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The Androgen Receptor Antagonizes Wnt/β-Catenin Signaling in Epidermal Stem Cells

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Activation of Wnt/β-catenin signaling in adult mouse epidermis leads to expansion of the stem cell compartment and redirects keratinocytes in the interfollicular epidermis and sebaceous glands (SGs) to differentiate along the hair follicle (HF) lineages. Here we demonstrate that during epidermal development and homeostasis there is reciprocal activation of the androgen receptor (AR) and β-catenin in cells of the HF bulb. AR activation reduced β-catenin-dependent transcription, blocked β-catenin-induced induction of HF growth, and prevented β-catenin-mediated conversion of SGs into HFs. Conversely, AR inhibition enhanced the effects of β-catenin activation, promoting HF proliferation and differentiation, culminating in the formation of benign HF tumors and a complete loss of SG identity. We conclude that AR signaling has a key role in epidermal stem cell fate selection by modulating responses to β-catenin in adult mouse skin.

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INTRODUCTION

Adult mammalian epidermis is maintained by self-renewing stem cells, which reside in distinct locations and give rise to progeny that differentiate along the lineages of the hair follicle (HF), sebaceous gland (SG), and interfollicular epidermis (IFE; Owens and Watt, 2003; Fuchs, 2009; Watt and Jensen, 2009). During normal epidermal homeostasis, each stem cell population produces the differentiating cells that are appropriate for its specific location (Kretzschmar and Watt, 2014). However, in response to injury or genetic manipulation, stem cells in any region of the epidermis have the ability to give rise to all differentiated epidermal lineages (Watt and Jensen, 2009; Arwert *et al.*, 2012)

Canonical Wnt signaling has a major role in regulating epidermal stem cell renewal and lineage selection (Blanpain *et al.*, 2007; Watt and Collins, 2008). The onset and duration of Wnt signaling can be controlled by expressing a 4-hydroxytamoxifen (4-OHT) inducible form of stabilized β-catenin under the control of the *keratin 14* (*K14*) promoter in the basal layer of transgenic mouse epidermis (K14ΔNβ-cateninER mice). Studies with this model have shown that a single application of 4-OHT induces existing HFs to enter the growth phase of the hair cycle (anagen), whereas repeated treatments result in expansion of the stem cell compartment and reprogramming of cells in the SG and IFE to form ectopic HFs (Van Mater *et al.*, 2003; Lo Celso *et al.*, 2004; Silva-Vargas *et al.*, 2005). The cells at the base of the SG are particularly sensitive to β-catenin activation, whereas the stem cells in the HF bulge are resistant (Baker *et al.*, 2010; Deschene *et al.*, 2014).

β-catenin is highly regulated through binding partners and cross-talk with other signaling pathways (Clevers and Nusse, 2012). One such partner is the androgen receptor (AR), which interacts with β-catenin in an androgen-dependent manner (Yang *et al.*, 2002; Song *et al.*, 2003). Androgen-bound AR can inhibit β-catenin target gene expression (Chesire and Isaacs, 2002; Pawlowski *et al.*, 2002) as a result of competition with TCF/LEF1 transcription factors for β-catenin binding (Mulholland *et al.*, 2003; Terry *et al.*, 2006).

In the epidermis, AR and enzymes of androgen metabolism are expressed in SGs and HFs (Randall *et al.*, 1993; Miyake *et al.*, 1994; Rosenfield *et al.*, 1998; Itami and Inui, 2005; Rosenfield, 2005). AR activation can promote MYC-induced SG differentiation (Cottle *et al.*, 2013) and inhibit hair growth (Naito *et al.*, 2008; Crabtree *et al.*, 2010). Regulation of androgen expression and AR signaling is implicated in androgenetic alopecia (AGA), a common form of hair loss that occurs in men and women (Paus and Cotsarelis, 1999), and a reciprocal relationship between β-catenin and AR signaling has been observed in co-cultures of human dermal papilla (DP) cells and HF stem cells isolated from healthy and

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Abbreviations: 4-OHT, 4-hydroxytamoxifen; AGA, androgenetic alopecia; AR, androgen receptor; CDP, CCAAT displacement protein; DP, dermal papilla; FAS, fatty acid synthase; HF, hair follicle; IFE, interfollicular epidermis; K14, keratin 14; SG, sebaceous gland; TRP2, tyrosinase-related protein 2; QRT-PCR, quantitative real-time reverse-transcriptase–PCR

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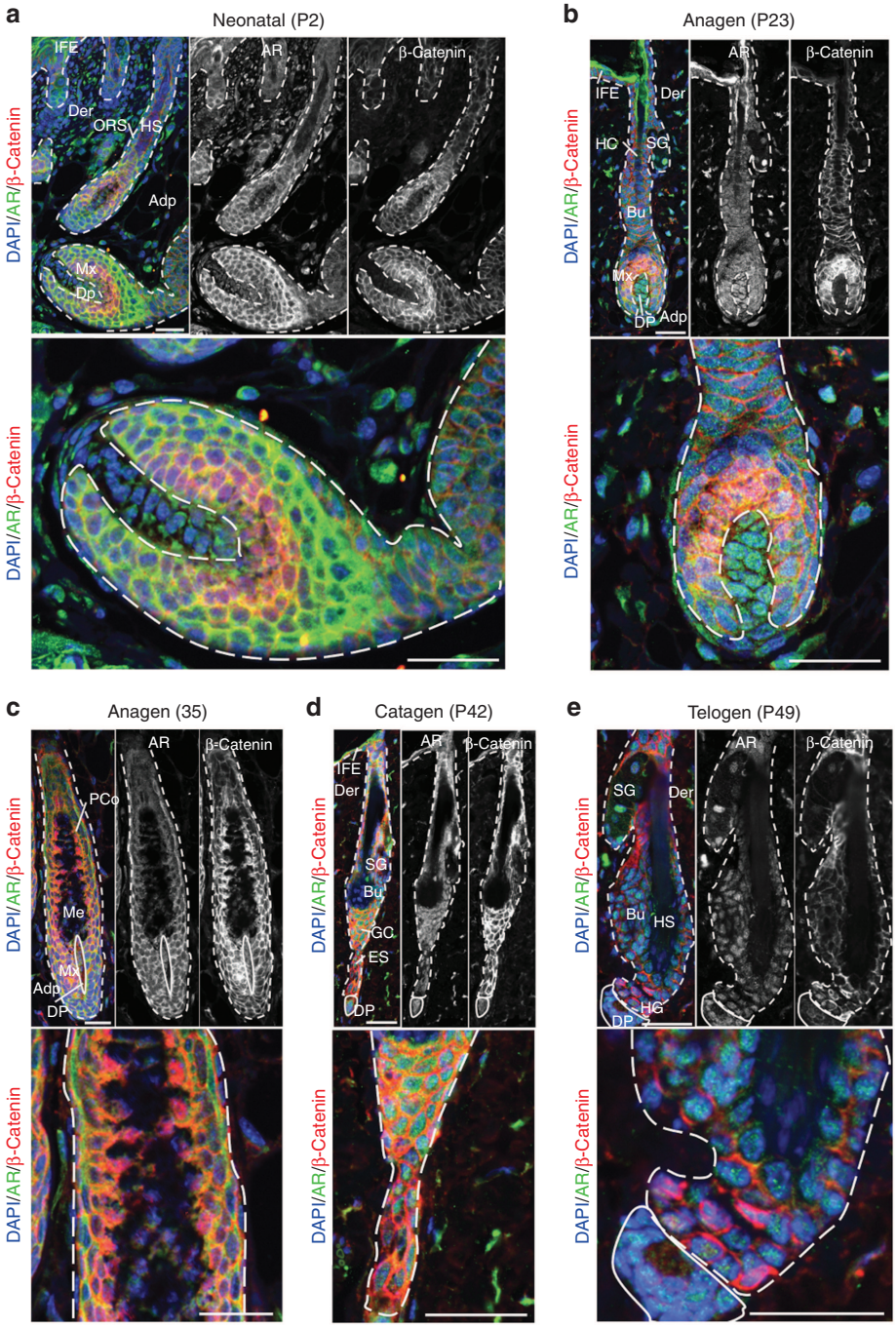

