The Androgen Receptor Antagonises Wnt/β-Catenin Signalling in Epidermal Stem Cells OPEN

Kai Kretzschmar, Denny L Cottle, Pawel J Schweiger, Fiona M Watt

Cite this article as: Kai Kretzschmar, Denny L Cottle, Pawel J Schweiger, Fiona M Watt, The Androgen Receptor Antagonises Wnt/β-Catenin Signalling in Epidermal Stem Cells, *Journal of Investigative Dermatology* accepted article preview 29 June 2015; doi: 10.1038/jid.2015.242.

Received 17 February 2015; revised 16 May 2015; accepted 1 June 2015; Accepted article preview online 29 June 2015

The androgen receptor antagonises Wnt/β-catenin signalling in epidermal stem cells

Kai Kretzschmar[1,2,6], Denny L. Cottle[2,3], Pawel J. Schweiger[2,4,5] and Fiona M. Watt[1*]

[1]Centre for Stem Cells and Regenerative Medicine, King's College London, UK

[2]Wellcome Trust-Medical Research Council Stem Cell Institute, University of Cambridge, UK

[3]Department of Biochemistry and Molecular Biology, Monash University, Australia

[4]Cancer Research UK Cambridge Research Institute, University of Cambridge, UK

[5]Present address: Biotech Research and Innovation Center (BRIC), University of Copenhagen, Denmark

[6]Present address: Hubrecht Institute-Royal Netherlands Academy of Arts and Sciences (KNAW) and University Medical Centre (UMC) Utrecht, The Netherlands

*Corresponding author:

King's College London Centre for Stem Cells and Regenerative Medicine, 28th Floor, Tower Wing, Guy's Hospital Campus, Great Maze Pond, London SE1 9RT, Telephone: +44 (0)20 7188 5608, Email: fiona.watt@kcl.ac.uk

Short title: Androgen receptor modulates epidermal β-catenin signalling

Key words: hair follicles, androgen receptor, beta-catenin, stem cells, lineage selection

Abbreviations: androgen receptor, AR; dermal papilla, DP; hair follicle, HF; 4-hydroxytamoxifen, 4-OHT; interfollicular epidermis, IFE; sebaceous gland, SG

ABSTRACT

Activation of Wnt/β-catenin signalling 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 tumours and complete loss of SG identity. We conclude that AR signalling plays a key role in epidermal stem cell fate selection by modulating responses to β-catenin in adult mouse skin.

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) (Fuchs, 2009; Owens and Watt, 2003; 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 (Arwert *et al.*, 2012; Watt and Jensen, 2009)

Canonical Wnt signalling plays 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 signalling can be controlled by expressing a 4-hydroxytamoxifen (4-OHT) inducible form of stabilised 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), while repeated treatments result in expansion of the stem cell compartment and reprogramming of cells in the SG and IFE to form ectopic HFs (Lo Celso *et al.*, 2004; Silva-Vargas *et al.*, 2005; Van Mater *et al.*, 2003). The cells at the base of the SG are particularly sensitive to β-catenin activation while 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 crosstalk with other signalling pathways (Clevers and Nusse, 2012). One such partner is the androgen receptor (AR), which interacts with β-catenin in an androgen-dependent manner (Song *et al.*, 2003; Yang *et al.*, 2002). 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 (Itami and Inui, 2005; Miyake *et al.*, 1994; Randall *et al.*, 1993; Rosenfield, 2005; Rosenfield *et al.*, 1998). AR activation can promote MYC-induced SG differentiation (Cottle *et al.*, 2013) and inhibit hair growth (Crabtree *et al.*, 2010; Naito *et al.*, 2008). Regulation of androgen expression and AR signalling 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 signalling has been observed in co-cultures of human dermal papilla (DP) cells and HF stem cells isolated from healthy and AGA individuals (Leiros *et al.*, 2012). These observations led us to investigate whether the AR modulates the responsiveness of epidermal cells to β-catenin activation.

RESULTS

Cellular localisation of androgen receptor and β-catenin in cycling hair follicles

Although the AR is expressed in adult skin (Cottle et al., 2013), it is only active when localised to the cell nucleus (Azzi et al., 2006). AR usually localises 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, P2) the AR was localised 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 localisation (Bennett *et al*., 2010).

