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# P-cadherin selectively stimulates hair follicle melanogenesis

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**Running title**

P-cadherin regulates hair pigmentation

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**Abstract**

P-cadherin serves as a major topobiological cue in mammalian epithelium. In human hair follicles (HFs), it is prominently expressed in the inner hair matrix that harbors the HF pigmentary unit. However, the role of P-cadherin in normal human pigmentation remains unknown. Since patients with mutations in the gene that encodes P-cadherin show hypotrichosis and fair hair, we explored the hypothesis that P-cadherin may control HF pigmentation. When P-cadherin was silenced in melanogenically active organ-cultured human scalp HFs, this significantly reduced HF melanogenesis and tyrosinase activity as well as gene and/or protein expression of gp100, stem cell factor, c-Kit, and microphthalmia-associated transcription factor (MITF), both *in situ and* in isolated human HF melanocytes. Instead, epidermal pigmentation was unaffected by P-cadherin knockdown in organ-cultured human skin. In hair matrix keratinocytes, P-cadherin silencing reduced plasma membrane β-catenin, while glycogen synthase kinase 3 beta (GSK3β) and phospho-β-catenin expression were significantly upregulated. This suggests that P-cadherin-GSK3β/Wnt signaling is required for maintaining expression of MITF to sustain intrafollicular melanogenesis. Thus, P-cadherin-mediated signaling is a novel, melanocyte subtype-specific topobiological regulator of normal human pigmentation, possibly via GSK3β-mediated canonical Wnt signaling.

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**Introduction**

Like other intercellular adhesion molecules, P-cadherin serves as an important topobiological tissue cue (Edelman, 1989; Engler *et al.*, 2009), that regulates cell-cell recognition and signaling, namely during epithelial morphogenesis, hair growth and tumor development (Bauer and Bosserhoff, 2006; Borghi and James Nelson, 2009; Edelman, 1989; Fanelli *et al.*, 2008; Goodwin and Yap, 2004; Hirai *et al.*, 1989; Paredes *et al.*, 2007; Taneyhill, 2008; Van Marck *et al.*, 2005). As a transmembrane glycoprotein composed of 5 extracellular domains that promote calcium ion-dependent cell-cell adhesion of adjacent keratinocytes P-cadherin forms a major component of adherens junctions (Fanelli *et al.*, 2008; Nagafuchi *et al.*, 1987; Takeichi, 1991; Takeichi *et al.*, 1988). Selective P-cadherin expression thus connects defined epidermal and hair follicle (HF) keratinocyte populations into functional epithelial units (Horiguchi *et al.*, 1994; Muller-Rover *et al.*, 1999; Shimoyama *et al.*, 1989a; Shimoyama *et al.*, 1989b).

The intracellular domain of P-cadherin interacts with p-120 catenin and β-catenin, which through α-catenin, anchors the adherens junctions to the actin cytoskeleton (Fanelli *et al.*, 2008; Furukawa *et al.*, 1997). Since cadherin-mediated cell adhesion and the canonical Wnt signaling depend on the same pool of β-catenin, P-cadherin may influence the canonical Wnt signaling pathway (for details see **Supplementary Text S1**) which is involved in many developmental processes (Dann *et al.*, 2001; Fanelli *et al.*, 2008; Linker *et al.*, 2005; Nelson and Nusse, 2004) including the development of both, normal melanocytes and malignant melanoma (Bellei *et al.*, 2010; Schepsky *et al.*, 2006; Takeda *et al.*, 2000; Yamaguchi *et al.*, 2009).

While the key role of E-cadherin in melanocyte biology (Gruss and Herlyn, 2001; Haass *et al.*, 2004; Haass *et al.*, 2005; Kuphal and Bosserhoff, 2006, 2011) and a role for P-cadherin in melanoblast development and melanoma progression are recognized (Bauer and Bosserhoff, 2006; Van Marck *et al.*, 2005), the functions of P-cadherin in adult human physiology remain poorly characterized (Cavallaro and Dejana, 2011). Interestingly, in humans and mice, P-

cadherin is prominently expressed in that part of the HF epithelium which harbors the HF pigmentary unit (Muller-Rover *et al.*, 1999; Samuelov *et al.*, 2012). Moreover, P-cadherin regulates HF morphogenesis in mice (Hirai *et al.*, 1989; Shimomura *et al.*, 2008). Most recently, we have shown that P-cadherin silencing inhibits hair fiber production and promotes HF regression (catagen induction) in human organ-cultured human HFs (Samuelov *et al.*, 2012). This corresponds well to the fact that mutations in the *CDH3* gene, which encodes for the P-cadherin protein, are associated with two distinct autosomal recessive disorders: hypotrichosis with juvenile macular dystrophy (*HJMD*;OMIM 601553) and ectodermal dysplasia, ectrodactyly and macular dystrophy (*EEM*;OMIM 225280) (Shimomura *et al.*, 2008). These patients display sparse and short hair (Bergman *et al.*, 2004; Shimomura *et al.*, 2008; Sprecher *et al.*, 2001).

Although hair pigmentation abnormalities have not been previously reported as part of the defining phenotype of HJMD patients, a retrospective clinical analysis of a large series of HJMD patients revealed that most of these patients, compared with their healthy siblings, display unusually fair hair, despite their dark pigmentation ethnic background (Bergman *et al.*, 2004; Indelman *et al.*, 2003; Shimomura *et al.*, 2008) (Figure 1a). Given that cadherins play a key role in the development of the neural crest (Pla *et al.*, 2001; Taneyhill, 2008), from where melanocytes arise and travel into precisely defined epithelial compartments of mammalian skin and its appendages (Peters *et al.*, 2002; Plonka *et al.*, 2009; Tobin, 2011) we speculated that the hair hypopigmentation phenomenon seen in HJMD patients may reflect an important, hitherto unappreciated role of P-cadherin in normal human HF pigmentation. Also, P-cadherin knockdown had not only inhibited human hair growth, but also had reduced human HF pigmentation in organ culture (Samuelov *et al.*, 2012). While this could have resulted from the expected catagen-associated switch-off of follicular melanogenesis (Kloepper *et al.*, 2010; Slominski *et al.*, 2005; Tobin, 2011), P-cadherin might also have exerted hair cycle-independent effects on human melanocytes.