Figure 1. Reciprocal epidermal androgen receptor (AR) and β-catenin activity. (**a**–**e**) Wild-type mouse skin stained for AR (green) and β-catenin (red). Gray scale images of AR and β-catenin labeling are also shown. Bar = 30 μm. Adp, adipocyte; Bu, bulge; Der, dermis; DP, dermal papilla; ES, epithelial strand; GC, germ capsule; HC, hair canal; HG, hair germ; HS, hair shaft; IFE, interfollicular epidermis; Me, medulla; Mx, matrix; ORS, outer root sheath; PCo, pre-cortex; SG, sebaceous gland.

AGA individuals (Leirós *et al.*, 2012). These observations led us to investigate whether the AR modulates the responsiveness of epidermal cells to β-catenin activation.

RESULTS

Cellular localization of AR and β-catenin in cycling HFs

Although the AR is expressed in adult skin (Cottle *et al.*, 2013), it is only active when localized to the cell nucleus (Azzi *et al.*, 2006). AR usually localizes to the cell nucleus in humans and rodents that have not undergone castration (Robel *et al.*, 1983). In line with this observation, in neonatal skin (postnatal day 2), the AR was localized to the nucleus in all epidermal and dermal cells, except for epidermal cells in the hair bulb, where it was present in the cytoplasm (Figure 1a). Although in some bulb cells the AR appeared to be concentrated at the cell periphery, the AR is

not reported to have a plasma membrane localization (Bennett *et al.*, 2010).

At postnatal day 2, β-catenin was enriched in the nucleus of cells that had cytoplasmic AR (Figure 1a). In all other epidermal cells, β-catenin was localized to the plasma membrane, as reported previously (Niemann *et al.*, 2002).

During early anagen (the growth phase of the hair cycle) of adult skin, nuclear β-catenin was confined to cells of the hair bulb (Figure 1b). At this stage, the AR was strongly localized in the nucleus of hair bulb cells adjacent to the DP, indicating active AR signaling, but also detectable in the cytoplasm (Figure 1b). During full anagen, β-catenin was nuclear in upper bulb cells of the follicle, whereas the AR was localized to the cytoplasm (Figure 1c). Conversely, nuclear β-catenin was absent in the base of catagen HFs (Figure 1d), indicating downregulation of canonical Wnt signaling. Here the AR was strongly localized to the nucleus, suggesting activated signaling. In telogen, β-catenin was only detectable at the cell membrane, whereas the AR was nuclear (Figure 1e). During all hair cycle stages, the AR was localized to the nucleus of DP cells and other dermal fibroblasts, whereas β-catenin was barely detectable in dermal cells (Figure 1a–e).

In summary, during phases of hair growth, β-catenin was strongly nuclear in the hair bulb, whereas it localized to the cell membrane and cytoplasm during hair regression (catagen) or rest (telogen). Conversely, the AR was mainly nuclear during telogen, catagen, and early anagen but predominantly cytoplasmic in the hair bulb adjacent to the DP during HF morphogenesis and full anagen.

To complement the immunolocalization studies, we examined AR and β-catenin levels in published gene expression profiles of back skin epidermal and dermal cells isolated by flow cytometry at different stages of HF development and homeostasis (Supplementary Figure S1a online; Collins *et al.*, 2011). The epidermal cells were α6 integrin-positive, undifferentiated cells from IFE, SG, and HF, whereas the dermal cells were Pdgfrα-positive cells from all dermal compartments, including the DP. In the epidermis, AR expression was highest in telogen keratinocytes, lower in anagen keratinocytes and keratinocytes from the skin with β-catenin-induced ectopic HFs, and least abundant in neonatal keratinocytes. In the dermis, AR levels were highest in adult telogen and anagen fibroblasts and lowest in neonatal fibroblasts and fibroblasts from the skin with ectopic HFs. β-catenin also showed dynamic expression in the epidermis and dermis, but the variation was less pronounced than in the case of the AR (Supplementary Figure S1a online). The Wnt/β-catenin target genes *Cd44*, *Tcf7l1*, *Tnc*, *Cux1*, *Wnt5a*, *Lgr5*, *Lef1,* and *Dlx3* were upregulated in the epidermis during HF growth (neonatal, anagen, ectopic HF skin) compared with telogen (Supplementary Figure S1b online), whereas AR target genes *Ppara*, *Fabp4*, *Abca1,* and *Scp2* (Schirra *et al.*, 2005) were consistently downregulated under these conditions (Supplementary Figure S1c online).

The changes in subcellular localization of AR and β-catenin and the trend toward an inverse relationship between the levels of AR and β-catenin target genes in keratinocytes and dermal fibroblasts during hair morphogenesis and cycling suggest a functional interaction between both proteins.