At P2, β-catenin was enriched in the nucleus of cells that had cytoplasmic AR (**Figure 1a**). In all other epidermal cells β-catenin was localised 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 localised in the nucleus of hair bulb cells adjacent to the DP, indicating active AR signalling, 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 localised to the cytoplasm (**Figure 1c**). Conversely, nuclear β-catenin was absent in the base of catagen HFs (**Figure 1d**), indicating down-regulation of canonical Wnt signalling. Here the AR was strongly localised to the nucleus, suggesting activated signalling. In telogen, β-catenin was only detectable at the cell membrane, while the AR was nuclear (**Figure 1e**). During all hair cycle stages AR was localised 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 localised to the cell membrane and cytoplasm during hair regression (catagen) or rest (telogen). Conversely, 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**) (Collins *et al.*, 2011). The epidermal cells were α6 integrin-positive, undifferentiated cells from IFE, SG and HF, while 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 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 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**). 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 to telogen (**Supplementary Figure S1b**), while AR target genes *Ppara*, *Fabp4*, *Abca1* and *Scp2* (Schirra *et al.*, 2005) were consistently downregulated under these conditions (**Supplementary Figure S1c**).

The changes in subcellular localisation of AR and β-catenin and the trend towards an inverse relationship between 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.

Androgen receptor modulates Wnt/β-catenin signalling 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), and 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). This suggested that cultured sebocytes would be a good model in which to examine whether in the epidermis, as in the prostate, AR negatively regulated Wnt signalling. We therefore transfected immortalised human sebocytes (Seb-E6E7 cells) (Cottle *et al.*, 2013; Lo Celso *et al.*, 2008) with the TOPFLASH Wnt activity reporter containing wild-type TCF binding sites, or with FOPFLASH 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 approximately 100-fold less potent than its active form, dihydroxytestosterone (DHT).

In Seb-E6E7 cells treated with the AR activator testosterone there was no significant decrease in luciferase reporter activity compared to the DMSO control. However, application of the anti-androgen bicalutamide caused a significant increase in luciferase activity, indicating that AR signalling negatively regulates endogenous Wnt/β-catenin activity (**Supplementary Figure S2**). 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*.

Androgen receptor modulates epidermal responses to β-catenin induced anagen and ectopic hair follicle formation

ΔK5ΔNβ-cateninER transgenic mice express a stabilised 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 analyse whether the intracellular localisation of AR was altered in this experimental setting, we stained back skin of 4-OHT treated ΔK5ΔNβ-cateninER transgenic mice with an antibody against the AR (**Supplementary Figure S3a**). Control (acetone only treated) tissue showed nuclear AR expression in all epidermal cells and all dermal fibroblasts, including DP cells (**Supplementary Figure S3a**). 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**). The AR was present in the cytoplasm of hair bulb cells, but remained nuclear in adjacent DP cells. Upon sustained epidermal stabilisation 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**).

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**). In K15ΔNβ-cateninER transgenic mice, in which stabilised β-catenin is specifically expressed in the HF bulge (Baker *et al.*, 2010), 4-OHT induced anagen resulted in AR localisation only in the cytoplasm of cells in the lower HF (**Supplementary Figure S3b**). Cells of the IFE and SG were unaffected and still had nuclear AR (**Supplementary Figure S3b**). 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 localised to the cytoplasm of all epidermal cells on 4-OHT treatment (**Supplementary Figure S3c**). Thus β-catenin induced anagen and ectopic HF formation correlated with re-localisation 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 localisation 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 signalling events. We treated ΔK5ΔNβ-cateninER transgenic mice daily with 4-OHT in combination with these compounds for up to 14 days (**Figure 2c and d**). Quantitative reverse transcription-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 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 (**Figures 2f, j, n and Supplementary Figures S3d** and **S4g-p**). The proportion of telogen HF was not statistically significant in acetone-treated skin compared to skin treated with 4-OHT and testosterone, which is consistent with the inhibitory effect of AR on β-catenin signalling (**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 Figures S4e and f**), 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 (**Figures 2h, 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 keratinised cells to form in the hair bulbs (**Figure 2l** and **Supplementary Figures S4a and b**). Full anagen induction was significantly reduced in skin treated with 4-OHT and bicalutamide compared to 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 (**Figures 2i, m, n** and **Supplementary Figures S4c and d**). All of the effects were observed in both male and female mice (e.g. **Supplementary Figure S4**).

