Wnt Signaling through the β-Catenin Pathway Is Sufficient to Maintain, but Not Restore, Anagen-Phase Characteristics of Dermal Papilla Cells

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Dermal papilla cells of the hair follicle can be maintained in an active, hair-inducing state *in vitro* when cocultured with cells secreting Wnt3a. By inducing cultured dermal papilla cells to secrete Wnt themselves, we demonstrate that this activity is a direct effect of Wnt signaling to dermal papilla cells. We further demonstrate that the effects of Wnt3a are exerted through activation of the β-catenin signal transduction pathway and do not require alternative Wnt transduction cascades. Once dermal papilla cells have lost hair-inducing properties *in vitro*, neither treatment with Wnt nor expression of a truncated and activating form of β-catenin is sufficient to restore these properties to the cultured cells.

The formation of the hair follicle depends on inductive signaling between its epithelial and mesenchymal components (Sengel, 1976). In the adult animal, the hair follicle undergoes cycles of regeneration and growth (anagen), degeneration (catagen), and quiescence (telogen) (Fuchs *et al*, 2001). Signaling between the epithelial and mesenchymal components is thought to play a critical role in this regeneration and growth process as well (Panteleyev *et al*, 2001). In an effort to understand the nature of those signals, a transgenic mouse that expresses green fluorescent protein (GFP) under the control of a fragment of the versican gene (*vers*-GFP) was generated so that dermal papilla (DP) cells could be isolated from the skin of newborn mice by fluorescence-activated cell sorting (Kishimoto *et al*, 1999). The inductive capacity of these cells was demonstrated in a skin reconstitution assay in which dissociated keratinocytes and dermal cells are grafted in a chamber on a nude mouse host (Kamimura *et al*, 1997). In this assay, the GFP-expressing cells have the ability to induce hair growth in a skin reconstitution assay, whereas the GFP-negative dermal population does not (Kishimoto *et al*, 1999). We have previously reported that these cells lose both the ability to induce hair growth and the expression of the GFP transgene when cultured *in vitro* in the absence of signals from the follicular epithelium (Kishimoto *et al*, 1999, 2000). Nevertheless, coculture with cells expressing Wnt3a, a secreted protein that is normally expressed by follicular epithelium, maintains both the hair inductive activity and the expression

of the GFP transgene in these cells for extended periods in culture (Kishimoto *et al*, 2000).

The Wnts are a family of secreted glycoproteins that exert their influence on responding cells by activating one or more of several signal transduction pathways within the cell (Slusarski *et al*, 1997; Nusse, 1999). Perhaps the best characterized of these is the β-catenin signal transduction cascade, which has been shown to play critical roles in follicle formation and regeneration (Andl *et al*, 2002; DasGupta and Fuchs, 1999; Noramly *et al*, 1999; Huelsken *et al*, 2001; DasGupta *et al*, 2002; Maretto *et al*, 2003; Van Mater *et al*, 2003). Although the details of the β-catenin signal transduction cascade are quite complex, a critical step in its activation is the inhibition of phosphorylation events on the N-terminal domain of β-catenin that normally target cytoplasmic β-catenin for degradation (Nusse, 1999). The stabilized protein can translocate to the nucleus and bind to members of the TCF family of DNA-binding proteins. The ability of this β-catenin/TCF complex to activate the transcription of adjacent genes depends on the C-terminal domain of β-catenin.

The activity of the β-catenin pathway in embryonic epidermis and subsequently in follicular epithelium is critical for follicle formation and regeneration (Huelsken *et al*, 2001; Andl *et al*, 2002). Transient activation of the pathway in this layer is sufficient to initiate the anagen phase (Van Mater *et al*, 2003). Nevertheless, there is some question about whether the β-catenin pathway is normally active in the DP of the anagen hair follicle, based largely on the dearth of reporter activity observed in DP cells of a transgenic line designed to detect transcriptional responses to activation of the β-catenin pathway (DasGupta and Fuchs, 1999). These observations suggest a model in which either Wnts act on the epithelial components of the follicle to generate

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secondary signals that act on the DP or Wnts act directly on the DP but by activating an alternative signal transduction cascade. The presence of feeder cells in the experiments demonstrating the ability of Wnt to maintain hair inductive activity in DP cells raised the possibility that Wnt might act on the feeder cells to generate a second signal that then acts on the DP cells. Thus the questions of whether Wnt act directly on DP cells and if so whether activation of the β-catenin signaling pathway is involved remained un-answered. Furthermore, analysis of the effects of Wnt on DP cells that had lost GFP expression in culture was complicated by the need to distinguish between this population and the feeder cells.