AR modulates Wnt/β-catenin signaling in cultured human sebocytes

In human and mouse skin, AR activity is highest in the SG, where it regulates lipid synthesis and accumulation (Imperato-McGinley *et al.*, 1993; Zouboulis *et al.*, 2007). We have recently shown that AR positively regulates murine SG differentiation in concert with c-MYC (Cottle *et al.*, 2013), a known Wnt/β-catenin target gene (He *et al.*, 1998). These observations suggested that cultured sebocytes would be a good model in which to examine whether in the epidermis, as in the prostate, the AR negatively regulated Wnt signaling. We therefore transfected immortalized human sebocytes (Seb-E6E7 cells; Lo Celso *et al.*, 2008; Cottle *et al.*, 2013) with the TOPFLASH Wnt activity reporter containing wild-type TCF binding sites, or with the FOPFLASH reporter containing mutant TCF binding sites as a negative control (van de Wetering *et al.*, 1996). Cells were co-transfected with a Renilla luciferase reporter to adjust for transfection efficiency. The testosterone concentration used (20 μM) has previously been shown to activate exogenous AR in AR-Luciferase assays (Cottle *et al.*, 2013). Testosterone is ~100-fold less potent compared with its active form, dihydroxytestosterone.

In Seb-E6E7 cells treated with the AR activator testosterone, there was no significant decrease in luciferase reporter activity compared with the DMSO control. However, application of the anti-androgen bicalutamide caused a significant increase in luciferase activity, indicating that AR signaling negatively regulates endogenous Wnt/β-catenin activity (Supplementary Figure S2 online). Application of a combination of testosterone and bicalutamide decreased luciferase reporter activity to the same level as the DMSO control, consistent with previous reports showing that testosterone and bicalutamide compete for AR binding (Furr and Tucker, 1996). We conclude that AR acts as an antagonist of Wnt/β-catenin-dependent transcription in epidermal cells *in vitro*.

AR modulates epidermal responses to β-catenin-induced anagen and ectopic HF formation

The ΔK5ΔNβ-cateninER transgenic mice express a stabilized form of β-catenin in the hair germ and SGs (Baker *et al.*, 2010), two sites of high AR activity in the skin (Figure 2a and b). To analyze whether the intracellular localization of AR was altered in this experimental setting, we stained the back skin of 4-OHT-treated ΔK5ΔNβ-cateninER transgenic mice with an antibody against the AR (Supplementary Figure S3a online). Control (acetone-only treated) tissue showed nuclear AR expression in all epidermal cells and all dermal fibroblasts, including DP cells (Supplementary Figure S3a online). In addition, transgene-negative mice treated with 4-OHT did not display any changes in AR expression (data not shown). After 4-OHT treatment of ΔK5ΔNβ-cateninER mice for 7 days (3 doses), HFs entered anagen (Supplementary Figure S3a online). The AR was present in the cytoplasm of hair bulb cells but remained nuclear in adjacent DP cells. Upon sustained