This hypothesis was probed by assessing the HF pigmentary unit (HFPU) of normal, melanogenically active human HFs in which P-cadherin had been knocked down in organ culture (Samuelov *et al.*, 2012). These gene silencing studies in microdissected, organ-cultured adult human scalp HFs (Kloepper *et al.*, 2010; Philpott *et al.*, 1990) compared exclusively HFs that were in the melanin-producing anagen VI stage of the hair cycle (Schneider *et al.*, 2009; Tobin, 2011). These analyses were complemented by P-cadherin knockdown experiments in full-thickness human skin and in isolated, cultured human HF melanocytes.

**Results**

**Follicular melanin production is reduced in patients with mutated P-cadherin**

As shown by routine histochemistry, the precortical hair matrix and the hair shafts of one HJMD-affected child from whom a biopsy was available, were markedly less melanized, compared to normally pigmented scalp HFs from healthy donors (Figure 1a-b). In addition, some degree of melanin incontinence into the follicular dermal papilla (DP) was visible (Figure 1a).

Although these findings could not be quantitatively validated (due to the unavailability of good longitudinal HF sections from a larger number of HJMD patients – an extremely rare pediatric genodermatosis), they suggested that: a) the normal level of human HF pigmentation may be reduced, and b) melanosome transfer into the keratinocytes of the precortical hair matrix may be disturbed in the absence of functional P-cadherin.

**P-cadherin expression in human anagen hair follicles is mostly restricted to its pigmentary unit**

In murine and human HFs, P-cadherin expression is most prominent in the innermost layer of hair matrix keratinocytes (Furukawa *et al.*, 1997; Muller-Rover *et al.*, 1999), i.e. in the epithelial HF compartment which harbors the HFPU (Tobin, 2011). Since double-immunofluorescence of a HF melanocyte-specific marker, gp100 (Silver locus protein/NKI/beteb) which optimally visualizes human HFPU melanocytes (Kloepper *et al.*,

2008; Singh *et al.*, 2008; Tobin, 2011) with P-cadherin could not be successfully established, P-cadherin immunoreactivity (IR) was assessed on sequential hair matrix cryosections. This confirmed our previous findings that P-cadherin expression is most prominent in the human follicular hair matrix (Samuelov *et al.*, 2012) which houses the HFPU and is in immediate contact with the follicular DP (Figure 1c-d*).*

**P-cadherin knockdown reduces melanin production in human anagen VI hair follicles**

P-cadherin knockdown at the mRNA and protein level can be achieved by lipofectamine-mediated siRNA transfection of anagen VI HFs (control: scrambled oligos), which inhibits human hair growth and prematurely induces apoptosis-driven HF regression (catagen) (Samuelov *et al.*, 2012). While routine histology already had suggested that P-cadherin silencing may reduce HF pigmentation (Samuelov *et al.*, 2012), this may simply have reflected the normal catagen-associated switch-off pigmentation (Slominski et al., 2005). Therefore, this preliminary observation was systematically followed-up in the current study by comparing only (melanogenically fully active) anagen VI HFs between test and control groups.

Quantitative Masson-Fontana histochemistry revealed that P-cadherin silenced anagen VI HFs had a significantly reduced melanin content in the HFPU region, compared to scrambled oligos-treated control HFs (p <0.01). A single transfection for 5-7h sufficed to obtain this effect (Figure 1e,f,g), and became more pronounced when HFs were transfected twice with P-cadherin siRNA (data not shown) (as this repeated transfection caused too much HF damage, all subsequent experiments were performed with a single short-term transfection). The hair cycle stage of each hair follicle was determined by Ki-67 staining in order to demonstrate the proliferation activity of the HM keratinocytes below Auber's line, a marker of anagen stage (Kloepper *et al.*, 2010).

**P-cadherin silencing reduces tyrosinase activity and expression *in situ***

Since tyrosinase is the rate-limiting key enzyme of melanogenesis and drives intrafollicular, anagen-coupled melanin synthesis (Slominski *et al.*, 2005; Tobin, 2011), we next investigated

how the reduced HF melanin content of P-cadherin-silenced anagen VI HFs correlated with tyrosinase activity and gene expression *in situ*. These analyses revealed that P-cadherin silencing significantly reduced both, tyrosinase activity *in situ* (Figure 2a-c) and tyrosinase mRNA steady-state level, compared to controls (Figure 2d).

**P-cadherin silencing reduces follicular gp100 expression**

These pigmentary effects of P-cadherin silencing were further explored by assessing the IR of gp100 in anagen VI HFs, since the presence of gp100 structural protein is critical for the production and maturation of the fibrillar structures within the melanosomes (Hoashi *et al.*, 2010; Kawakami *et al.*, 2008; Singh *et al.*, 2008; Valencia *et al.*, 2006). Moreover, gp100 is further cleaved into several fragments and forms the fibrillar matrix of these organelles (Berson *et al.*, 2003; Kushimoto *et al.*, 2001; Yasumoto *et al.*, 2004). Gp100 plays a role in the maturation of stage II melanosomes. Shortly after its processing in stage II melanosomes, gp100 is further cleaved into several fragments which form the fibrillar matrix of the organelle (Berson *et al.*, 2003; Kushimoto *et al.*, 2001; Yasumoto *et al.*, 2004). The NKI/beteb antibody, which was used in the current study, recognizes cleaved gp100 when present in mature (i.e. stage III/VI) melanosomes (Singh *et al.*, 2008; Yasumoto *et al.*, 2004).