To address these issues, a second transgenic line was employed that expresses the tva receptor for type A avian retroviruses under the control of the *β-actin* promoter (*act*-tva) (Fisher *et al*, 1999). Expression of this transgene renders the mouse cells susceptible to infection by the RCASBP(A)-based avian retroviruses (Petropoulos and Hughes, 1991), although these vectors are replication-defective in mouse cells. This approach allowed the use of proven retroviruses to confer Wnt gene expression or β-catenin pathway activation in the absence of Wnt gene expression, on the purified populations derived from the bitransgenic *vers*-GFP;*act*-tva strain.

In this study we report that low-level expression of Wnt3a by a subset of cells in the enriched DP cell population is sufficient to maintain both GFP expression and hair-inductive activity. We further show that the effects of Wnt treatment result from activation of the β-catenin pathway in the responding cells. Expression of a stabilized form of β-catenin that activates the pathway in the absence of a Wnt signal is sufficient to maintain GFP expression in this population. Nevertheless, after GFP expression is lost from the cultured cells, neither exposure to Wnt3a nor expression of the stabilized β-catenin is sufficient to reactivate the GFP transgene whose expression correlates with the ability to induce hair follicle formation.

Results

These experiments employ a transgenic mouse line, *vers*-GFP, that expresses GFP in the DP under the control of a short segment of the human versican gene (Kishimoto *et al*, 1999). To simplify the manipulation of gene expression in the cells derived from these mice, this line was crossed to the *β-actin*-tva transgenic line so that the cells could be infected by the RCASBP(A) retroviruses (Fisher *et al*, 1999). Routine analysis of the *vers*-GFP;*act*-tva mice showed GFP expression in the DP, but extensive expression in the upper dermis and dermal sheath of the hair follicle as well (data not shown). Examination of the singly transgenic *vers*-GFP mice revealed an indistinguishable pattern (Fig 1). Independently maintained lines of the same strain show a similar profile and do not provide an alternative source of pure DP cells (data not shown). Thus although sorting for GFP expression enriches for DP cells, cells of the upper dermis that are not in hair follicles are likely to comprise a substantial fraction of the purified population employed in this study. Despite this difference from previous descrip-
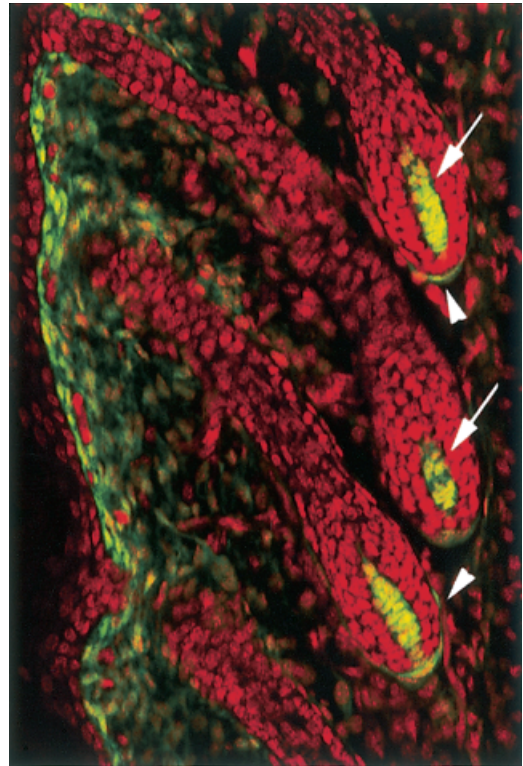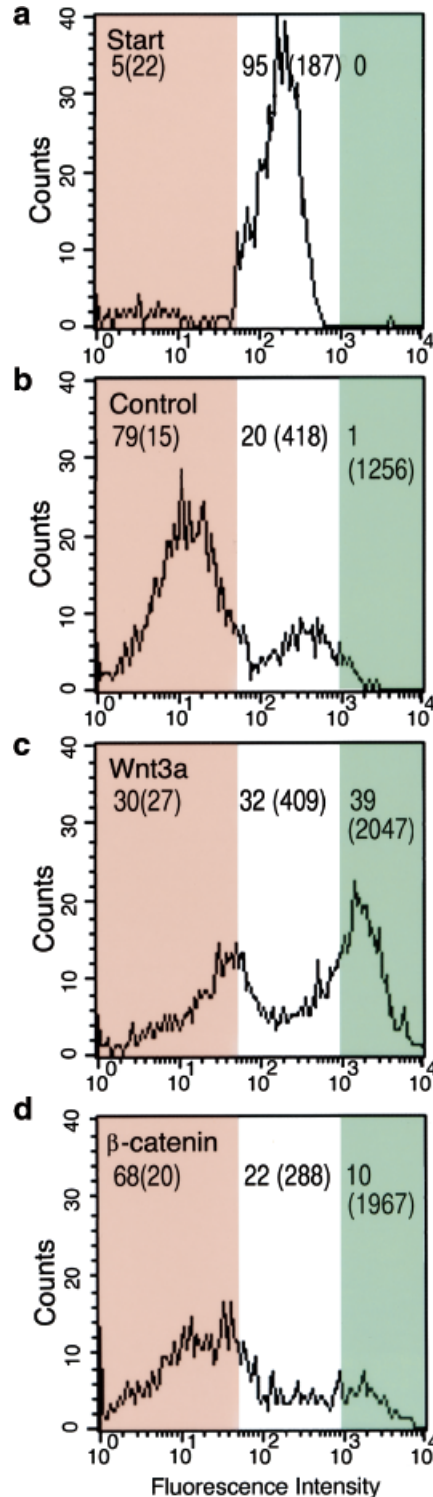