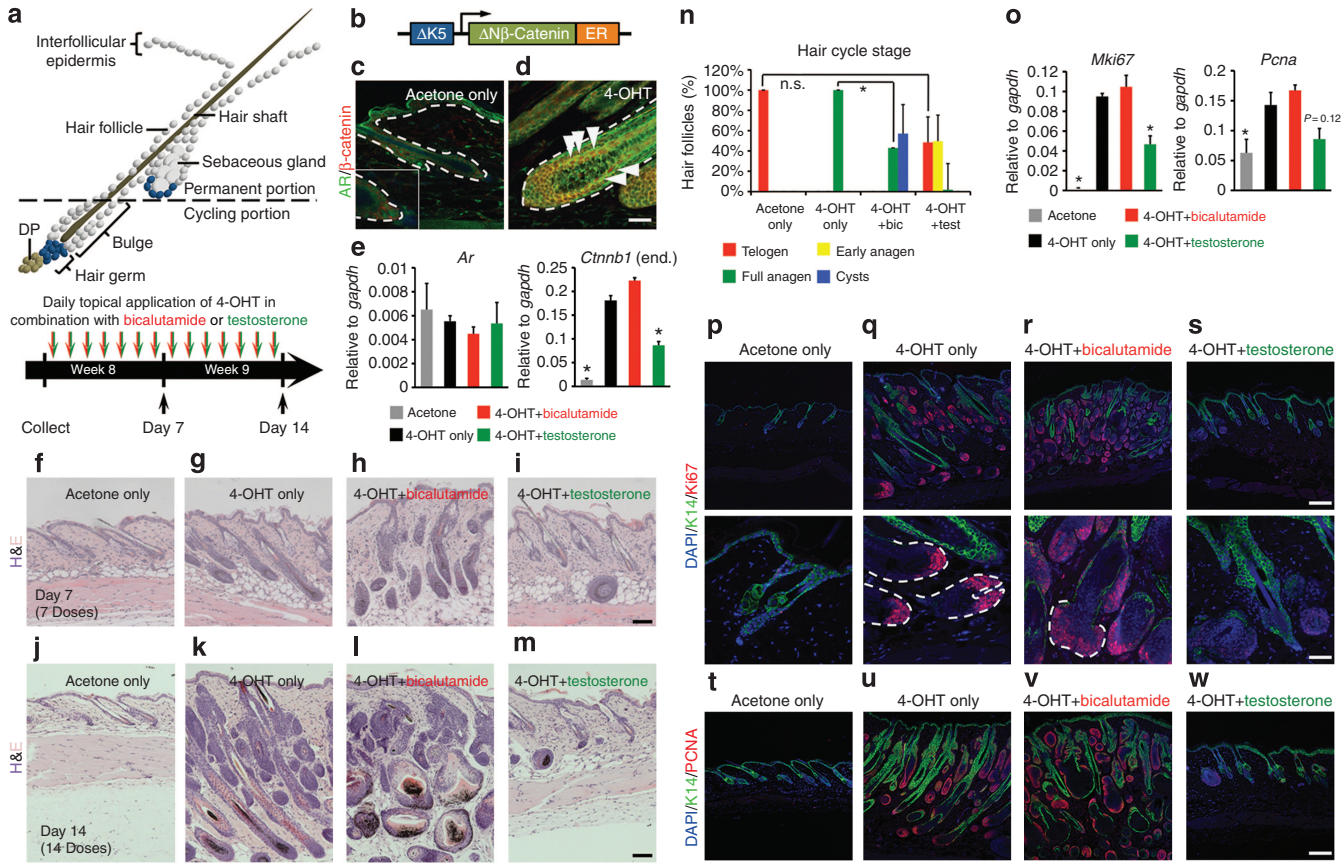

Figure 2. Androgen receptor (AR) activity modulates β-catenin-induced anagen and ectopic hair follicle formation. (**a**) Schematic showing transgene expression (blue cells) in ΔK5ΔNβ-cateninER mice. (**b**) Schematic of ΔK5ΔNβ-cateninER transgene. (**c**) Back skin from ΔK5ΔNβ-cateninER mice treated for 14 days with acetone or 4-hydroxytamoxifen (4-OHT), labeled with antibodies to AR (green) and β-catenin (red). Arrowheads indicate nuclear β-catenin. (**d**) Treatment regime. (**e**) Relative expression (normalized to *Gapdh*) of *Ar* (left panel) and *Ctnnb1* (right panel) in whole back skin. Data are averages ± SEM from 3 to 4 mice. Asterisks denote significant difference relative to 4-OHT only ($P<0.05$). (**f**–**m**) Back skin of ΔK5ΔNβ-cateninER mice after 7 or 14 days of treatment stained with hematoxylin and eosin (H&E). (**n**) % hair follicles in each stage of hair cycle after 14 days of treatment. Data are averages ± SEM from 3 mice per condition. *$P<0.05$ between experimental groups as indicated, and NS indicates non-significance. (**o**) Relative expression (normalized to *Gapdh*) of *Mki67* and *Pcna* in whole back skin. Data are averages ± SEM from 3 to 4 mice. Asterisks denote significant difference relative to 4-OHT alone ($P<0.05$). (**p**–**w**) Back skin of ΔK5ΔNβ-cateninER mice treated for 14 days and labeled with antibodies to Ki67 (red in **p**–**s**), PCNA (red in **t**–**w**), and keratin 14 (K14; green), counterstained with DAPI to label nuclei (blue). Bars = 200 μm (**f**–**m** and **p**–**s** upper panels) and 80 μm (**c** and **p**–**s** lower panels, **t**–**w**). Bic, bicalutamide; test, testosterone.

epidermal stabilization of β-catenin with 4-OHT for 21 days (9 doses), ectopic HFs formed from most SGs, which showed strong cytoplasmic AR (Supplementary Figure S3a online).

In order to confirm that these effects were a specific response to β-catenin activation, we also stained back skin sections of two other ΔNβ-cateninER transgenic mouse lines (Supplementary Figure S3b and c online). In K15ΔNβ-cateninER transgenic mice, in which stabilized β-catenin is specifically expressed in the HF bulge (Baker *et al.*, 2010), 4-OHT-induced anagen resulted in AR localization only in the cytoplasm of cells in the lower HF (Supplementary Figure S3b online). Cells of the IFE and SG were unaffected and still had nuclear AR (Supplementary Figure S3b online). In K14ΔNβ-cateninER transgenic mice, in which β-catenin is activated in the entire epidermal basal layer and the SG (Lo Celso *et al.*, 2004), AR was localized to the cytoplasm of all epidermal cells on 4-OHT treatment (Supplementary Figure S3c online). Thus, β-catenin-induced anagen and ectopic HF formation correlated with re-localization of the AR from the nucleus to the cytoplasm but only in those cells that expressed activated β-catenin. This is consistent with the changes in AR localization that occur during the normal hair growth cycle (Figure 1a–f).

Epidermal AR activity can be modulated through topical application of the androgen testosterone or the anti-androgen bicalutamide (Cottle *et al.*, 2013). The concentration of bicalutamide used was insufficient to induce anagen in wild-type skin so that we could examine the specific impact of transgene-dependent signaling events. We treated ΔK5ΔNβ-cateninER transgenic mice daily with 4-OHT in combination with these compounds for up to 14 days (Figure 2b–d). Quantitative real-time reverse-transcriptase–PCR (QRT-PCR) showed that *Ar* mRNA expression was similar in all conditions (Figure 2e), indicating that AR activity rather than expression was altered. In contrast, expression of endogenous *Ctnnb1* mRNA was upregulated in the skin

treated with 4-OHT or 4-OHT and bicalutamide and downregulated by testosterone treatment (Figure 2e).

Transgenic mice treated with acetone (carrier), bicalutamide or testosterone alone, or wild-type mice treated with 4-OHT in combination with either drug, remained in telogen (Figure 2f, j, and n and Supplementary Figures S3d and S4g–p online). The proportion of telogen HF was not significantly different in acetone-treated skin compared with skin treated with 4-OHT and testosterone, which is consistent with the inhibitory effect of AR on β-catenin signaling (Figure 2n). In contrast, 4-OHT application to transgenic mice induced anagen within 7 days (Figure 2g and n) and conversion of SGs into ectopic HFs within 14 days (Figure 2k and Supplementary Figure S4e and f online), as reported previously (Baker *et al.*, 2010). Combined application of 4-OHT and bicalutamide stimulated existing HFs to enter anagen earlier than treatment with 4-OHT alone (Figure 2h and n and data not shown). Ectopic HFs formed within 14 days (Figure 2l), but there was a striking impairment of hair shaft formation causing large cysts filled with keratinized cells to form in the hair bulbs (Figure 2l and Supplementary Figure S4a and b online). Full anagen induction was significantly reduced in skin treated with 4-OHT and bicalutamide compared with 4-OHT alone, reflecting the formation of HF cysts (Figure 2n). Conversely, treating transgenic mice with 4-OHT in combination with testosterone for 14 days retarded anagen induction and blocked ectopic HF formation (Figure 2i, m, and n and Supplementary Figure S4c and d online). All of the effects were observed in both male and female mice (e.g., Supplementary Figure S4 online).

β-catenin-induced anagen and ectopic HF formation are correlated with the stimulation of epidermal cell proliferation, as evaluated by increased expression of Ki67 and proliferating cell nuclear antigen (Lo Celso *et al.*, 2004; Baker *et al.*, 2010; Figure 2o–w). Similarly, combined treatment of bicalutamide and 4-OHT showed increased expression of both markers (Figure 2o, r, and v). In contrast, application of testosterone in combination with 4-OHT decreased Ki67 and proliferating cell nuclear antigen expression (Figure 2o, s and w).

We conclude that AR negatively regulates β-catenin-induced proliferation, anagen induction, and ectopic HF formation in mouse skin.