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

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

Androgen receptor activation blocks β-catenin target gene expression and conversion of sebaceous glands into ectopic hair follicles

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 LEF1 (**Figure 3a**). Immunolocalisation 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**). 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**

Supplemental Figure S5c). 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 signalling. The increases in *Bmp4* and *Ptgs2* were not statistically significant, but both genes were significantly downregulated upon testosterone treatment (**Supplementary Figure S5c**). 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 antagonised by testosterone (**Supplementary Figure S5c**). The observation that testosterone increased *FlnA* expression in the presence of 4-OHT is consistent with the conclusion that AR signalling antagonised β-catenin signalling. Since 4-OHT treatment led to a major reduction in *FlnA*, it is not surprising that there was no further effect of bicalutamide.

CCAAT displacement protein (CDP) is expressed in the HF bulb during Wnt driven anagen (Silva-Vargas *et al.*, 2005) (**Figure 3c**). On bicalutamide treatment the expanded ORS 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 3e**). Staining for glycogen synthase kinase 3β (GSK3β), 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 to 4-OHT alone, suggesting that the activity of the β-catenin destruction complex was decreased.

Androgen receptor activation blocks conversion of sebaceous glands into ectopic hair follicles

In ΔK5ΔNβ-cateninER back skin ectopic HFs arise exclusively from the SGs and this correlates with loss of expression of the fatty acid synthase (FAS), encoded by *Fasn*, which is an AR target gene expressed in differentiating sebocytes (**Figure 4a, b**) (Cottle *et al.*, 2013). Treatment with bicalutamide and 4-OHT also led to loss of FAS and conversion of the SG to CDP-positive ectopic HFs (**Figure 4a-c, e**).

Conversely, 4-OHT and testosterone treated back skin from ΔK5ΔNβ-cateninER mice showed preservation of FAS-positive sebocytes and reduction in ectopic HFs (**Figure 4a-c, e**). QRT-PCR analysis confirmed that expression of *Fasn* and another sebocyte marker, *Fabp5,* was reduced in bicalutamide treated skin, indicating 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**), explaining the increase in *Fasn* expression in transgenic mice treated with 4-OHT only (**Figure 4d**). B lymphocyte-induced maturation protein 1 (BLIMP1), which is expressed by terminally differentiated keratinocytes in several epidermal compartments (Cottle *et al.*, 2013; Kretzschmar *et al.*, 2014), was no longer expressed in sebocytes following combined application of 4-OHT and bicalutamide (**Supplementary Figure S5b**).

Androgen receptor 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; Driskell *et al.*, 2012). Epidermal β-catenin activation in adult skin reprograms the dermis to a neonatal state characterised by increased fibroblast proliferation and density, and extracellular matrix (ECM) remodelling (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; Muller-Rover *et al.*, 2001). Sustained β-catenin activation in combination with AR inhibition led to an increase in platelet-derived growth factor receptor (PDGFR)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 dramatically increased around cysts induced by combined 4-OHT and bicalutamide treatment (**Figure 5b**). Next, we visualised mature (bright pink) and immature collagen (blue) using Herovici's method (**Figure 5c**). ECM remodelling occurred in the presence of bicalutamide but was inhibited by testosterone (**Figure 5c**). The DP of anagen HFs expressed Corin (**Figure 5e**) (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 H&E 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 back skin of transgenic mice treated with 4-OHT showed expression of the melanocyte marker tyrosinase-related protein 2 (TRP2) 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 stabilisation, but also regulates the effects of epidermal β-catenin activation on melanocytes and dermal fibroblasts (**Figure 5g**). 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 signalling 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 signalling 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, while β-catenin activation led to inhibition of AR activity, as evaluated by relocation of AR from nucleus to cytoplasm, which is associated with inhibition of AR signalling (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 localisation of AR and β-catenin during the hair cycle suggests that their interaction may be predominantly indirect. The downregulation of GSK3β on AR inhibition would, for example, affect β-catenin protein stability, while 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. Amongst negative regulators of Wnt/β-catenin signalling in hair follicle stem cells, microRNAs (miRs) such as miR-214 have been identified (Ahmed *et al.*, 2014). Interestingly, in the prostate of castrated rats miR-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 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 characterised by ECM remodelling and fibroblast proliferation (Collins *et al.*, 2011). All of these effects, both cell-autonomous and non-cell-autonomous, were inhibited by AR signalling.