Figure 1
GFP expression in the skin of the *vers*-GFP mouse. GFP (*green*) is expressed in the DP (*arrow*), dermal sheath (*arrowhead*), and cells of the upper dermis in the skin of a *vers*-GFP transgenic mouse 3 d after birth.

tions, the biologic properties of this dermal/DP population purified on the basis of GFP expression, henceforth referred to as purified dermal (PD) cells, remain unchanged. The GFP-positive population has potent hair-inductive activity, whereas the GFP-negative population does not (data not shown). The expression of the tva receptor in these cells does not alter those properties (data not shown). For simplicity, the GFP-positive populations in the previous work will also be referred to as PD cells, although those populations may have been more highly enriched for DP cells.

PD cells maintained in culture lose GFP expression and hair-inductive activity over the course of several passages (Kishimoto *et al*, 1999). Nevertheless, PD cells cocultured with cells expressing Wnt3a or Wnt7a retain both GFP fluorescence and hair-inductive capacity (Kishimoto *et al*, 2000). To confirm that this was a direct effect of Wnt on the PD cells, pure PD cultures were infected with a retrovirus encoding Wnt3a at a MOI of 0.1 and maintained in culture (Fig 2). These cultures showed a rapid increase in the mean GFP fluorescence intensity per cell (data not shown), and a larger fraction of the cells maintained GFP expression for extended periods in culture when compared to uninfected cells or cells infected with a control retrovirus (Fig 2 and data not shown). For example, after 17 d in culture, 70% of the cells in the Wnt3a-infected culture continued to express GFP and the mean fluorescence intensity for the entire population was 926 (Fig 2*c*). In contrast, only 22% of the cells in the control culture were GFP-positive and the mean fluorescence for the entire population was 100 (Fig 2*b*). As

