermis and in placode cells for hair follicle induction; however, the significance of Wnt signaling in dermal condensates in their interaction with placode cells for progression of hair follicle formation remained unresolved. Our present study utilizes the $Tbx18^{Cre}$ mouse model to specifically ablate β-catenin in DP precursor cells at E14.5, thus abrogating canonical Wnt signaling in the dermal niche compartment at the earliest stages of hair follicle development. The results from this work demonstrate that Wnt signaling within the dermal compartment is required for morphogenesis of 1^{st} wave guard hair follicles, in addition to implementing precise technology that allows genetic targeting of the dermal niche during hair development.

Studies of Wnt signaling in early embryonic skin have previously revealed widespread activation of Wnt signaling in E10.5–13.5 dermis (Chen et al., 2012; Zhang et al., 2009) and more focused activity in dermal condensates at E14.5 (Chen et al., 2012; Zhang et al., 2009). To examine the patterns of Wnt signaling in relation to the development of all hair follicle types, we quantitatively assessed BATGAL Wnt reporter activity in both epidermis and dermis of newly forming follicles from E14.5 onwards. Histological examination of embryo back skins conclusively demonstrates that Wnt signaling is active in both compartments during the earliest stages of placode-condensate crosstalk at all three waves of hair morphogenesis. Interestingly, although many nascent follicles display positive LacZ staining in both placode cells and the DP niche, follicles with only one labeled compartment can also be observed, indicating dynamic Wnt activation during hair morphogenesis.

Targeted β-catenin ablation at E14.5 compromised Wnt signaling in dermal condensate cells and severely impaired hair follicle formation, thus emphasizing the requirement for functional Wnt/β-catenin signaling in the dermal niche during the 1^{st} wave of hair morphogenesis. Based on hair follicle type counts from P10 backskin, 2^{nd} wave auchene hair formation appeared to be compromised as well. At first it was surprising to observe 2^{nd} wave awl and 3^{rd} wave zigzag hair follicles forming normally in knockout embryos, but further analysis in a BATGAL reporter background revealed Wnt signaling was not completely abrogated in these corresponding dermal condensates during the earliest formation of these hair types. Therefore we cannot evaluate the requirement of Wnt/β-catenin signaling in these hair types at that stage. However, signaling activity was missing in the DPs of 2^{nd} and 3^{rd} wave follicles in P5 cKO pups, resulting in postnatal hair shaft phenotypes consistent with previous studies exploring the role of Wnt/β-catenin in the DP niche during postnatal hair growth (Enshell-Seijffers et al., 2010).

The significant reduction in 1^{st} wave guard hair formation following dermal condensate β-catenin ablation is the most striking finding in our cKO embryos. Importantly, the mouse genetic tools we use allow us to target the dermal condensate at a specific stage during hair morphogenesis, so we can exactly address the role of Wnt signaling in early hair follicle formation. Recent studies examining the role of Wnt signaling in back skin and hair

development used widely-acting genetic tools to ablate β-catenin broadly throughout the dermis at an earlier embryonic time point, before hair follicles were initiated, and subsequently blocked all stages of hair morphogenesis. Our tools address the importance of Wnt signaling in DP niche precursor cells in a more specific place and time to definitively establish the crucial role of this molecular pathway in promoting early hair formation once placode and condensate have already appeared.

To better understand how Wnt/β-catenin signaling in the DP niche drives hair morphogenesis, we used genome-wide analysis to compare gene expression differences between wild-type and knockout dermal condensate cells. The results from this study identify Fgf and Activin signaling as putative downstream targets of Wnt signaling in the mesenchyme, as expression of factors from both pathways are decreased in cKO condensate cells. Several studies have highlighted the crucial role of Fgf signaling in hair follicle development, including Fgf10 knockout mice that fail to grow whiskers (Ohuchi et al., 2003) and FGFR2 null mice that similarly display deficient hair growth (De Moerlooze et al., 2000; Petiot et al., 2003). In this study, Fgf10 expression is diminished in β-catenin depleted condensate cells, suggesting that the intersection of Fgf and Wnt signaling could be important for mediating the instructive effects of condensate niche cells during early hair morphogenesis. Future studies will be necessary to determine the extent and dynamic nature of this interaction.