Indeed, P-cadherin silenced HFs showed significantly lower gp100 IR *in situ* compared to controls. This was seen both, when measuring total IR or the number of gp100+ cells in defined reference areas (Figure 2e-h). Also, by confocal microscopy, the number of dendrites per gp100+ cells as well as their length, in P-cadherin siRNA-treated HFs were significantly reduced compared to controls (Figure 2i-k). This did not result from increased HF melanocyte apoptosis (see **supplementary text S2** and Supplementary Figure S1). Moreover, the steady-state level of SILV mRNA, which encodes the gp100 protein (Du *et al.*, 2003), was significantly reduced in P-cadherin silenced HFs, as measured by quantitative qRT-PCR (Figure 2l).

Gp100 has been advocated as a marker for assessing melanosome transfer from melanocytes to adjacent keratinocytes (Singh *et al.*, 2010; Singh *et al.*, 2008). Therefore, we have followed-up the above gp100 data by transmission electron microscopy (TEM) of

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representative HF ultra-thin sections. This ultrastructural analysis showed that the HFPU of P-cadherin silenced anagen hair bulbs contained HF melanocytes that had accumulated an excessive amount of melanosomes; instead, only a very low number of melanosomes was seen to have been successfully transferred to adjacent hair matrix keratinocyets (Supplementary Figure S2). Apart from this phenomenon, however, the ultrastructural appearance and amount of melanocytes and melanosomes in test and control HFs were normal. This ultrastructural evidence raises the possibility that P-cadherin expression might be involved in orderly melanosome transfer within the human HFPU. However, this hypothesis would have to be further explored by formal melanosome tracking assays, which were outside of the scope of the current study.

**P-cadherin appears dispensable for human epidermal melanogenesis**

P-cadherin expression is also found in human epidermis, where it reportedly is expressed only in the basal layer, while E-cadherin is expressed throughout the epidermis (Fujita *et al.*, 1992; Furukawa *et al.*, 1997; Hirai *et al.*, 1989; Magerl *et al.*, 2001; Muller-Rover *et al.*, 1999). Recently, however, we found, that intraepidermal P-cadherin IR in human epidermis is more widespread than previously reported and actually predominates in the stratum spinosum and stratum granulosum of the epidermis (Samuelov *et al.*, 2012). In some individuals, P-cadherin IR was even *reduced* in the basal layer of human epidermis (Samuelov *et al.*, 2012) and thus does not correlate with the preferential basal/suprabasal location of epidermal melanocytes in human skin. Therefore, we next assessed whether P-cadherin expression is also required for normal human epidermis by P-cadherin knockdown in full-thickness adult human skin organ culture (Samuelov *et al.*, 2012).

Comparing P-cadherin siRNA- or scrambled oligo-transfected human skin punches (2 mm), no significant quantitative difference in terms of melanin content, gp100 IR and tyrosinase activity was seen between test and control samples (Supplementary Figure S3). Therefore, in human skin, the topobiological dependence of melanogenesis on adequate P-cadherin expression is melanocyte subpopulation-specific and appears to be restricted to melanocytes residing in that epithelial skin compartment where P-cadherin expression is most prominent

and where E-cadherin is reduced or even absent (Muller-Rover *et al.*, 1999): the innermost hair matrix.

**P-cadherin silencing inhibits melanogenesis, tyrosinase activity and microphthalmia-associated transcription factor expression in isolated human hair follicle melanocytes**

Since P-cadherin is expressed on both melanocytes and keratinocytes in the HFPU, we wanted to clarify whether the reduced pigmentation after P-cadherin silencing depends on melanocyte-keratinocyte interactions within the HFPU or whether P-cadherin silencing also inhibits melanogenesis in the absence of keratinocytes, i.e. in isolated, cultured human HF melanocytes (HFMs) *in vitro*. After having confirmed successful P-cadherin knockdown in isolated, cultured HFMs by P-cadherin immunostaining (Figure 3a-c*)* we found that P-cadherin silenced HFMs have a reduced melanin content and tyrosinase activity compared to controls (Figure 3d-k). MITF expression, strongly cytoplasmic and slightly intranuclear, was downregulated in isolated HFMs *in vitro* by P-cadherin knockdown (Figure 3l-n). No significant difference was found in gp100 expression between P-cadherin vs. scrambled-oligos-treated HFMs (data not shown). This suggests that the P-cadherin-dependence of normal intrafollicular melanogenesis reflects a direct P-cadherin effect on HF melanocytes.

**MITF, SCF, and c-Kit expression are down regulated by P-cadherin silencing**

As a first attempt at elucidating molecular mechanisms that may underlie the observed P-cadherin dependence of human HF pigmentation, the expression of the transcription factor, microphthalmia-associated transcription factor (MITF), widely acclaimed as the "master regulator" of pigmentation (McGill *et al.*, 2006; Nishimura *et al.*, 2005; Vachtenheim and Borovansky, 2010) was examined. Likewise, we assessed the levels of stem cell factor (SCF), a key growth factor that controls melanocyte survival and anagen-dependent melanogenesis in the HF (Botchkareva *et al.*, 2001; Slominski *et al.*, 2005; Tobin, 2011) and its high affinity receptor, c-Kit, in the innermost hair matrix.