Figure 2

Effects of Wnt and β-catenin pathway activation on GFP expression in PD cells. (*a–d*) Histogram views of GFP fluorescence per cell. The number of cells with each intensity (Y axis) are shown as the function of the log of the measured fluorescence in the GFP channel (X axis). The percentage of the population found in the GFP-negative (less than 50 units of fluorescence shown in *red*), starting GFP-positive (between 50 and 1000 units shown in *white*), and the GFP-high (greater than 1000 units shown in *green*) populations are indicated, and the mean fluorescence intensity of each population is indicated in parentheses. (*a*) 95% of freshly sorted PD cells express levels of GFP between 50 and 1000 units. Essentially none of the freshly isolated cells show greater than 1000 units of fluorescence (*green zone*). (*b*) After 17 days of culture, only 22% of the control population remains GFP-positive/high. The remaining cells exhibit less than 50 units of GFP expression. Cells infected with retroviruses encoding Wnt3a (*c*) or truncated β-catenin (*d*) show elevated expression per cell (means averaged across the entire population of 926 and 268, respectively). These cultures also have increased numbers of cells that continue to express GFP after 17 d in culture (71 and 32%, respectively), including significant populations that exhibit more than 1000 units of fluorescence (*green zone*, 39 and 10%, respectively). (*e,f*) Immunohistochemical detection of infected cells. (*e*) The majority of GFP-expressing cells (*green*) in the cultures inoculated with the Wnt3a virus are not infected, as indicated by staining for a viral antigen (*red*). (*f*) In contrast, cells expressing high levels of GFP (*green*) also express viral proteins (*red*) as well in the cultures infected with the virus expressing a truncated form of β-catenin.

expected for the effects of a secreted protein, this activity was not cell autonomous. Both infected and uninfected cells continue to express GFP under this treatment (Fig 2e). Furthermore, over 39% of the Wnt-treated cells exhibited a fluorescence intensity of greater than 1000, whereas 1% or less of either the starting population or the control culture showed this high level of fluorescence (Fig 2a–c).

The Wnt-expressing PD cultures also retain the ability to induce follicle formation *in vivo* in the nude mouse graft assay. These cells consistently showed greater ability to induce hair growth than the control populations in four independent experiments (Fig 3a,b and data not shown). Although Wnt-treated cells performed well in this assay, freshly isolated PD cells that had not been cultured usually induced better hair growth than the cultured Wnt-treated cells (Fig 3a,c and data not shown).

The presence of a TCF-binding site in the versican-regulatory elements driving the expression of the GFP transgene suggested that the activity of Wnt3a is mediated by the β-catenin signal transduction pathway. Nevertheless,
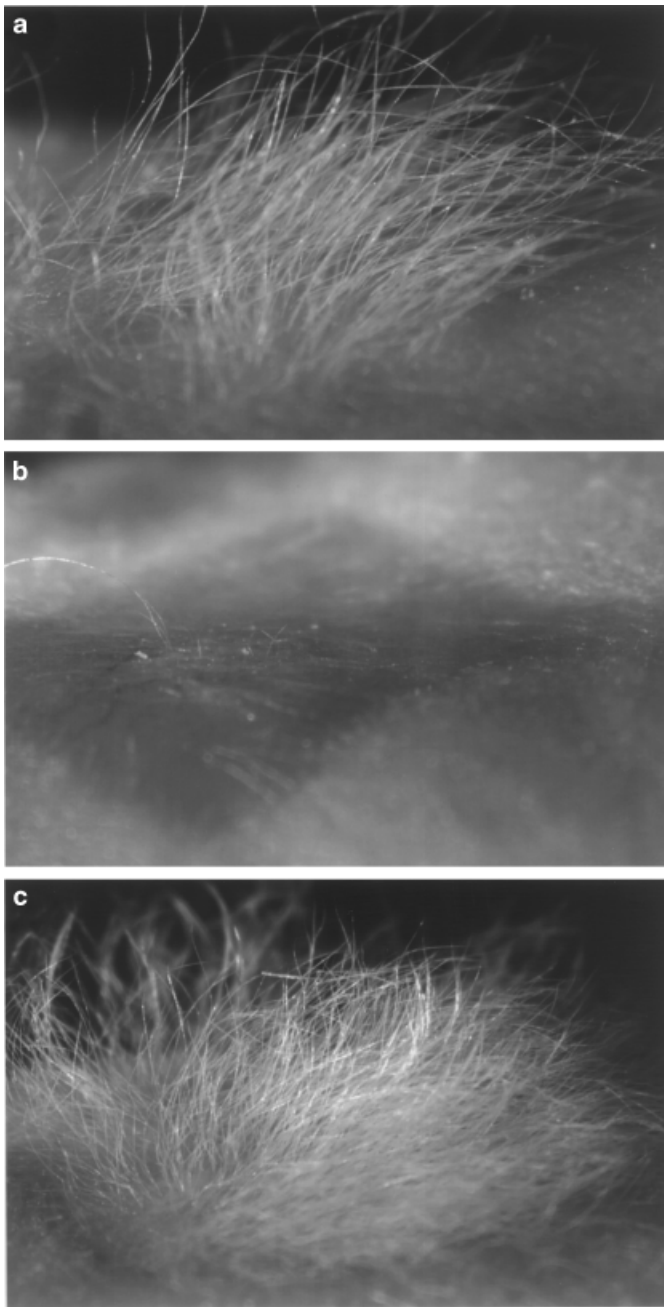
Figure 3
Forced expression of Wnt3a in PD cells *in vitro* maintains the ability to induce hair growth. Cultures in which GFP expression has been maintained by infection with the Wnt3a virus induce the formation of hair in the skin reconstitution assay (*a*), whereas corresponding cultures maintained in the absence of exogenous Wnt3a expression do not (*b*). Freshly isolated PD cells induce more robust hair growth in this assay (*c*).