This study additionally identifies decreased Inhba expression in β-catenin depleted condensate cells. Inhba forms dimeric complexes, also known as Activin A, previously implicated in tooth and hair follicle morphogenesis (Ferguson et al., 1998; McDowall et al., 2008). These morphogens from the TGF-β family appear to modulate hair follicle formation, which has been demonstrated by the generation of Activin knockout mice that lack whiskers at birth (Matzuk et al., 1995b). Interestingly, when the Activin receptor type IB is knocked out specifically in the epidermis, mice display a hair loss phenotype that worsens with age (Qiu et al., 2011), suggesting that DP Activin signaling to a receptive epidermis might be an important component of the epithelial-mesenchymal crosstalk that drives hair maintenance. Although the Activin signaling pathway has clearly been linked to hair follicle formation and maintenance, the detailed molecular mechanisms behind its action have yet to be deciphered.

While this study emphasizes the requirement for Wnt signaling in the dermal niche during hair follicle morphogenesis by specifically ablating β-catenin in dermal condensate cells during early hair formation, the interconnections between Wnt activation and the concurrent or sequential activation of other major signaling pathways remain to be explored. Mechanistically, this study provides further evidence that the modulation of multiple major signaling pathways in dermal-epidermal crosstalk is crucial for driving hair morphogenesis during embryonic skin development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Ablation of Wnt/β-catenin signaling in hair follicle dermal condensates
- Progression of hair follicle formation after induction is impaired
- Genome-wide gene expression analysis of β-catenin null dermal papilla precursors
- Identification of Fgf/Activin ligands as potential downstream Wnt pathway effectors

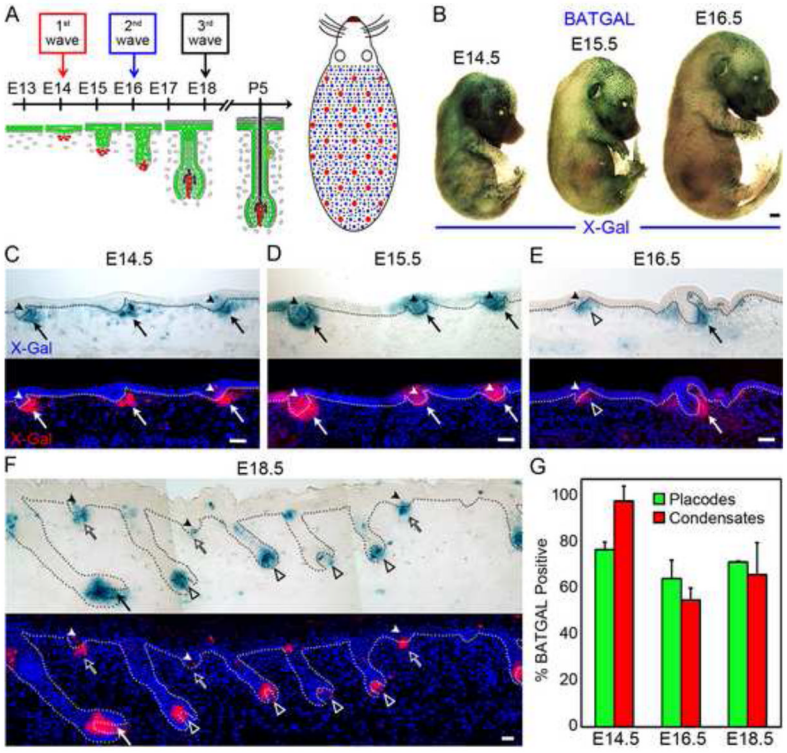

Figure 1. Active Wnt/β-catenin signaling in placodes and dermal condensates of all three hair follicle waves

(**A**) Schematic representation of mouse hair development in three consecutive waves. The 1st wave starts at E14.5 forming guard hair follicles. 2nd wave awl/auchene hairs form at E16.5. Zigzag hair follicles form at E18.5 in the 3rd wave. Right: Illustration of spatial distribution of guard (red), awl/auchene (blue) and zigzag (black) hair follicles in backskin. (**B**) Whole-mount X-Gal staining of Wnt reporter BATGAL embryos at indicated time points. Note blue dots in a typical hair follicle distribution pattern. (**C, D**) Wnt reporter activity in embryonic skin sections. LacZ is strongly expressed in hair placodes (arrowheads) and DP condensates (arrows). Lower panels: Pseudo-colored image of LacZ staining overlaid with DAPI to highlight nuclei. The dotted line marks basement membrane between epidermis and dermis. (**E**) At E16.5, LacZ is expressed in DP niches of both 1st (arrows) and 2nd (open arrowheads) wave hair follicles. (**F**) X-Gal stained sections from BATGAL embryos at E18.5. Wnt reporter activity is also detectable in DP precursor cells of 3rd wave zigzag follicles (open arrows). (**G**) Quantification of Wnt signaling. BATGAL is expressed in hair placodes and dermal condensates of all follicle types in all three waves. Note that Wnt signaling is detectable in placodes, DP condensates or both, suggesting dynamic Wnt activity in both compartments. Scale bars: 1mm in B; 50μm in C–F.