This showed a significant downregulation of SCF, both at the protein and gene level by P-cadherin silencing (Figure 4a-d). Moreover, c-Kit IR was also reduced (Figure 4e-g). In

addition, the number of MITF+ cells around the DP (Figure 4h-j) and MITF steady-state transcript levels (Figure 4k) were significantly reduced in mRNA extracts from P-cadherin siRNA-treated HFs compared to scrambled oligos-treated anagen VI hair bulbs. Asmentioned, MITF was also strongly downregulated in isolated HFMs *in vitro* following P-cadherin silencing (Figure 3l-n). This suggests that the greatly reduced melanogenesis and disturbed melanosome transfer within the HFPU after P-cadherin silencing may result from a decline in MITF/SCF/c-Kit-mediated signaling.

**MITF down-regulation may result from inhibition of the canonical Wnt signaling by P-cadherin silencing**

Previously, we had shown that the hair growth-inhibitory effects of P-cadherin silencing are associated with an inhibition of canonical Wnt signaling (Samuelov *et al.*, 2012). Since MITF also is a well-known target gene of β-catenin/Wnt signaling (Bellei *et al.*, 2010; Takeda *et al.*, 2000), we hypothesized that the inhibition of melanogenesis by P-cadherin silencing may also result from changes in the cytoplasmic pool and stabilization of β-catenin, thus reducing the expression of β-catenin target genes. In fact, β-catenin IR was significantly down-regulated in the innermost hair matrix of P-cadherin silenced HFs. Instead, no significant differences in β-catenin IR were found in the outer root sheath (ORS), where P-cadherin is co-expressed with E-cadherin (Muller-Rover *et al.*, 1999) (Figure 5a-c). In contrast, phospho-β-catenin and glycogen synthase kinase 3 beta (GSK3β) were significantly increased in P-cadherin silenced HFs (Figure 5d-i). Therefore, the reduced MITF expression may result from intramelanocytic Wnt inhibition, followed by MITF down regulation, and ultimately by reduction of tyrosinase activity, gp100 expression and melanin synthesis.

To further correlate the reduced MITF expression with Wnt signaling inhibition, we cultured P-cadherin-silenced HFs with the potent, non-specific GSK3β inhibitor, lithium chloride (LiCl). As we had reported before, this completely reversed the effect of P-cadherin silencing on the transcription of the key β-catenin target gene, axin2 (Samuelov *et al.*, 2012). Interestingly, the MITF mRNA steady state-level was not reversed (and was even further downregulated) by LiCl-mediated GSK3β inhibition (Figure 5j), while phospho-β-catenin IR in anagen VI HFs was

normalized    by    LiCl    in    P-cadherin    silenced    HFs    (Supplementary    Figure    S4).

Proopiomelanocortin (POMC) transcription and alpha-melanocyte stimulating hormone (α-

MSH) and adrenocorticotropic hormone (ACTH) protein IR in situ were essentially unchanged

(Supplementary Figure S5). These observations suggest that P-cadherin knockdown inhibits

HF pigmentation primarily through the GSK3β/Wnt pathway, while POMC, α-MSH, and ACTH

(i.e. key neuroendocrine controls of human HF pigmentation (Paus, 2011; Slominski *et al.*,

2005)) appear to be unaffected.

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**Discussion**

Here, we provide the first evidence that a member of the cadherin family, P-cadherin, is important for the control of normal human hair pigmentation *in situ*. Using the HF as a physiologically and clinically relevant model system for testing normal adult human melanocyte functions within their natural tissue context (Paus, 2011; Tobin, 2011), we show that P-cadherin plays an essential regulatory role in the control of human HF melanogenesis. In contrast, human epidermal pigmentation *in situ* is independent of P-cadherin-mediated signaling. Such a strikingly differential dependence of melanocytes within the same organ (here: human skin) on one member of the cadherin family has not previously been reported. Thus, P-cadherin provides a crucial topobiological cue for HF melanocytes, as opposed to their epidermal counterparts. It deserves to be explored whether P-cadherin also is important in human retinal pigment epithelium (see **supplementary text S3**).

While P-cadherin has not previously been implicated in the biology of adult, differentiated human melanocytes *in vitro* or *in vivo*, the hair pigmentation phenotype of HJMD patients had encouraged the hypothesis that P-cadherin may control human HF melanocyte functions. Our findings verify this hypothesis, and raise the question how P-cadherin exerts its effects on mature human HF melanocytes *in situ* and *in vitro*.

Since classical melanogenesis-promoting neurohormones, which are prominently produced within the human HF epithelium (Paus, 2011; Slominski *et al.*, 2005; Tobin, 2011) appears to be unaffected by P-cadherin silencing, the Wnt pathway offers one reasonable candidate mechanism. Wnt signaling has already been implicated in the human hair *growth*-modulatory effects of P-cadherin (Samuelov *et al.*, 2012) and is up-stream of MITF (Bellei *et al.*, 2010; Takeda *et al.*, 2000). MITF controls the production of key melanogenic enzymes (Aksan and Goding, 1998; Bellei *et al.*, 2010; Bertolotto *et al.*, 1998a; Bertolotto *et al.*, 1998b; Yasumoto *et al.*, 1994) and melanosomal matrix proteins such as gp100 (Du *et al.*, 2003). Moreover, Wnt signaling is essential for neural crest and melanocyte development, and mice lacking Wnt1 and Wnt3a, which trigger the canonical pathway resulting in β-catenin target gene expression, suffer from pigmentary defects (Ikeya *et al.*, 1997). In contrast, inhibition of

canonical Wnt signaling downregulates melanocyte density and differentiation via reducing MITF expression (Yamaguchi *et al.*, 2007). Wnt/β-catenin signaling furthermore upregulates MITF (Bellei *et al.*, 2010; Takeda *et al.*, 2000) . Moreover, β-catenin functionally interacts with the MITF protein and then activates MITF-specific target genes (Schepsky *et al.*, 2006) (see supplementary Figure S6a).