Wnt signals are also transduced by other cascades whose independent or coordinate activity with the β-catenin pathway could be required to maintain PD cells in an active state. To test the sufficiency of activation of the β-catenin pathway, a retrovirus encoding a truncated form of β-catenin lacking the N- and C-terminal domains was employed (Capdevila *et al*, 1998). This truncated β-catenin construct activates the β-catenin cascade by stabilizing endogenous β-catenin proteins in the absence of an

extracellular Wnt signal (Noramly *et al*, 1999; DasGupta *et al*, 2002). Infection with this virus was sufficient to elevate and maintain GFP expression in the PD cells. After 17 d of culture, PD cells infected at an MOI of 0.2 showed a 2.7-fold increase in mean fluorescence intensity of the entire population when compared to controls and 32% of the cells remained GFP-positive. Ten percent of this population showed mean fluorescence levels above 1000 and accounted for the difference between the culture infected with the β-catenin virus and the control culture. The remaining 90% of the cells in this culture showed a mean fluorescence intensity and distribution between the GFP-negative and GFP-positive categories that was nearly indistinguishable (Fig 2*b,d* and data not shown). This observation suggested that the activity of the truncated β-catenin virus was cell autonomous. Immunohistochemical analysis confirmed that all intensely fluorescent cells were infected (50 of 50 scored), whereas uninfected cells exhibited at most low levels of GFP expression (Fig 2*f* and data not shown). This suggests that the activity exerted by Wnt3a is mediated by the β-catenin pathway and is direct. Activation of the pathway does not result in the generation of other paracrine signals that maintain activity in surrounding cells.

Although both Wnt3a and truncated β-catenin activated the β-catenin pathway and increased GFP expression, not all cells in the culture continued to express GFP. Productive infection and effective expression of the transgenes takes several days, during which some of the originally GFP-positive cells cease expressing GFP. These observations suggested that once GFP expression had been lost, neither Wnt3a exposure nor expression of the truncated β-catenin construct was sufficient to reactivate expression. To test the capacity of Wnt signaling to rejuvenate PD cells that had lost GFP expression, purified PD cells expressing GFP were cultured for 2 wk in the absence of Wnt3a and the GFP-negative population derived from them was purified by fluorescence-activated cell sorting. These GFP-negative PD cells were infected with the Wnt3a virus, the β-catenin virus, or a control. In freshly isolated, GFP-positive cells Wnt3a and β-catenin both caused an increase in mean fluorescence intensity of over twofold 5 d after infection (data not shown). Nevertheless, 5 d after infection of those PD cells that had lost GFP expression before infection, GFP expression was unaffected. Despite productive infection, less than 1% of the cells were GFP-positive in all cultures. Even when the MOI was increased threefold, no effect on GFP expression was observed. Thus cells that have lost GFP expression in culture also lose the ability to respond to Wnt3a by activating the β-catenin pathway and restoring the gene expression associated with freshly purified PD cells.