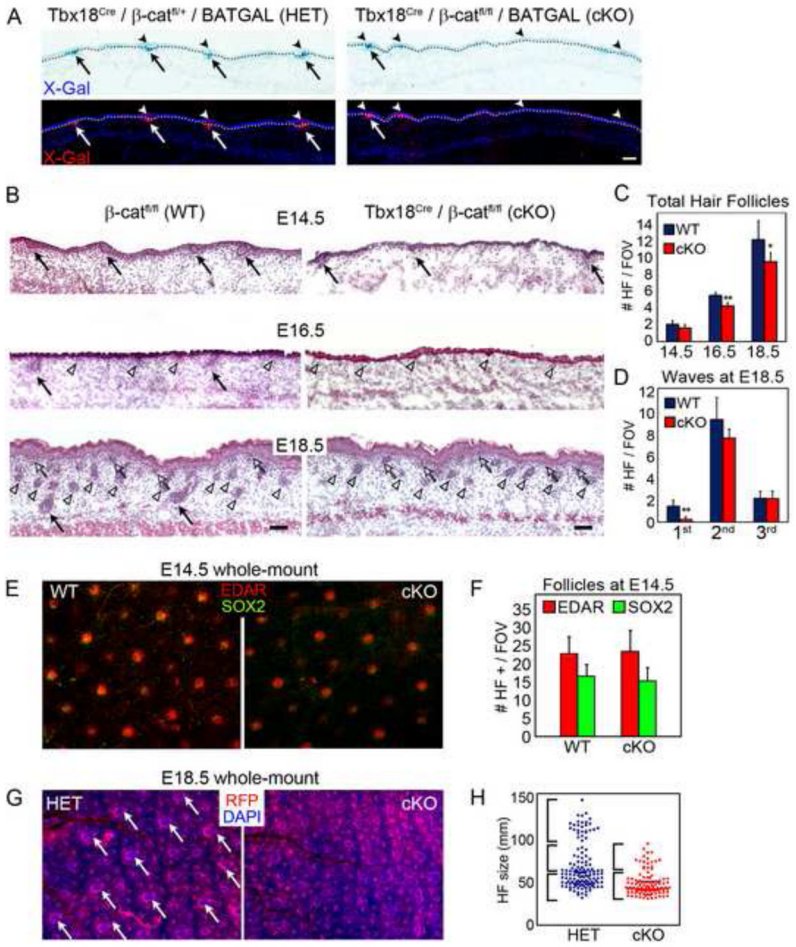

Figure 2. Embryonic β-catenin ablation in dermal condensates impairs 1st wave guard hair follicle formation

(**A**) Tbx18^{Cre} mediated β-catenin ablation in embryonic DP precursor cells in dermal condensates at E14.5. Cre and floxed lines were triple crossed with Wnt reporter BATGAL mice. Shown are sections of whole-mount X-gal stained embryos confirming strong reduction of Wnt activity in dermal condensates (arrows) of β-catenin cKO. Note that placodes (arrowheads) remained Wnt reporter positive. Bottom: X-gal stained sections and counterstaining with DAPI to highlight nuclei. Dotted line marks basement membrane between epidermis and dermis. (**B**) Hematoxylin and eosin stained sections of DP conditional β-catenin knockout (cKO) and wild-type (WT) backskin at indicated time points. Induction of 1st wave guard hair follicles (arrows) is not affected at E14.5, but their downgrowth and formation at E16.5 and E18.5 was strongly diminished. Formation of 2nd wave (open arrowheads) and 3rd wave hair follicles (open arrows) was unaffected, consistent with the lack of efficient β-catenin ablation in these follicle types. (**C**) Quantification of total hair follicle numbers. (**D**) Quantification of hair follicle types from the three waves at E18.5. Note 1st wave guard hair follicles are significantly reduced. (**E**) E14.5 whole-mount immunofluorescence staining for dermal condensate marker SOX2 and placode marker EDAR. (**F**) Quantification of SOX2-positive dermal condensates and EDAR-positive placodes. (**G**) E18.5 whole-mount skins of β-catenin heterozygous (HET) control and cKO embryos crossed with Lef1-RFP reporter mice to highlight DPs. Note that large 1st wave guard hair follicles (arrows) are missing in cKO. (**H**) Quantification of hair follicle sizes. Largest 1st wave guard hair follicles are absent in cKO skins. Scale bars: 100μm.