Prolonged activation of β-catenin in the epidermis promotes the formation of benign HF tumours called trichofolliculomas (Gat *et al.*, 1998; Lo Celso *et al.*, 2004; Narhi *et al.*, 2008). The effect of sustained β-catenin stabilisation in the presence of AR inhibition was to cause defects in hair shaft formation and development of cysts resembling trichofolliculomas (**Figure 3d**) (Palmer *et al.*, 2008). Since 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 signalling 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, since CD44 has previously been identified as a component of tumour stroma that promotes tumour growth and spread (Edward *et al.*, 2005). Thus our findings may also provide insights into the formation of HF tumours and other Wnt-driven cancers.

In summary, we have identified the AR as an intracellular regulator of epidermal β-catenin signalling 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 signalling 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 hair follicles, 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 & 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 (Baker *et al.*, 2010; Lo Celso *et al.*, 2004). At the start of every experiment mice were 7 to 8 weeks old and in telogen (Stenn and Paus, 2001). The ΔNβ-cateninER transgene was activated by topical application of 4-OHT (Sigma) as described previously (Baker *et al.*, 2010). To modulate AR signalling, 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 Collins *et al.* (2011) and deposited under accession number GSE32966 on NCBI's Gene Expression Omnibus (GEO) website were analysed with GeneSpring GX11 (Agilent).

Human sebocyte culture, transfection and luciferase assays

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

RNA extraction and quantitative RT-PCR (qRT-PCR)

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

Histology, immunohistochemistry and imaging

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

Quantitation and statistical analysis

Quantitation of changes in the epidermis is described in the Supplementary Material. Statistical analysis was performed using the unpaired Student's *T* test. *p*-values of less than 0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank everyone who provided suggestions and advice. We acknowledge the technical assistance of the core facilities of the Wellcome Trust-Medical Research Council (MRC) Stem Cell Institute. This work was funded by the MRC, Wellcome Trust and the European Union Framework 7 programme. KK was the recipient of an MRC PhD studentship. PJS was the recipient of a Cancer Research UK PhD studentship. FMW gratefully acknowledges financial support from the Department of Health via the National Institute for Health Research comprehensive Biomedical Research Centre award to Guy's & St Thomas' National Health Service Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust.

REFERENCES

Ahmed MI, Alam M, Emelianov VU, *et al.* (2014) MicroRNA-214 controls skin and hair follicle development by modulating the activity of the Wnt pathway. *The Journal of cell biology* 207:549-67.

Arwert EN, Mentink RA, Driskell RR, *et al.* (2012) Upregulation of CD26 expression in epithelial cells and stromal cells during wound-induced skin tumour formation. *Oncogene* 31:992-1000.

Azzi L, El-Alfy M, Labrie F (2006) Gender differences and effects of sex steroids and dehydroepiandrosterone on androgen and oestrogen alpha receptors in mouse sebaceous glands. *The British journal of dermatology* 154:21-7.

Baker CM, Verstuyf A, Jensen KB, *et al.* (2010) Differential sensitivity of epidermal cell subpopulations to beta-catenin-induced ectopic hair follicle formation. *Dev Biol* 343:40-50.

Bennett NC, Gardiner RA, Hooper JD, Johnson DW, Gobe GC (2010) Molecular cell biology of androgen receptor signalling. *Int J Biochem Cell Biol.* 42:813-827.

Blanpain C, Horsley V, Fuchs E (2007) Epithelial stem cells: turning over new leaves. *Cell* 128:445-58.

Castoria G, D'Amato L, Ciociola A, *et al.* (2011) Androgen-induced cell migration: role of androgen receptor/filamin A association. *PloS one* 6:e17218.

Chesire DR, Isaacs WB (2002) Ligand-dependent inhibition of beta-catenin/TCF signaling by androgen receptor. *Oncogene* 21:8453-69.

Clevers H, Nusse R (2012) Wnt/beta-catenin signaling and disease. *Cell* 149:1192-205.

Collins CA, Kretzschmar K, Watt FM (2011) Reprogramming adult dermis to a neonatal state through epidermal activation of beta-catenin. *Development* 138:5189-99.

Cottle DL, Kretzschmar K, Schweiger PJ, *et al.* (2013) c-MYC-induced sebaceous gland differentiation is controlled by an androgen receptor/p53 axis. *Cell Rep* 3:427-41.

Crabtree JS, Kilbourne EJ, Peano BJ, *et al.* (2010) A mouse model of androgenetic alopecia. *Endocrinology* 151:2373-80.