Therefore, our data raise the possibility that P-cadherin knockdown reduces expression of MITF, the "master regulator" of melanogenesis (Vachtenheim and Borovansky, 2010), through increased β-catenin degradation and downregulation of cytoplasmic β-catenin available for Wnt signaling. As a consequence, melanogenesis, tyrosinase activity, gp100 production and melanosome transfer to keratinocytes are all inhibited (see hypothetical scenario summarized in Supplementary Figure S6b). Interestingly, non-specific GSK3β inhibition by LiCl upregulated transcription of the prototypic β-catenin target gene, Axin2 (Samuelov *et al.*, 2012), while the mRNA level of MITF remained low. This suggests that, besides the canonical Wnt signaling pathway, other GSK3β-related mechanisms may also contribute to regulating MITF at the mRNA level, e.g. SCF/c-Kit-mediated signaling (for discussion, see supplementary text S4) and LiCl treatment did not suffice to reverse the effect of P-cadherin silencing on MITF expression.

Since the control of MITF expression is rather complex and is also regulated by other signaling pathways besides GSK3β/Wnt (e.g. MAPK and CREB phosphorylation through SCF) (Nakajima *et al.*, 2012), this may explain the absence of MITF mRNA upregulation after inhibiting GSK3β. In contrast, the transcript levels of Axin2, a specific  β-catenin target gene (Lovatt and Bijlmakers, 2010), are restored after culturing P-cadherin-silenced HFs in the presence of a GSK3β inhibitor (Samuelov *et al.*, 2012). Attenuated MITF- and/or SCF/c-Kit signaling may also underlie the reduced HF melanocyte dendricity observed upon P-cadherin silencing (for discussion, see **supplementary text S5**).

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The melanocyte subpopulation-specific control of human pigmentation by a cadherin family member identified here not only opens a new frontier in general pigment and cell adhesion biology, but is also clinically relevant: it suggests novel strategies for the therapeutic modulation of human HF pigmentation (including hair graying) via targeting P-cadherin-mediated signaling. Our data also underscore that P-cadherin silencing in human HFs provides an intriguing preclinical research model for dissecting the as yet incompletely understood pathobiological consequences of loss of function-mutations of the *CDH3* gene in HJMD and EEM syndromes for human tissue pathology.

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**Materials and Methods**

**Tissue collection, organ culture, and P-cadherin knockdown**

The same human anagen VI HFs from five patients undergoing routine face-lift surgery whose

growth response to P-cadherin silencing we had previously examined (after ethics approval

and informed patient consent) (Samuelov *et al.*, 2012) were re-analyzed in the current study

for pigmentary phenomena. Since the current study constitutes a systematic re-analysis of the

same human HF and skin samples reported before in Samuelov et al. (2012), see this study

for details on tissue collection, organ culture, and P-cadherin knockdown. In the current study,

only anagen VI (melanogenically active) HFs were included in all the experiments.

**Hair follicle melanocyte culture**

HFMs cultures were established as described (Kauser *et al.*, 2005) (for details, see

supplementary data).

**Melanin histochemistry**

Quantitative melanin histochemistry by Masson–Fontana stain was performed as described

(Gaspar *et al.*, 2010) (for details see supplementary data).

**Immunohistochemistry (LSAB – Peroxidase)**

P-cadherin, β -catenin and phospho- β -catenin IR was detected by LSAB-peroxidase

immunohistochemistry as described in the supplementary data and (Samuelov *et al.*, 2012).

**Immunofluorescence microscopy and quantitative immunohistomorphometry**

Immunfluorescence stainings for β –catenin, phospho- β –catenin and GSK3β were

performed as described before (Samuelov *et al.*, 2012).

Immunfluorescence microscopy for SCF, gp100, c-Kit, MITF, ACTH, α-MSH and quantitative

immunohistomorphometry are described in the supplementary data.

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**Tyrosinase activity and melanocyte apoptosis *in situ***

The tyramide-based tyrosinase assay was used for measuring tyrosinase activity *in situ* as previously described (Han *et al.*, 2002; Kloepper *et al.*, 2010), while HF melanocyte apoptosis was examined by gp100/TUNEL double-immunofluorescence (see supplementary data).

**Histology**

Archival scalp skin sections of one 5 year old female HJMD patient were stained with hematoxylin and eosin (Samuelov *et al.*, 2012). Scalp skin biopsy had been obtained for diagnostic purposes after informed consent.

**Quantitative Real-Time PCR (qRT-PCR)**

qRT-PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems/Life Technologies, Foster City, CA, USA) using the 5' nuclease assay as described before (Bodo *et al.*, 2005; Dobrosi *et al.*, 2008; Toth *et al.*, 2009) (for primers and details, see supplementary data and (Samuelov *et al.*, 2012).

**Confocal microscopy and transmission electron microscopy**

Confocal microscopy and transmission electron microscopy were performed as described (Samuelov *et al.*, 2012).

**For methodological details, see supplementary information.**

**Conflict of interest**

The authors state no conflict of interest.

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**Figure legends**

**Figure 1. Follicular melanin production is reduced in patients with HJMD, P-cadherin expression in normal human anagen VI hair follicles overlaps the hair follicle pigmentary unit and reduces melanin content in P-cadherin silenced anagen VI hair follicles**

(a) Scalp skin biopsy obtained from HJMD patient. Hematoxylin Eosin (H&E) staining demonstrates a vellus hair follicle (HF) with no evidence of melanin content in the hair shaft (HS) and precortical hair matrix (HM) (magnification x400). The arrowhead points to melanin granules in the dermal papilla, indicating melanin incontinence.