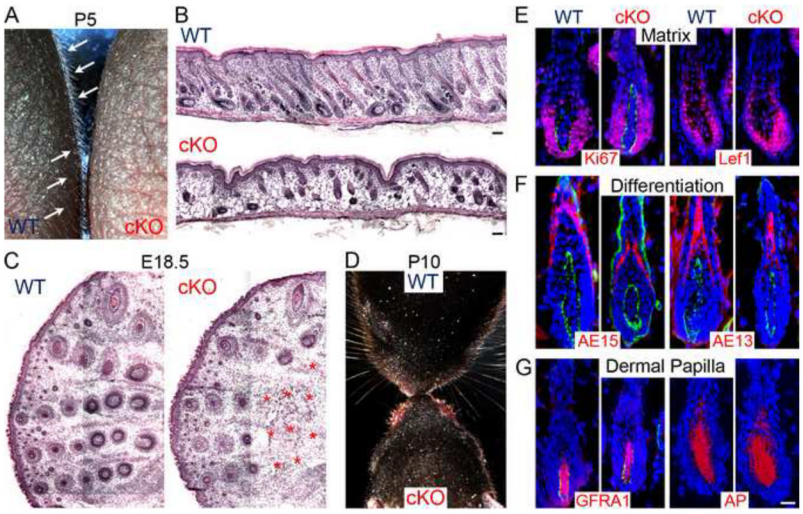

Figure 3. Impaired 1st wave guard and whisker hair follicle formation, but normal differentiation markers in developed hair follicles during postnatal growth

(**A**) Image of WT and cKO back skins at P5. Note absence of guard hair shafts in cKO. White arrows highlight outgrowing guard hair shafts in WT. (**B**) H&E sections of P5 back skins. Note thinner skin and fewer hair follicles in cKO. (**C**) H&E sections of whisker pads at E18.5. Several whiskers failed to form in cKO embryos (asterisks). (**D**) Top view of WT and cKO pups at P10. External whiskers are missing in cKO. (**E–G**) Immunofluorescence stainings of proliferation and differentiation markers in P5 WT and cKO awl hair follicles. (**E**) Ki67 labeling of proliferating matrix cells and Lef1 distribution was comparable between WT and cKO mice. (**F**) Unaltered AE15 and AE13 differentiation marker expression. Itgb4 (green) highlights basement membrane. (**G**) DP marker GFRA1 and AP are unperturbed. Scale bars: 100μm in B; 25μm in G.