Deschene ER, Myung P, Rompolas P, *et al.* (2014) beta-Catenin activation regulates tissue growth non-cell autonomously in the hair stem cell niche. *Science* 343:1353-6.

Driskell RR, Clavel C, Rendl M, *et al.* (2011) Hair follicle dermal papilla cells at a glance. *J Cell Sci* 124:1179-82.

Driskell RR, Giangreco A, Jensen KB, *et al.* (2009) Sox2-positive dermal papilla cells specify hair follicle type in mammalian epidermis. *Development* 136:2815-23.

Driskell RR, Juneja VR, Connelly JT, *et al.* (2012) Clonal growth of dermal papilla cells in hydrogels reveals intrinsic differences between Sox2-positive and -negative cells in vitro and in vivo. *J Invest Dermatol* 132:1084-93.

Edward M, Gillan C, Micha D, *et al.* (2005) Tumour regulation of fibroblast hyaluronan expression: a mechanism to facilitate tumour growth and invasion. *Carcinogenesis* 26:1215-23.

Fuchs E (2009) Finding one's niche in the skin. *Cell stem cell* 4:499-502.

Furr BJ, Tucker H (1996) The preclinical development of bicalutamide: pharmacodynamics and mechanism of action. *Urology* 47:13-25; discussion 9-32.

Gat U, DasGupta R, Degenstein L, *et al.* (1998) De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. *Cell* 95:605-14.

Hansen LS, Coggle JE, Wells J, *et al.* (1984) The influence of the hair cycle on the thickness of mouse skin. *The Anatomical record* 210:569-73.

He TC, Sparks AB, Rago C, *et al.* (1998) Identification of c-MYC as a target of the APC pathway. *Science* 281:1509-12.

Imperato-McGinley J, Gautier T, Cai LQ, *et al.* (1993) The androgen control of sebum production. Studies of subjects with dihydrotestosterone deficiency and complete androgen insensitivity. *J Clin Endocrinol Metab* 76:524-8.

Inui S, Itami S (2013) Androgen actions on the human hair follicle: perspectives. *Experimental dermatology* 22:168-71.

Itami S, Inui S (2005) Role of androgen in mesenchymal epithelial interactions in human hair follicle. *J Investig Dermatol Symp Proc* 10:209-11.

Kretzschmar K, Cottle DL, Donati G, *et al.* (2014) BLIMP1 is required for postnatal epidermal homeostasis but does not define a sebaceous gland progenitor under steady-state conditions. *Stem Cell Reports* 3:620-33.

Kretzschmar K, Watt FM (2014) Markers of epidermal stem cell subpopulations in adult mammalian skin. *Cold Spring Harb Perspect Med* 4.

Leiros GJ, Attorresi AI, Balana ME (2012) Hair follicle stem cell differentiation is inhibited through cross-talk between Wnt/beta-catenin and androgen signalling in dermal papilla cells from patients with androgenetic alopecia. *The British journal of dermatology* 166:1035-42.

Lo Celso C, Berta MA, Braun KM, *et al.* (2008) Characterization of bipotential epidermal progenitors derived from human sebaceous gland: contrasting roles of c-Myc and beta-catenin. *Stem Cells* 26:1241-52.

Lo Celso C, Prowse DM, Watt FM (2004) Transient activation of beta-catenin signalling in adult mouse epidermis is sufficient to induce new hair follicles but continuous activation is required to maintain hair follicle tumours. *Development* 131:1787-99.

Mangerini R, Argellati F, Pfeffer U, *et al.* (2012) Effects of bicalutamide and 4OH-tamoxifen on androgen-regulated gene expression in the LNCaP cell line. *Anticancer research* 32:5323-9.

Miyake K, Ciletti N, Liao S, *et al.* (1994) Androgen receptor expression in the preputial gland and its sebocytes. *J Invest Dermatol* 103:721-5.

Mooso BA, Vinall RL, Tepper CG, *et al.* (2012) Enhancing the effectiveness of androgen deprivation in prostate cancer by inducing Filamin A nuclear localization. *Endocrine-related cancer* 19:759-77.

Mulholland DJ, Read JT, Rennie PS, *et al.* (2003) Functional localization and competition between the androgen receptor and T-cell factor for nuclear beta-catenin: a means for inhibition of the Tcf signaling axis. *Oncogene* 22:5602-13.