(b) H&E staining of normally pigmented scalp HF of healthy human individual. The arrowheads indicate melanin in the HM and the HS.

(c,d) The pictures represent sequential sections of the same normal anagen VI HF. (c) Positive NKIbeteb (gp100) immunoeactivity (IR) in an anagen VI HF. (d) Positive P-cadherin IR in the innermost cell layer of matrix keratinocytes. Dotted lines represent the area of P-cadherin (d) and NKIbeteb (c) expression. Note the overlap expression of P-cadherin with the HF pigmentary unit. Since the P-cadherin antibody works only with highly sensitive immunohistochemistry method, double staining of NKIbeteb and P-cadherin could not be applied.

(e) The graph represents histomorphometic analysis of Masson-Fontana staining, which revealed significant downregulation of melanin content in P-cadherin siRNA-treated HFs compared to scrambled oligos-treated ones. Similar results were obtained after two transfections with P-cadherin siRNA vs. scrambled oligos (data not shown). Data was pooled from two highly comparable experiments. **p<0.01, n=12, *Mann Whitney test.*

(f,g) Note the reduced melanin content in P-cadherin siRNA-treated anagen VI HFs compared to scrambled oligos-treated HFs. Each Masson-Fontana staining picture is accompanied by the corresponding Ki-67 staining of the same HF in order to demonstrate the proliferation activity of the HM keratinocytes below Auber's line, a marker of anagen stage (Kloepper *et al.*, 2010). The dotted lines represent Auber's line.

DP – dermal papilla; HF – hair follicle; HJMD - hypotrichosis with juvenile macular dystrophy; HM – hair matrix; HS – hair shaft; IHM – innermost hair matrix; IR – immunoreactivity; ORS – outer root sheath; PCHM – precortical hair matrix sheath; RD – reticular dermis; SC – subcutaneous fat.

**Figure 2. P-cadherin knockdown downregulates tyrosinase activity and gp100 at the mRNA and protein level**

(a-c) Reduced tyrosinase activity In anagen VI P-cadherin siRNA-treated hair follicles (HFs), (b) compared to scrambled oligos-treated HFs (a). Dotted lines represent reference areas of measurements. Data was pooled from two highly comparable experiments in which HFs were transfected once with P-cadherin siRNA vs. scrambled oligos. The same results were achieved when HFs were transfected twice during the four day culture. $**p<0.01$, *Mann Whitney test,* n=10-13.

(d) mRNA level of TYR in P-cadherin siRNA vs. scrambled oligos treated HFs. $***p<0.001$, *Student's t test for unpaired samples.*

(e-f) Reduced gp100 immunoreactivity (IR) and number of gp100+ cells in P-cadherin siRNA-treated hair follicles (HFs) (f) compared to scrambled oligos-treated ones (e). White dotted lines represent reference areas of measurement. Yellow dotted lines represent the basement membrane.

(g) Histomorphometric analysis of gp100 IR. Data was pooled from two highly comparable experiments. $*p<0.05$, *Mann Whitney test,* n=13-16.

(h) Reduced gp100+ cells in the P-cadherin siRNA-treated HFs. Data was pooled from two highly comparable experiments. $*p<0.05$, *Mann Whitney test,* n=13-16.

(i-k) Reduced number of dendrites per gp100+ cell in P-cadherin siRNA-treated HFs (j) compared to scrambled oligos-treated ones (i), as was measured by confocal microscopy. Yellow dotted lines represent the basement membrane. Note the fewer and shorter dendrites in the P-cadherin silenced HFs compared to control (yellow arrow heads). Data was pooled from two highly comparable experiments. $**p<0.01$, *Mann Whitney test,* n=96-119.

(l) Reduced gp100 transcript level in the P-cadherin siRNA-treated HFs. $***p<0.001$, *Student's t test for unpaired samples.*

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In all mRNA results (d, I) transcript levels were compared to glyceraldehyde 3-phosphate dehydrogenase, peptidylprolyl isomerase A or β-actin.

DP – dermal papilla; HS – hair shaft; IHM – innermost hair matrix; IR – immunoreactivity.

**Figure 3. P-cadherin knockdown reduces melanogenesis, tyrosinase activity and MITF in isolated follicular melanocytes**

(a-c) Reduced P-cadherin expression in hair follicle melanocytes (HFMs) treated with P-cadherin siRNA (b) compared to HFMs treated with scrambled oligos (a). C represents negative control with retrieving the primary antibody.

(d-g) Reduced melanin content by Masson-Fontana staining in P-cadherin silenced HFMs (e) compared to HFMs treated with scrambled-oligos (d). F represents negative control without silver nitrate.

(h-k) P-cadherin silenced HFMs (i) demonstrate reduced tyrosinase activity compared to scrambled oligos-treated HFMs (h). Tyrosinase expression was also significantly reduced in the P-cadherin silenced HFMs (data not shown).

(l-n) Reduced microphthalmia-associated transcription factor expression in HFMs treated with P-cadherin siRNA compared to scrambled oligos-treated HFMs. N represents negative control with retrieving the primary antibody.

MITF - microphthalmia-associated transcription factor; P-cad. – P-cadherin; Scr. – scrambled.

**Figure 4. P-cadherin knockdown significantly downregulates stem cell factor, c-Kit and microphthalmia-associated transcription factor significantly**

(a-c) Reduced stem cell factor (SCF) expression in P-cadherin silenced HFs (b) compared to control (a). Data was pooled from two highly comparable experiments in which HFs were transfected once with P-cadherin siRNA vs. scrambled oligos. Dotted lines represent reference areas of measurements. White lines encircle the dermal papilla (DP). *** p<0.0001, *Mann Whitney test*, n=15-17.