AR activation blocks β-catenin target gene expression and conversion of SGs into ectopic HFs

We next examined the effects of AR modulation on expression of β-catenin target genes and markers of HF and SG differentiation. Application of 4-OHT alone or in combination with bicalutamide resulted in high levels of expression of *LEF1*, which is a well-established epidermal β-catenin target gene, in the hair bulb and ectopic HFs (Figure 3a), whereas on combined application of 4-OHT and testosterone most HFs lacked detectable levels of *LEF1* (Figure 3a). Immunolocalization of SOX-9 (Nowak *et al.*, 2008) and K15 within the bulge was unaffected by treatment with 4-OHT alone or in combination with bicalutamide or testosterone (Figure 3b and Supplementary Figure S5a online). However, total epidermal *Sox9* mRNA levels were increased by 4-OHT alone or in combination with bicalutamide and decreased on testosterone treatment (Figure 3f). The same effects were observed on mRNA levels of other β-catenin target genes (*Tcf7l1, Tcf7l2, Cux1, Cdh3, Axin2, Ccnd1, c-Myc, Wnt5a, Frmd4a,* and *Bmp2*; Figure 3f and Supplementary Figure S5c online). Although *Ccnd1* is a well-established Wnt/β-catenin target gene, it has also been reported to be an AR target gene in mouse skin (Schirra *et al.*, 2005), making it a particularly appropriate readout of the antagonist effect of AR on Wnt/β-catenin signaling. The increases in *Bmp4* and *Ptgs2* were not statistically significant, but both genes were significantly downregulated upon testosterone treatment (Supplementary Figure S5c online). Conversely, Filamin A (*FlnA*), which is required for AR activation in response to androgen stimulation (Castoria *et al.*, 2011; Mooso *et al.*, 2012), was significantly downregulated by β-catenin activation, an effect that was antagonized by testosterone (Supplementary Figure S5c online). The observation that testosterone increased *FlnA* expression in the presence of 4-OHT is consistent with the conclusion that AR signaling antagonized β-catenin signaling. As 4-OHT treatment led to a major reduction in *FlnA*, it is not surprising that there was no further effect of bicalutamide.

CDP (CCAAT displacement protein) is expressed in the HF bulb during Wnt-driven anagen (Silva-Vargas *et al.*, 2005; Figure 3c). On bicalutamide treatment, the expanded outer root sheath and cysts developing from the hair bulb were strongly positive for CDP (Figure 3c). The cysts expressed the hair shaft marker K31, which is indicative of HF differentiation (Figure 3d). Staining for glycogen synthase kinase 3β, an antagonist of β-catenin and component of its degradation complex (Clevers and Nusse, 2012), was reduced in HF cysts by combined application of 4-OHT and bicalutamide compared with 4-OHT alone, suggesting that the activity of the β-catenin destruction complex was decreased (Figure 3e).

AR activation blocks conversion of SGs into ectopic HFs

In ΔK5ΔNβ-cateninER back skin ectopic HFs arise exclusively from the SGs, and this correlates with loss of expression of fatty acid synthase (FAS), encoded by *Fasn*, which is an AR target gene expressed in differentiating sebocytes (Figure 4a and b; Cottle *et al.*, 2013). Treatment with bicalutamide and 4-OHT also led to a loss of FAS and conversion of the SG to CDP-positive ectopic HFs (Figure 4a–c and e). Conversely, 4-OHT and testosterone-treated back skin from ΔK5ΔNβ-cateninER mice showed preservation of FAS-positive sebocytes and a reduction in ectopic HFs (Figure 4a–c and e). QRT-PCR analysis confirmed that expression of *Fasn* and another sebocyte marker, *Fabp5,* was reduced in bicalutamide-treated skin, indicating the loss of sebocyte differentiation (Figure 4d). In addition to being expressed in the SG, FAS was expressed in the cuticle layer of anagen HFs (Supplementary Figure S5d online), explaining the increase in *Fasn* expression in transgenic mice treated with 4-OHT only (Figure 4d). B lymphocyte–induced maturation protein 1, which is expressed by terminally differentiated keratinocytes in several epidermal compartments (Cottle *et al.*, 2013; Kretzschmar *et al.*, 2014), was no longer expressed in