Muller-Rover S, Handjiski B, van der Veen C, *et al.* (2001) A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. *J Invest Dermatol* 117:3-15.

Naito A, Sato T, Matsumoto T, *et al.* (2008) Dihydrotestosterone inhibits murine hair growth via the androgen receptor. *Br J Dermatol* 159:300-5.

Narayanan R, Jiang J, Gusev Y, *et al.* (2010) MicroRNAs are mediators of androgen action in prostate and muscle. *PloS one* 5:e13637.

Narhi K, Jarvinen E, Birchmeier W, *et al.* (2008) Sustained epithelial beta-catenin activity induces precocious hair development but disrupts hair follicle down-growth and hair shaft formation. *Development* 135:1019-28.

Niemann C, Owens DM, Hulsken J, *et al.* (2002) Expression of DeltaNLef1 in mouse epidermis results in differentiation of hair follicles into squamous epidermal cysts and formation of skin tumours. *Development* 129:95-109.

Nishimura EK, Jordan SA, Oshima H, *et al.* (2002) Dominant role of the niche in melanocyte stem-cell fate determination. *Nature* 416:854-60.

Nowak JA, Polak L, Pasolli HA, *et al.* (2008) Hair follicle stem cells are specified and function in early skin morphogenesis. *Cell stem cell* 3:33-43.

Owens DM, Watt FM (2003) Contribution of stem cells and differentiated cells to epidermal tumours. *Nat Rev Cancer* 3:444-51.

Palmer HG, Anjos-Afonso F, Carmeliet G, *et al.* (2008) The vitamin D receptor is a Wnt effector that controls hair follicle differentiation and specifies tumor type in adult epidermis. *PloS one* 3:e1483.

Paus R, Cotsarelis G (1999) The biology of hair follicles. *N Engl J Med* 341:491-7.

Pawlowski JE, Ertel JR, Allen MP, *et al.* (2002) Liganded androgen receptor interaction with beta-catenin: nuclear co-localization and modulation of transcriptional activity in neuronal cells. *J Biol Chem* 277:20702-10.

Rabbani P, Takeo M, Chou W, *et al.* (2011) Coordinated activation of Wnt in epithelial and melanocyte stem cells initiates pigmented hair regeneration. *Cell* 145:941-55.

Randall VA, Thornton MJ, Messenger AG, *et al.* (1993) Hormones and hair growth: variations in androgen receptor content of dermal papilla cells cultured from human and red deer (Cervus elaphus) hair follicles. *J Invest Dermatol* 101:114S-20S.

Robel P, Eychenne B, Blondeau JP, *et al.* (1983) Androgen receptors in rat and human prostate. *Hormone research* 18:28-36.

Rosenfield RL (2005) Hirsutism and the variable response of the pilosebaceous unit to androgen. *J Investig Dermatol Symp Proc* 10:205-8.

Rosenfield RL, Deplewski D, Kentsis A, *et al.* (1998) Mechanisms of androgen induction of sebocyte differentiation. *Dermatology* 196:43-6.

Schirra F, Suzuki T, Richards SM, *et al.* (2005) Androgen control of gene expression in the mouse meibomian gland. *Invest Ophthalmol Vis Sci* 46:3666-75.

Schneider MR (2012) Genetic mouse models for skin research: strategies and resources. *Genesis* 50:652-64.

Silva-Vargas V, Lo Celso C, Giangreco A, *et al.* (2005) Beta-catenin and Hedgehog signal strength can specify number and location of hair follicles in adult epidermis without recruitment of bulge stem cells. *Dev Cell* 9:121-31.

Song LN, Herrell R, Byers S, *et al.* (2003) Beta-catenin binds to the activation function 2 region of the androgen receptor and modulates the effects of the N-terminal domain and TIF2 on ligand-dependent transcription. *Mol Cell Biol* 23:1674-87.

Stenn KS, Paus R (2001) Controls of hair follicle cycling. *Physiol Rev* 81:449-94.

Terry S, Yang X, Chen MW, *et al.* (2006) Multifaceted interaction between the androgen and Wnt signaling pathways and the implication for prostate cancer. *J Cell Biochem* 99:402-10.

Van de Wetering M, Castrop J, Korinek V, *et al.* (1996) Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Mol Cell Biol* 16:745-52.