The fact that these cells fail to reactivate GFP expression in response to expression of truncated β-catenin (Fig 4) or exposure to LiCl, which activates the β-catenin pathway by inhibiting GSK3-β (data not shown), demonstrates that at least one block to GFP reactivation lies downstream of the stabilization of β-catenin. To explore this further, we examined the expression of the members of the Tcf/Lef family of DNA-binding proteins that β-catenin interacts with to activate the transcription of adjacent genes. RT PCR analysis of the freshly isolated PD population showed
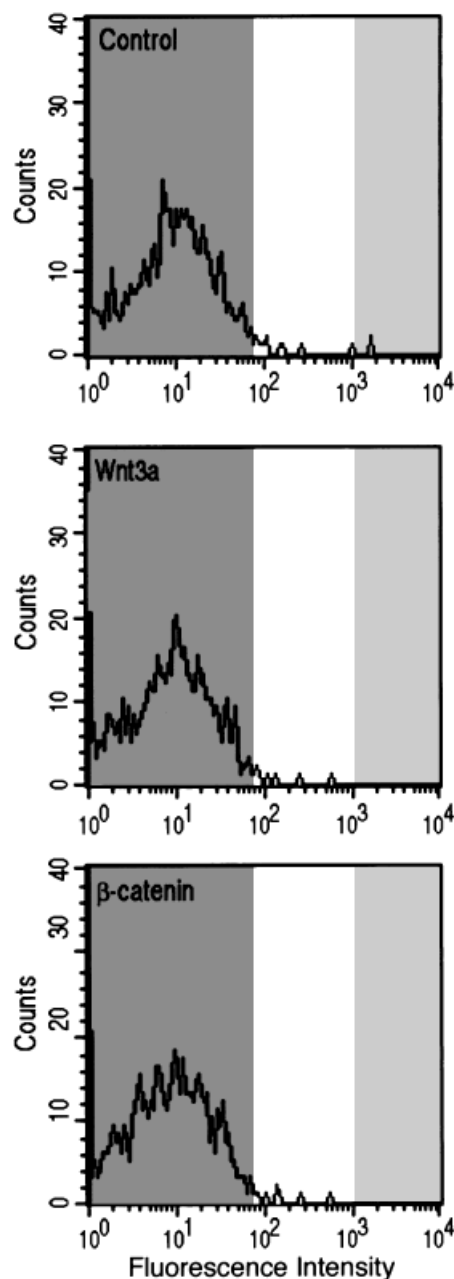
Figure 4
Infection with a retrovirus encoding either Wnt3a or truncated β-catenin does not restore GFP expression to initially GFP-positive PD cells that have lost that expression in culture. Histogram views of GFP fluorescence per cell as described in the legend to Fig. 2. Control (*a*), Wnt3a infected (MOI, 0.25) (*b*), and β-catenin infected (MOI, 0.5) (*c*) show indistinguishable profiles of GFP expression.

Figure 5
Expression of Tcf family members in fresh and cultured PD cells. Semiquantitative RT PCR analysis of Tcf-1, Tcf-3, Tcf-4, and Lef-1 transcript levels in RNA isolated from freshly isolated GFP-positive cells (*F +*) and from cells derived from them that have lost GFP expression *in vitro* (*C–*). Bands corresponding to alternative splice forms of TCF-1 (423 and 330 bp) are observed in freshly isolated cells, as are the single expected bands of 323 and 428 bp for Tcf-3 and Lef-1, respectively. Three expected isoforms of Tcf-4 (569, 557, and 500 bp) are observed although the 569- and 557-bp bands are not resolved on this gel. β-Actin serves as a control for input cDNA levels.

strong expression of Tcf-4, moderate levels of Lef-1 and Tcf-1, and lower levels of Tcf-3 mRNA (Fig 5). Transcripts of Tcf-1, Tcf-3, and Lef-1 were reduced below the level of detection in cells that had lost GFP expression, whereas Tcf-4 levels were substantially reduced but still readily detected under these conditions (Fig 5).

Discussion

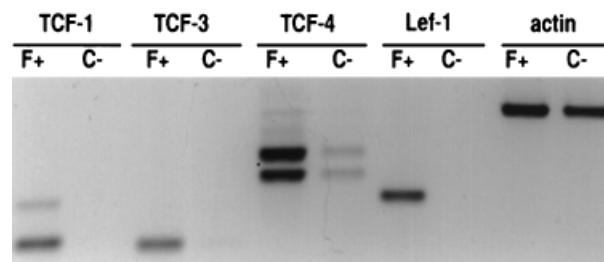The results reported here confirm that Wnt 3a can maintain the hair-inductive properties of DP cells when they are cultured *in vitro* and demonstrate Wnt3a acts directly on the purified population. This purified population contains cells from the upper dermis as well as DP cells, and we cannot conclude that Wnt3a acts directly on DP cells from these experiments alone. Nevertheless, activation of the β-catenin pathway within the cells is sufficient to mediate the effect of Wnt on GFP expression and does so in a cell-autonomous fashion. Previous work has shown that the ability to induce hair growth segregates with GFP expression in these cultured cells (Kishimoto *et al*, 1999). In aggregate, these observations strongly suggest that Wnts act directly on the DP cells to activate the β-catenin pathway and maintain them in the inductive state.