(d) mRNA level of SCF in P-cadherin siRNA vs. scrambled oligos treated HFs. **p<0.01,
*Student's t test for unpaired samples.*

(e-g) Reduced c-Kit expression in P-cadherin silenced HFs (f) compared to control (e).Data
was pooled from two highly comparable experiments in which HFs were transfected once with
P-cadherin siRNA vs. scrambled oligos. Dotted lines represent reference areas of
measurements.  ** p<0.01, *Mann Whitney test*, n=11-14.

(h-j) Reduced number of microphthalmia-associated transcription factor (MITF) positive cells
around the DP of P-cadherin silenced HFs (i) compared to control (h). Data was pooled from
two highly comparable experiments in which HFs were transfected once with P-cadherin
siRNA vs. scrambled oligos. Arrow represents MITF+ cells around the DP.  **p<0.01, *Mann
Whitney test*, n=11-16.

(k) mRNA level of MITF. **p<0.01, *Student's t test for unpaired samples.*

In all mRNA results (d, k) transcript levels were compared to glyceraldehyde 3-phosphate
dehydrogenase, peptidylprolyl isomerase A or β-actin.

DP – dermal papilla; HS – hair shaft; IHM – innermost hair matrix; IR – immunoreactivity;
MITF - microphthalmia-associated transcription factor; SCF – stem cell factor; TYR –
tyrosinase.

**Figure 5. P-cadherin silencing in anagen hair follicles increases degradation of β-
catenin and reduces the expression of microphthalmia-associated transcription factor**

(a-c) Reduced β-catenin immunoreactivity (IR) in anagen hair follicles (HFs) after P-cadherin
siRNA treatment, specifically in the innermost hair matrix (IHM). No significant difference was
found in the outer root sheath. Data was pooled from two highly comparable experiments.
***p<0.0001, *Mann Whitney test*, n=14-22.

(d-f) Upregulation of glycogen synthase kinase 3 beta (GSK3β) IR in the innermost cell layer
of matrix keratinocytes after P-cadherin siRNA treatment. Data was pooled from two highly
comparable experiments. **p<0.01, *Mann Whitney test*, n=9-15.

(g-i) Increased phospho-β-catenin IR in anagen HFs after P-cadherin siRNA treatment, specifically in the IHM. Data was pooled from two highly comparable experiments. **p<0.01, *Mann Whitney test*, n=14-16.

(j) Microphthalmia-associated transcription factor (MITF) mRNA level is significantly downregulated in P-cadherin silenced HFs. Culturing P-cadherin silenced HFs with the potent GSK3β inhibitor, lithium chloride (LiCl), did not reverse the effect of P-cadherin silencing in terms of MITF transcript level, although MITF is a known target gene of β-catenin. *p<0.05; ***p<0.001, *Student's t test for unpaired samples.* The transcript levels were compared to glyceraldehyde 3-phosphate dehydrogenase, peptidylprolyl isomerase A or β-actin.

DP – dermal papilla; GSK3β - Glycogen synthase kinase 3 beta; HF – hair follicle; IHM – innermost hair matrix; IR – immunoreactivity; LiCl - lithium chloride; MITF - microphthalmia-associated transcription factor; ORS – outer root sheath; P-cad. – P-cadherin; P- β-catenin – phospho-β-catenin; Scr. – scrambled.