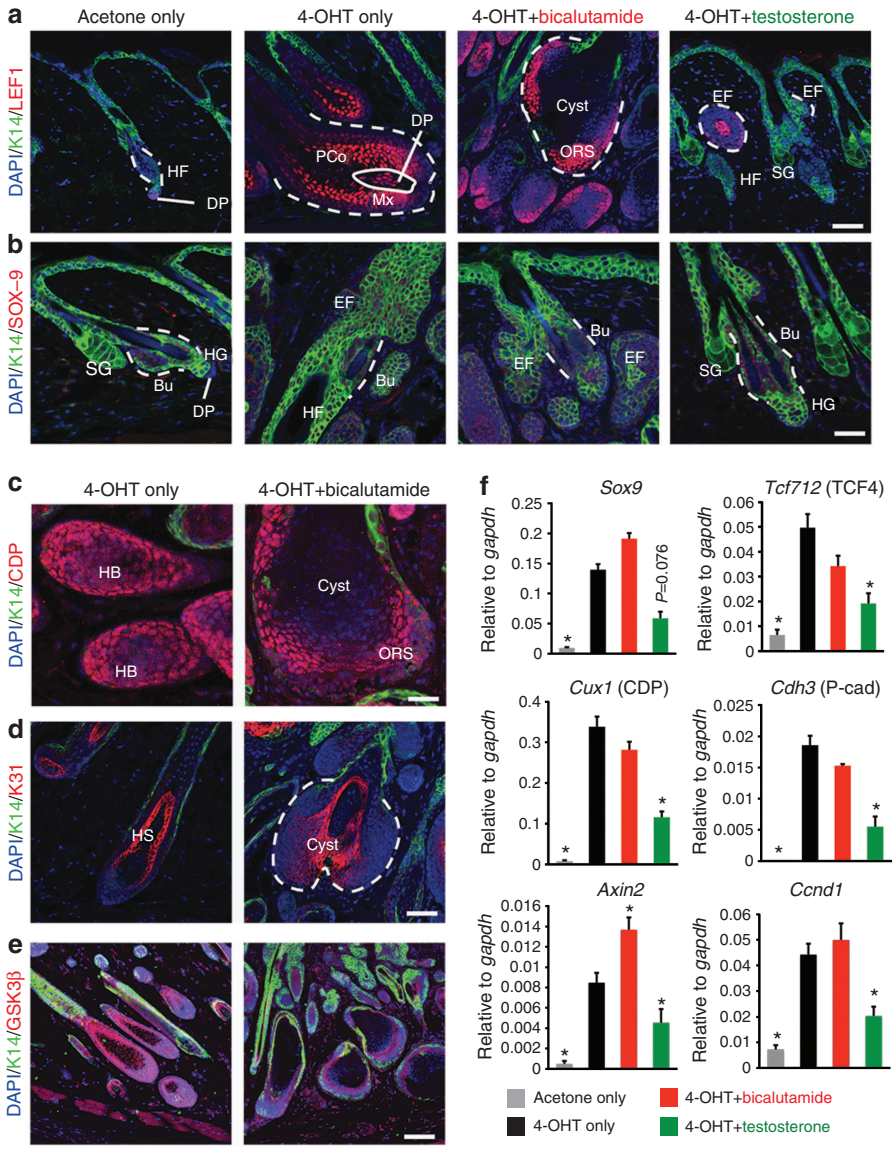

Figure 3. Androgen receptor (AR) activity modulates β-catenin target gene expression and anagen induction. (**a**–**e**) Back skin of ΔK5ΔNβ-cateninER mice treated for 14 days as indicated and labeled with antibodies to LEF1 (red in **a**), SOX-9 (red in **b**), CCAAT displacement protein (CDP; red in **c**), keratin 31 (K31; red in **d**), glycogen synthase kinase 3β (GSK3β; red in **e**), and K14 (green), counterstained with DAPI (blue). (**f**) Relative expression (normalized to *Gapdh*) of *Sox9*, *Tcf7l2*, *Cux1*, *Cdh3*, *Axin2*, and *Ccnd1* in whole back skin. Data are means ± SEM from 3 to 4 mice. Asterisks denote significant difference relative to 4-hydroxytamoxifen (4-OHT) alone ($P < 0.05$). Bars = 200 μm (**e**) and 80 μm (**a**–**d**). Bu, bulge; DP, dermal papilla; EF, ectopic hair follicle; HB, hair bulb; HF, hair follicle; HG, hair germ; HS, hair shaft; Mx, matrix; PCo, Pre-cortex; ORS, outer root sheath; SG, sebaceous gland.

sebocytes following combined application of 4-OHT and bicalutamide (Supplementary Figure S5b online).

AR modulates the effects of epidermal β-catenin on the dermis

Hair cycling and ectopic HF formation are controlled by interactions between the epidermis and dermis (Driskell *et al.*, 2011; 2012). Epidermal β-catenin activation in adult skin reprograms the dermis to a neonatal state characterized by increased fibroblast proliferation and density, and extracellular matrix remodeling (Collins *et al.*, 2011). Dermal thickness in ΔK5ΔNβ-cateninER skin increased on treatment with 4-OHT alone or in combination with bicalutamide (Figure 5g), as has been reported for wild-type anagen skin (Hansen *et al.*, 1984; Müller-Röver *et al.*, 2001). Sustained β-catenin activation in combination with AR inhibition led to an increase in platelet-derived growth factor receptor-α-positive (Figure 5a) and Vimentin-positive (Figure 5d) fibroblasts adjacent to ectopic HFs. In addition, the hyaluronic acid receptor CD44, which is a Wnt/β-catenin target gene (Wielenga *et al.*, 1999), was markedly increased around cysts induced by combined 4-OHT and bicalutamide treatment (Figure 5b). Next, we visualized mature (bright pink) and immature collagen (blue) using Herovici's method (Figure 5c). Extracellular matrix remodeling occurred in the presence of bicalutamide but was inhibited by testosterone (Figure 5c). The DP of anagen HFs expressed Corin (Figure 5e;

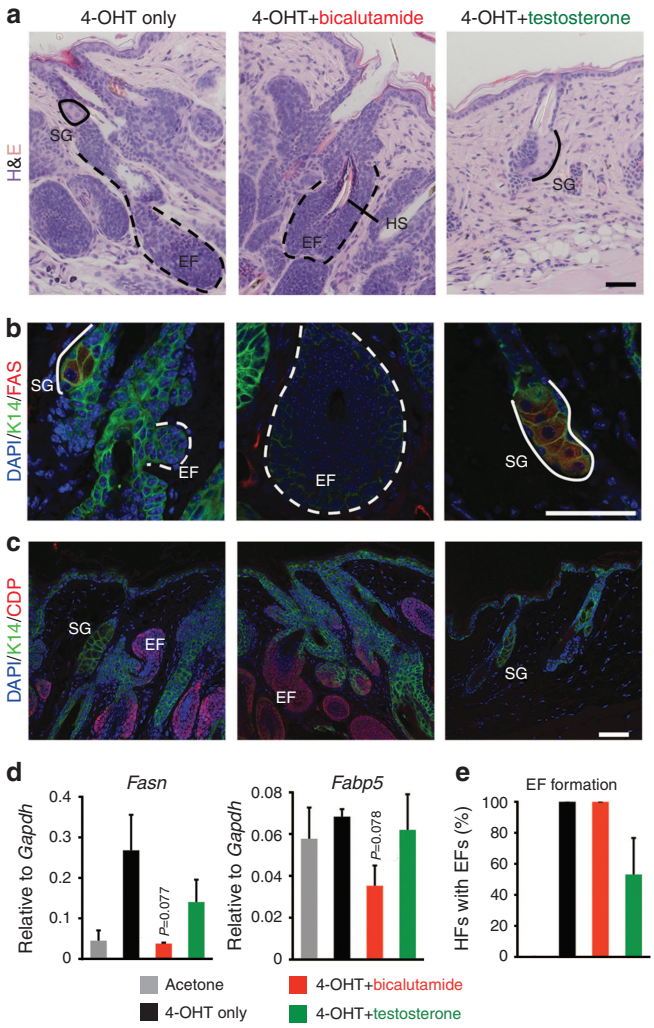

Figure 4. Androgen receptor (AR) inhibition blocks β-catenin-induced ectopic hair follicle formation. (**a**–**c**) Back skin of ΔK5ΔNβ-cateninER mice treated for 14 days and stained with hematoxylin and eosin (**a**) or antibodies to fatty acid synthase (FAS; red in **b**), CCAAT displacement protein (CDP; red in **c**), and keratin 14 (K14; green in **b** and **c**), counterstained with DAPI (blue in **b** and **c**). (**d**) Relative expression (normalized to *Gapdh*) of *Fasn* and *Fabp5* in whole back skin. Data are averages ± SEM from 3 to 4 mice per condition. Asterisks denote significant difference relative to 4-hydroxytamoxifen (4-OHT) only ($P < 0.05$). (**e**) Quantitation of hair follicles with associated ectopic hair follicles after 14 days of 4-OHT treatment. Data are averages ± SEM from 3 mice per condition. Bar = 80 μm. EF, ectopic hair follicle; HS, hair shaft; SG, sebaceous gland.

Driskell *et al.*, 2009). However, Corin was not expressed in the cysts that were induced by 4-OHT in combination with bicalutamide (Figure 5e). Quantitation of hematoxylin and eosin stained images revealed that only about 25% of hair bulbs had associated DP (Figure 5h), suggesting that cyst formation caused disintegration of the normal hair bulb.