Nevertheless, when cultured PD cells have lost expression of the GFP transgene whose expression correlates with the ability to induce hair, exposure to Wnt3a or expression of a truncated β-catenin construct are insufficient to restore GFP expression. These findings are significant for several reasons.

Wnt signaling in the follicle *in vivo* Although the presence of a TCF-binding site in the transgene construct suggested that the activity of Wnt might be mediated by the β-catenin pathway (Kishimoto *et al*, 2000), these results demonstrate that activation of the pathway is sufficient to maintain transgene expression in the DP. There had been some question about whether this pathway is normally active in the DP during the anagen phase of the hair cycle, in part because expression of lacZ was not readily detected in the DP of a TOPGAL transgenic mouse that expresses lacZ in response to activation of the β-catenin pathway because the gene is driven by a minimal promoter flanked by TCF-binding sites (DasGupta and Fuchs, 1999). Nevertheless, analysis of an analogous transgenic strain, BAT-gal, which also expresses lacZ under the control of a minimal promoter flanked by TCF-binding sites, has subsequently revealed clear evidence of β-catenin pathway activation in the DP *in vivo* (Maretto *et al*, 2003). In conjunction with our findings, these data suggest that activation of the β-catenin pathway in the papilla is critical for the maintenance of the

inductive properties required for the regeneration of the follicle and growth of the hair shaft.

Expansion of active DP cells *in vitro* The ability of engrafted DP cells to promote follicle neogenesis, or augment the growth of existing follicles, has made them the focus of strategies to promote hair growth. That strategy has been impeded by the difficulty of obtaining sufficient numbers of cells that exhibit these inductive properties. Our previous work had suggested that purified DP cells could be expanded in culture in the presence of Wnt3a to generate populations of DP cells suitable for autograft therapy. These findings extend that observation by demonstrating that Wnt may exert that effect directly on DP cells and by demonstrating that activation of the β-catenin pathway is Wnts-sufficient to achieve that end. Because Wnt both are difficult to obtain in large quantities and might have undesirable effects if carried along in an autograft procedure, the use of drugs designed to directly impact the β-catenin signaling pathway will likely prove a preferable approach to maintaining DP cell activity *in vitro*.

Signal integration during the hair cycle These results also demonstrate that Wnt/β-catenin signaling is sufficient to maintain these activities *in vitro*, but not to restore them to cells that have lost them. The loss of the ability to respond to Wnt, as assessed by the failure to activate the GFP reporter, may also have implications for signal integration during the hair cycle. Our work on the formation of the follicle during embryogenesis suggests that regulating the capacity to respond to Wnt is thought to play a key role in follicle formation (B.A. Morgan, unpublished). Increased expression of the Tcf family member Lef-1 is normally associated with formation of the epidermal placode, and recent work suggests that additional inductive signals from follicular mesenchyme may be required to render epithelial cells competent to activate the β-catenin signal transduction cascade in response to Wnt (Jamora *et al*, 2003). The work reported here demonstrates that the ability to respond to Wnt is compromised in the cultured DP cells. Changes in Tcf family member gene expression may explain this loss in Wnt responsiveness, although other changes in the expression of Wnt signal transduction components may also be involved. We speculate that this change may reflect a normal course of events during the hair cycle and that loss of the ability to respond to Wnt may be part of the process by which DP cell gene expression is altered during the transition to the catagen and telogen phases. By understanding the mechanisms by which this ability is lost, and the signals required to restore it, we will come closer to understanding the interplay between follicular epithelium and DP during the hair cycle.

Materials and Methods

Mouse strains Heterozygous *vers*-GFP mice maintained on an FVB background were bred with homozygous *act*-tva females, and *vers*-GFP-positive skins were identified using a Leica fluorescence dissecting scope. GFP-positive cells were purified as described previously (Kishimoto *et al*, 1999).