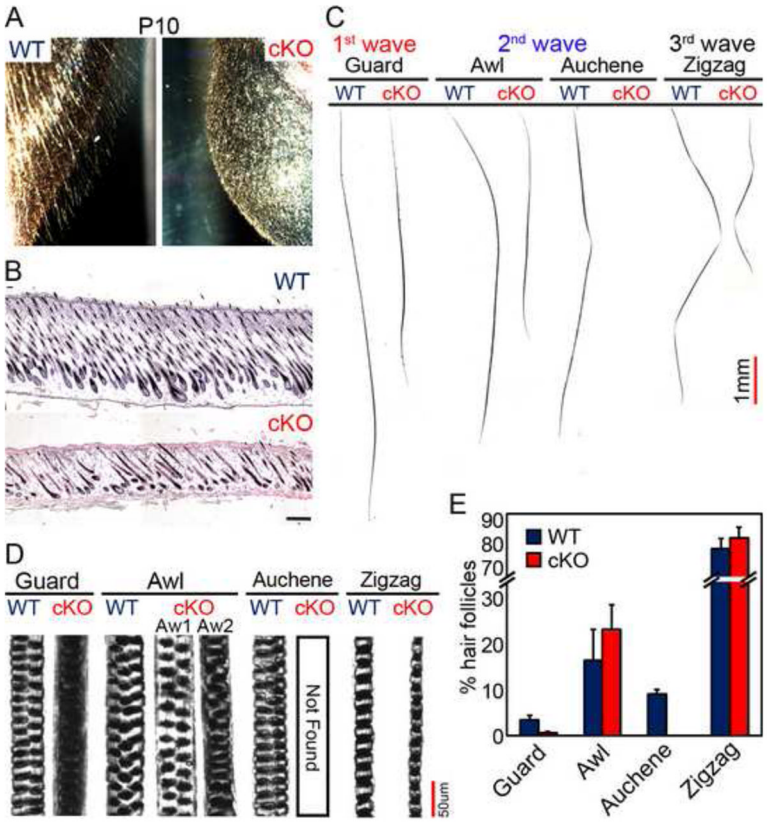

Figure 4. Hair shafts are shorter during postnatal hair growth

(**A**) Hair growth is strongly reduced in β-catenin cKO mice compared to WT. Note that guard hairs are absent from the lower backskin. (**B**) H&E sections showed thinner skin with fewer follicles in cKO. (**C**) Overall length of hair shafts from all three waves is shorter in cKO. Note that 2nd wave auchene hairs were missing. (**D**) Morphological analysis of hair shafts from all hair types. Awl hair contained two different subtypes in cKO; auchene hair was absent. (**E**) Quantification of all hair shaft types. Guard hairs were strongly reduced; auchene hairs were absent in cKO, but awl hair numbers were increased.

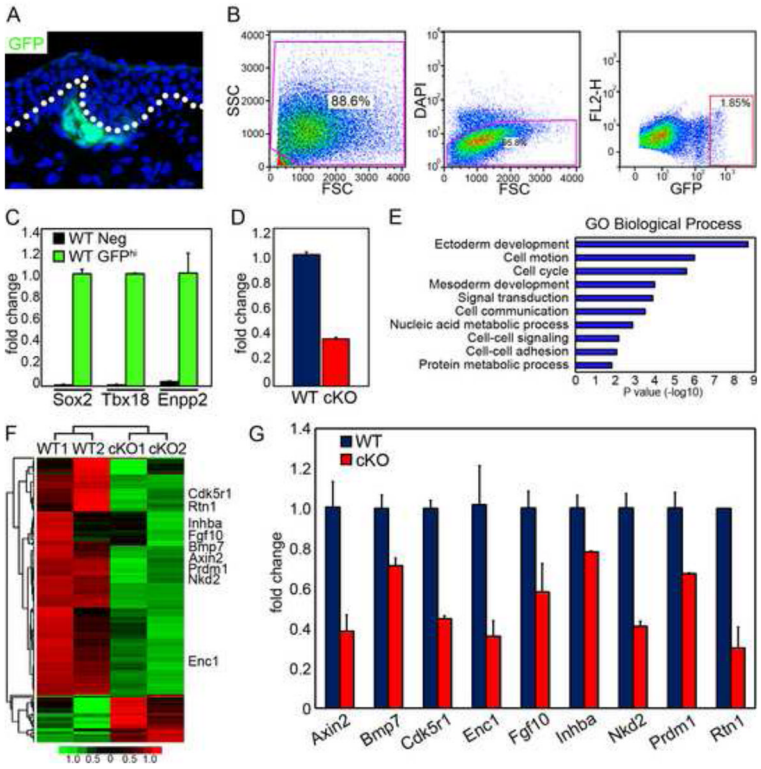

Figure 5. Ablation of Wnt/β-catenin signaling alters DP precursor gene expression, including signaling molecules Fgf10 and Inhba

(**A**) Section of E15.5 Sox2^{GFP} embryo backskin. Sox2 is specifically expressed in DP condensates. Dotted line marks basement membrane separating epidermis and dermis. (**B**) FACS plots. Single cells from embryonic Sox2^{GFP} skin preparations were DAPI stained to exclude dead cells. DP precursor cells were sorted as the GFP^{hi} cell population. (**C**) Real-time PCR of signature genes in sorted cells. GFP^{hi} cells were highly enriched for Sox2, Tbx18 and Enpp2. (**D**) Real-time PCR confirmed 70% β-catenin knockout efficiency in three sorts. (**E**) Analysis of enriched gene ontology (GO) categories. (**F**) Heat map analysis of altered gene expression in microarrays from WT and cKO sorted DP condensates. (**G**) Real-time PCR verification of downregulated genes in cKO. Expression levels were normalized to Gapdh and presented relative to WT. Data were from two independent sorts.