Melanoblasts reside in the secondary hair germ and bulge and are required for hair pigmentation during anagen (Nishimura *et al.*, 2002). Melanoblasts are stimulated to proliferate through epidermal β-catenin activation (Rabbani *et al.*, 2011). Anagen HFs in the back skin of transgenic mice treated with 4-OHT showed expression of the melanocyte marker TRP2 (tyrosinase-related protein 2) in melanocytes within the DP-associated matrix of the hair bulge (Figure 5f). Sustained β-catenin activation in transgenic mice treated with 4-OHT and bicalutamide showed that TRP2-positive melanocytes were relocated to the periphery of HF cysts (Figure 5f). Surprisingly, TRP2-positive cells were also present in ectopic HFs, suggesting a further maturation than previously achieved (Figure 5f).

These data show that AR activity not only controls epidermal sensitivity to β-catenin stabilization but also regulates the effects of epidermal β-catenin activation on melanocytes and dermal fibroblasts (Figure 5i). Taken together, our results suggest that AR acts as a negative regulator of both the cell-autonomous and non-cell-autonomous effects of epidermal Wnt/β-catenin signaling in adult mouse skin.

DISCUSSION

Previous studies have shown that AR activity regulates the hair cycle and contributes to the pathogenesis of AGA (Paus and Cotsarelis, 1999). AR knockout mice have a disturbed hair growth cycle (Naito *et al.*, 2008), as do mice in which AR is overexpressed in the epidermis (Crabtree *et al.*, 2010). We now demonstrate that one mechanism by which AR regulates the hair cycle is by inhibiting Wnt/β-catenin signaling in adult mouse epidermis.

AR and β-catenin exhibited partially inverse activity and expression during the hair cycle. AR negatively regulated β-catenin transcriptional activity and target gene expression, whereas β-catenin activation led to inhibition of AR activity, as evaluated by relocation of AR from the nucleus to the cytoplasm, which is associated with inhibition of AR signaling (Azzi *et al.*, 2006; Cottle *et al.*, 2013). These observations point to the existence of a feedback loop that controls the hair cycle. The partially reversed subcellular localization of AR and β-catenin during the hair cycle suggests that their interaction may be predominantly indirect. The downregulation of glycogen synthase kinase 3β on AR inhibition would, for example, affect β-catenin protein stability, whereas the decreased expression of Filamin A as a result of β-catenin activation would reduce AR activation in response to androgen stimulation (Castoria *et al.*, 2011; Mooso *et al.*, 2012).

The fact that we did not see any phenotypic effects of AR modulation *in vivo* in the absence of exogenous β-catenin activation further supports the view that AR negatively regulates β-catenin target genes by indirect mechanisms. Among negative regulators of Wnt/β-catenin signaling in HF stem cells, microRNAs such as microRNA-214 have been identified (Ahmed *et al.*, 2014). Interestingly, in the prostate of castrated rats, microRNA-214 expression is positively correlated by androgen action (Narayanan *et al.*, 2010), suggesting one mechanism of indirect regulation of β-catenin expression through AR activity. Collectively, these results suggest that the