Hair induction assay The graft assay was performed essentially as described (Kishimoto *et al*, 1999). All experiments included a positive control of freshly isolated GFP-positive cells plus keratinocytes. For analysis of Wnt-treated cells, freshly isolated GFP-positive cells were split into two populations and were infected at an multiplicity of infection (MOI) of 0.1 with either RCASBP(A)Wnt3a (Kengaku *et al*, 1998) or a control virus. They were cultured for four passages (12–17 days) before grafting. Four separate experiments were performed employing between 2 and 5 million cells per graft. All experiments gave qualitatively similar results; the example shown employed 5 million cells per graft. For reconfirmation of the preferential ability of GFP-positive cells to induce hair growth, GFP-negative cells were plated for 1 d to identify viable cells because of their lower plating efficiency. Negative cells were resorted and 5×10^5 cells were grafted. One million GFP-positive cells were used as the positive control to compensate for the lower viability (approximately 50%) of freshly isolated cells. All procedures were approved by the Institutional Review Board of Massachusetts General Hospital.

Analysis of GFP expression Skin was harvested from the middorsum of postnatal day 3 mouse pups, fixed overnight at 4°C in 4% paraformaldehyde-PBS, pH 7.0, dehydrated through graded sucrose, embedded in OCT, and frozen over liquid nitrogen. Seven-micrometer cryosections were stained with anti-GFP (Upstate Biotechnology, Lake Placid, NY) followed by secondary detection with TRITC-conjugated anti-rabbit (The Jackson Laboratory, Bar Harbor, ME) and stained with TOPRO 3 (Molecular Probes, Eugene, OR). Images were collected on a Leica confocal microscope with sequential scanning of the TOPRO3/GFP and TRITC channels. GFP fluorescence patterns were indistinguishable from immunohistochemical detection of GFP.

For purified cells, GFP expression levels were quantified using a FACScan and CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Mean fluorescence intensity was calculated from the gated population that excluded events with a forward scatter below 120 that were deemed to be debris. A minimum of 5000 cells were analyzed from each treatment.

Retrovirus preparation and infection RCASBP(A) replication-competent retroviruses encoding mouse Wnt3a (Kengaku *et al*, 1998), a truncated β-catenin construct (Capdevila *et al*, 1998), or no insert were prepared as described (Morgan and Fekete, 1996), and titer was determined on DF1 cells. These titers were used to estimate the MOI. Actual infection efficiency on *vers*-gfp;*act*-tva mouse cells was determined by immunohistochemical detection of viral protein using an anti-p27 antisera (SPAFAS) diluted 1:1800 followed by secondary detection with a TRITC-conjugated anti-rabbit IgG diluted 1:250 (The Jackson Laboratory).

RT-PCR total RNA was isolated from fresh GFP-positive cells or cultured, resorted GFP-negative cells derived from them using Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using Superscript first-strand synthesis system (Invitrogen). Semiquantitative RT-PCR was performed with 30 cycles of 94°C for 30 s, 61°C for 60 s, and 72°C for 45 s. β-Actin was used as the internal control using 22 cycles. The primers used are listed below. Parentheses indicate the amplified segment of the indicated GenBank file and the expected size of the products. The sizes of expected alternatively spliced isoforms of Tcf-1 and Tcf-4 are indicated: mouse Tcf-1 5′-CCA GCT TTC TCC ACT CTA CGA-3′ and 5′-TTC TTA GCC TCC TTC TCT GCC-3′ (nucleotides (nt) 321–743 of NM_009331, 423 bp; alternative splicing isoform, 330 bp; van de Wetering *et al*, 1996); mouse Tcf-3 5′-GGA TGA GGT CAA GTC GTC CCT GG-3′ and 5′-GCG ACC TTG TGT CCT TGA CTG TA-3′ (nt 181–503 of NM_009332, 323 bp); mouse Tcf-4 5′-GTT TGG AAG AAG CGG CCA AGA GG-3′ and 5′-GAT TGG GTA CAC AGG CTG ACC TT-3′ (nt 521–1020 of AF107298, 500 bp; alternative splicing isoforms, 557 and 569 bp; Douglas *et al*, 2001); mouse Lef-1 5′-GAG ATC AGT CAT CCC GAA GAG G-3′ and 5′-GGA CAT GCC TTG CTT GGA GTT G-3′ (nt 1101–1538 of

NM_010703, 438 bp); and β-actin as described previously (Kishimoto *et al*, 2000).

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