Van Mater D, Kolligs FT, Dlugosz AA, *et al.* (2003) Transient activation of beta -catenin signaling in cutaneous keratinocytes is sufficient to trigger the active growth phase of the hair cycle in mice. *Genes Dev* 17:1219-24.

Watt FM, Collins CA (2008) Role of beta-catenin in epidermal stem cell expansion, lineage selection, and cancer. *Cold Spring Harb Symp Quant Biol* 73:503-12.

Watt FM, Jensen KB (2009) Epidermal stem cell diversity and quiescence. *EMBO Mol Med* 1:260-7.

Whitfield J, Littlewood T, Evan GI, *et al.* (2015) The estrogen receptor fusion system in mouse models: a reversible switch. *Cold Spring Harbor protocols* 2015:pdb top069815.

Wielenga VJ, Smits R, Korinek V, *et al.* (1999) Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. *Am J Pathol* 154:515-23.

Yang J, Tan C, Darken RS, *et al.* (2002) Beta-catenin/Tcf-regulated transcription prior to the midblastula transition. *Development* 129:5743-52.

Zouboulis CC, Chen WC, Thornton MJ, *et al.* (2007) Sexual hormones in human skin. *Horm Metab Res* 39:85-95.

FIGURE LEGENDS

Figure 1. Reciprocal epidermal AR and β-catenin activity.

(**a-e**) Wild type mouse skin stained for AR (green) and β-catenin (red). Grey scale images of AR and β-catenin labelling are also shown. Adp, adipocytes; ANA, anagen; Bu, bulge; CAT, catagen; Der, dermis; DP, dermal papilla; ES, epithelial strand; GC, germ capsule; HC, hair canal; HG, hair germ; HS, hair shaft; IFE, interfollicular epidermis; IRS, inner root sheath; Me, medulla; Mx, matrix; ORS, outer root sheath; PCo, pre-cortex; pro, promoter; SG, sebaceous gland; TEL, telogen. Scale bar = 30 μm.

Figure 2. Androgen receptor 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-OHT labelled with antibodies to AR (green) and β-catenin (red). Arrowheads indicate nuclear β-catenin. (**d**) Treatment regime. (**e**) Relative expression (normalised to *Gapdh*) of *Ar* (left panel) and *Ctnnb1* (right panel) in whole back skin. Data are averages ± SEM from 3-4 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 haematoxylin 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. Asterisk denotes significant difference ($p<0.05$) between experimental groups as indicated and n.s. indicates non-significance. (**o**) Relative expression (normalised to *Gapdh*) of *Mki67* and *Pcna* in whole back skin. Data are averages ± SEM from 3-4 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 labelled with antibodies to

Ki67 (red in p-s), PCNA (red in t-w) and K14 (green) counterstained with DAPI to label nuclei (blue). Abbreviations: bic, bicalutamide; test, testosterone. Scale bars = 200 μm (f-m, p-s upper panels), 80 μm (c, p-s lower panels, t-w).

Figure 3. 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 labelled 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), GSK3β (red in e) and K14 (green), counterstained with DAPI (blue). (**f**) Relative expression (normalised to *Gapdh*) of *Sox9*, *Tcf7l2*, *Cux1*, *Cdh3*, *Axin2* and *Ccnd1* in whole back skin. Data are means ± SEM from 3-4 mice. Asterisks denote significant difference relative to 4-OHT alone ($p<0.05$). Scale bars = 200 μm (e) and 80 μm (a-d).

Figure 4. 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 H&E (a) or antibodies to FAS (red in b), CDP (red in c) and K14 (green in b and c), counterstained with DAPI (blue in b and c). (**d**) Relative expression (normalised to *Gapdh*) of *Fasn* and *Fabp5* in whole back skin. Data are averages ± SEM from 3-4 mice per condition. Asterisks denote significant difference relative to 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. Scale bars = 80 μm.

Figure 5. AR activity modulates of β-catenin effects on dermis and melanocytes.

(**a-f**) Back skin of ΔK5ΔNβ-cateninER mice treated for 14 days and stained with Herovici's method (c) or labelled 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 K14 (green

in a, b, d-f) counterstained with DAPI (blue in a, c-e). Arrowheads denote ectopic hair follicles (c) and regions magnified in the inserts (e). (**g, h**) Quantitation of dermal thickness (g) and hair bulbs with associated dermal papillae (h) after 14 days of 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). Scale bars = 200 μm (b, c, f); 100 μm (a, b) and 50 μm (e).