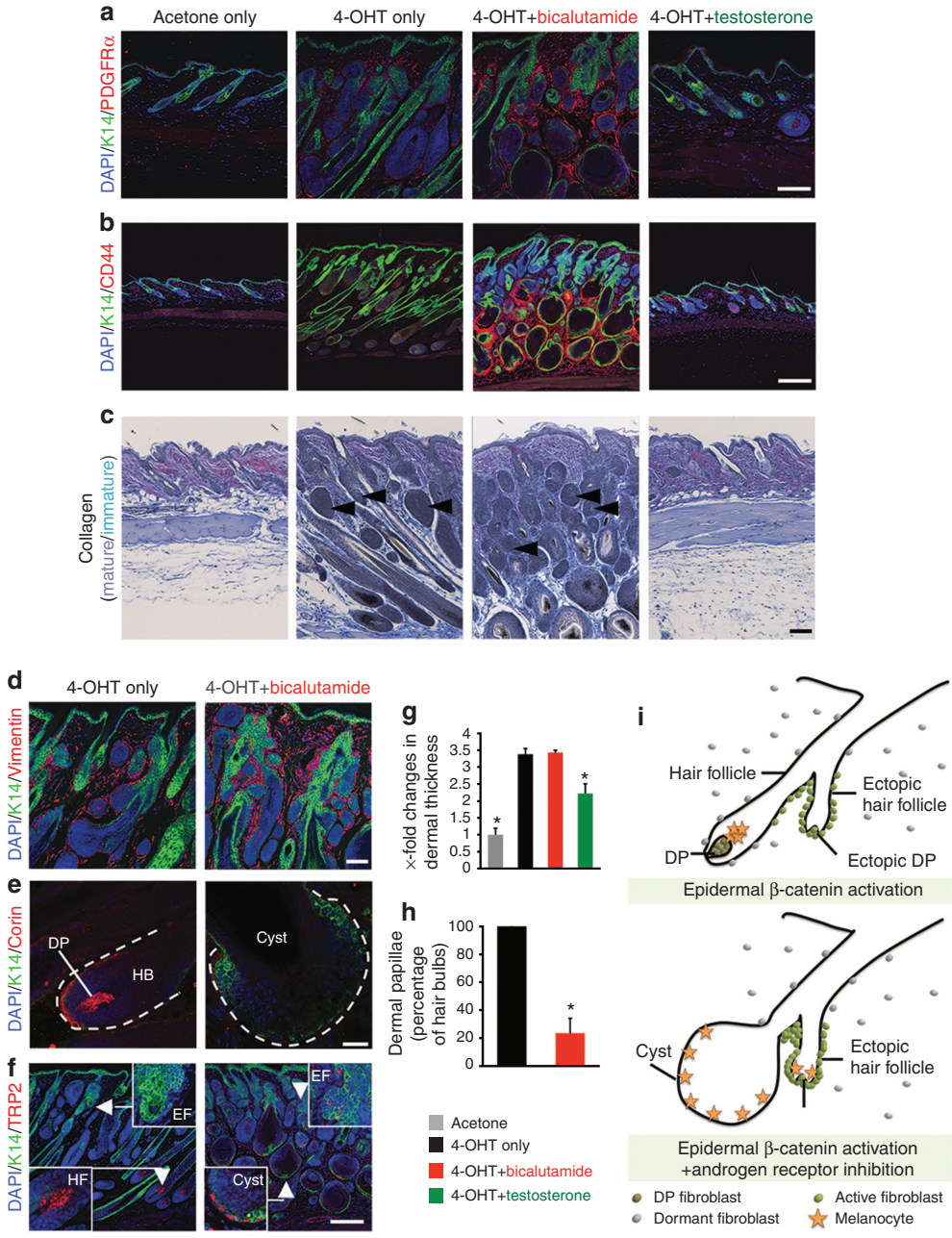

Figure 5. Androgen receptor (AR) activity modulates of β-catenin effects on the dermis and melanocytes. (**a**–**f**) Back skin of ΔK5ΔNβ-cateninER mice treated for 14 days and stained with Herovici's method (**c**) or labeled with antibodies against platelet-derived growth factor receptor α (PDGFRα; red in **a**), CD44 (red in **b**), Vimentin (red in **d**), Corin (red in **e**), tyrosinase-related protein 2 (TRP2; red in **f**), and keratin 14 (K14; green in **a**, **b** and **d**–**f**), counterstained with DAPI (blue in **a**, **c**–**e**). Arrowheads denote ectopic hair follicles (**c**) and regions magnified in the inserts (**f**). (**g** and **h**) Quantitation of dermal thickness (**g**) and hair bulbs with associated dermal papillae (**h**) after 14 days of 4-hydroxytamoxifen (4-OHT) treatment. Data are averages ± SEM from 3 mice per condition. Asterisks denote significant difference relative to 4-OHT only ($P < 0.05$). (**i**) Summary of phenotypes following β-catenin activation and/or AR inhibition through bicalutamide application in non-keratinocyte cell types (dermal fibroblasts and melanocytes). Bars = 200 μm (**b**, **c** and **f**); 100 μm (**a** and **b**), and 50 μm (**e**). DP, dermal papilla; EF, ectopic hair follicle; HB, hair bulb; HF, hair follicle.

interaction between AR and β-catenin could be both transcriptional and post-translational.

Epidermal β-catenin activation not only stimulates expansion of the stem cell compartment and differentiation along the HF lineages (Watt and Collins, 2008) but also stimulates melanocyte differentiation (Rabbani *et al.*, 2011) and reprogramming of the dermis to a neonatal state characterized by extracellular matrix remodeling and fibroblast proliferation (Collins *et al.*, 2011). All of these effects, both cell-autonomous and non-cell-autonomous, were inhibited by AR signaling.

Prolonged activation of β-catenin in the epidermis promotes the formation of benign HF tumors called trichofolliculomas (Gat *et al.*, 1998; Lo Celso *et al.*, 2004; Närhi *et al.*, 2008). The effect of sustained β-catenin stabilization in the presence of AR inhibition was to cause defects in hair shaft formation and development of cysts resembling trichofolliculomas (Figure 3d; Pálmer *et al.*, 2008). As AR inhibition by bicalutamide increases Wnt pathway activity *in vitro* and also causes a significant increase in expression of β-catenin target genes such as *Axin2* and *Tcf1l7* (TCF3), we believe that cyst formation in our model is triggered by a further upregulation of Wnt/β-catenin signaling through the increased activity of the ΔNβ-catenin transgene. The cyst phenotype is compatible with the concept that proliferation becomes to some extent uncoupled from differentiation, due to AR inhibition resulting in increased Wnt activity. The strong upregulation of CD44 by the combination of 4-OHT and bicalutamide is also interesting, as CD44 has previously been identified as a component of tumor stroma that promotes tumor growth and spread (Edward *et al.*, 2005). Thus, our findings may also provide insights into the formation of HF tumors and other Wnt-driven cancers.

In summary, we have identified the AR as an intracellular regulator of epidermal β-catenin signaling in adult mice. The mechanistic insights are potentially relevant to human HF disorders and diseases, such as AGA and trichofolliculomas, where the balance between antagonistic AR and β-catenin signaling may be disturbed. Nevertheless, there are undoubtedly differences between AR activity in mouse and human skin. None of the effects in mouse skin were gender specific, in agreement with our previous study (Cottle *et al.*, 2013). This is in contrast to the role of AR in human HFs, where the effects of AR appear to be context- and gender-dependent: androgens stimulate beard growth beginning with the onset of male puberty but inhibit scalp hair growth in AGA (Inui and Itami, 2013).

MATERIALS AND METHODS

Generation and experimental treatment of mice

Experiments were performed in accordance with the UK Government Animals (Scientific Procedures) Act 1986 and subject to institutional ethics approval. All transgenic mice have been described previously (Lo Celso *et al.*, 2004; Baker *et al.*, 2010). At the start of every experiment mice were 7–8-weeks old and in telogen (Stenn and Paus, 2001). The ΔNβ-cateninER transgene was activated by topical application of 4-OHT (Sigma St. Louis, MO) as described previously (Baker *et al.*, 2010). To modulate AR signaling, mice were treated daily for up to 14 days with 4-OHT (in 100 μl acetone) alone or in combination with 2 mg bicalutamide (Casodex, Sigma; dissolved in 100 μl acetone) or 2 mg testosterone (Sigma; dissolved in 100 μl acetone; Cottle *et al.*, 2013). In experiments in which the effect of 4-OHT alone was compared with 4-OHT and testosterone or bicalutamide, the volume of 4-OHT applied to mice treated with 4-OHT alone was made up to 200 μl with acetone. The effects of the drugs on mouse skin have been validated previously Cottle *et al.*, 2013). Wild-type littermates and acetone-treated transgenic mice were used as controls in all experiments. At least three mice, matched in gender and age (littermates), were treated per condition. No gender-specific effects were observed.

Gene expression analysis

For microarray analysis of dermal fibroblasts and epidermal keratinocytes isolated from different HF stages, gene expression profiles published in the study by Collins *et al.* (2011) and deposited under accession number GSE32966 on NCBI's Gene Expression Omnibus (GEO) website were analyzed with GeneSpring GX11 (Agilent, Santa Clara, CA).

Human sebocyte culture, transfection, and luciferase assays

The Seb-E6E7 line of immortalized human SG cells has been described elsewhere (Lo Celso *et al.*, 2008; Cottle *et al.*, 2013). Details of transfection methods, constructs, and luciferase analysis are provided in the Supplementary Materials online.

RNA extraction and QRT-PCR

RNA isolation (using the Trizol method), cDNA synthesis, and QRT-PCR were performed as described in the Supplementary Materials online.

Histology, immunohistochemistry, and imaging

Tissue samples for sections were fixed overnight in 4% paraformaldehyde and embedded in paraffin wax. Antibodies and labeling procedures are described in the Supplementary Materials online. Image analysis was performed using a Leica TCS SP5 confocal microscope (Wetzlar, Germany) (fluorescence microscopy) or a Zeiss Axiophot microscope equipped with a Zeiss AxioCam HRc camera (Oberkochen, Germany) (hematoxylin and eosin and Herovici staining).

Quantitation and statistical analysis

Quantitation of changes in the epidermis is described in the Supplementary Materials online. Statistical analysis was performed using the unpaired Student's *t*-test. *P*-values of <0.05 were considered statistically significant.

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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