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Figure 1
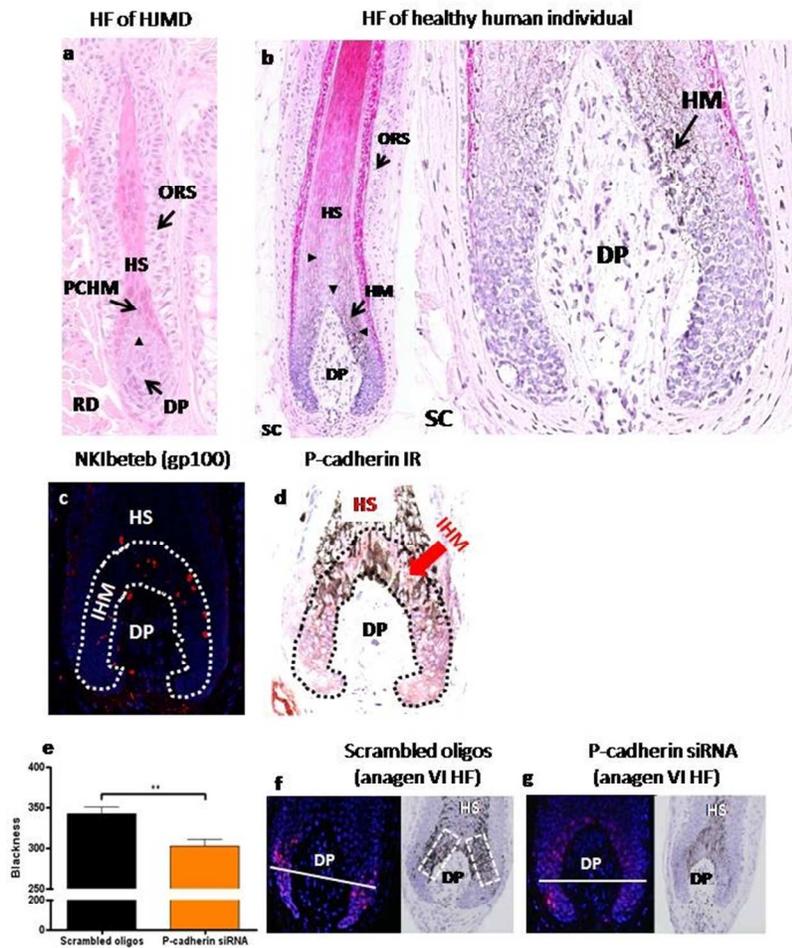
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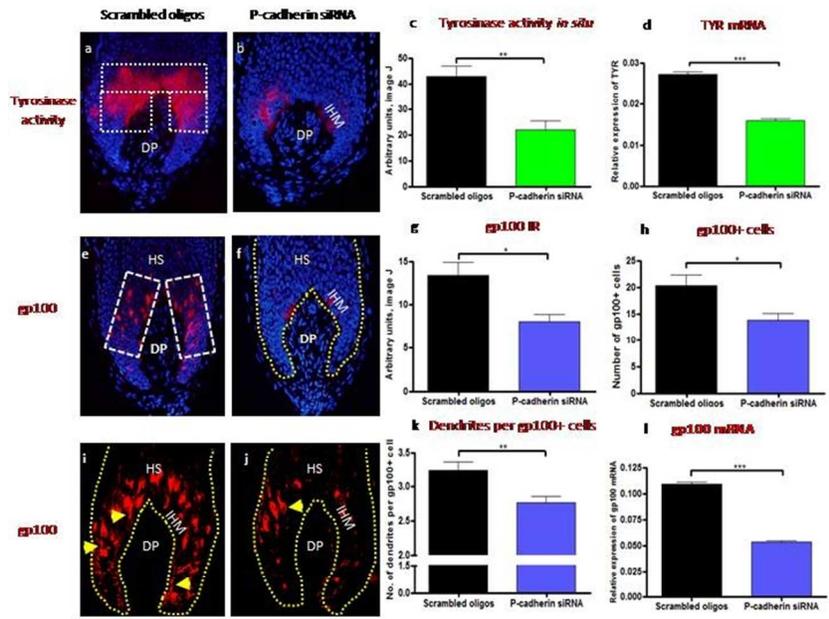57
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Figure 2



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Figure 3

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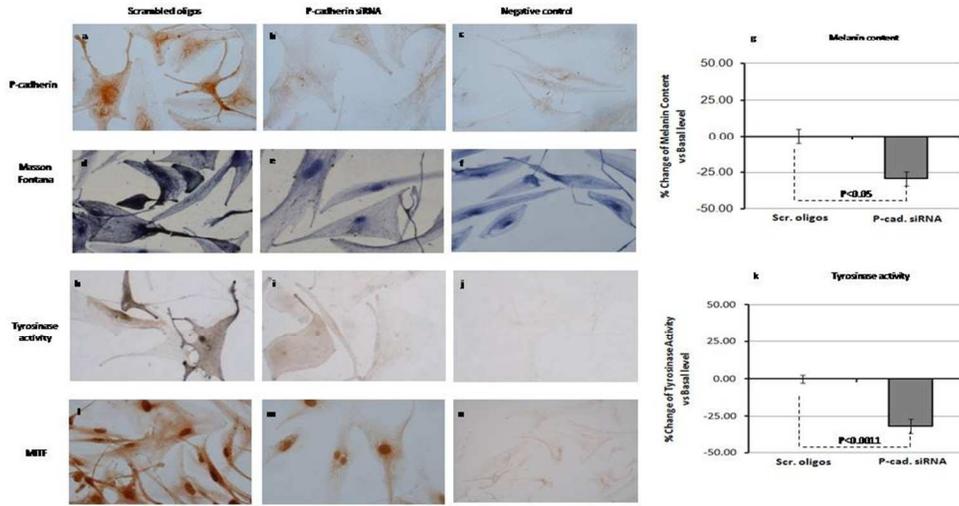44
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Figure 4
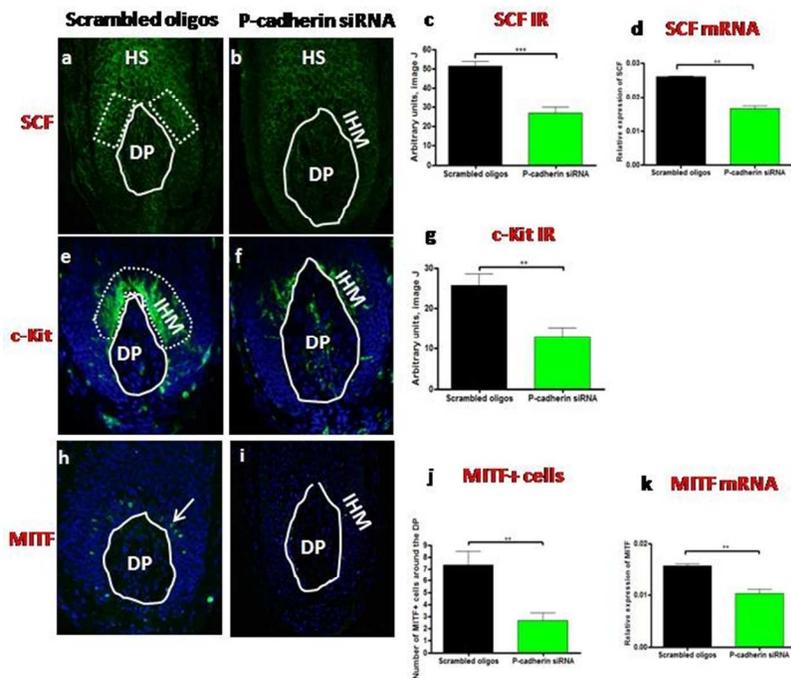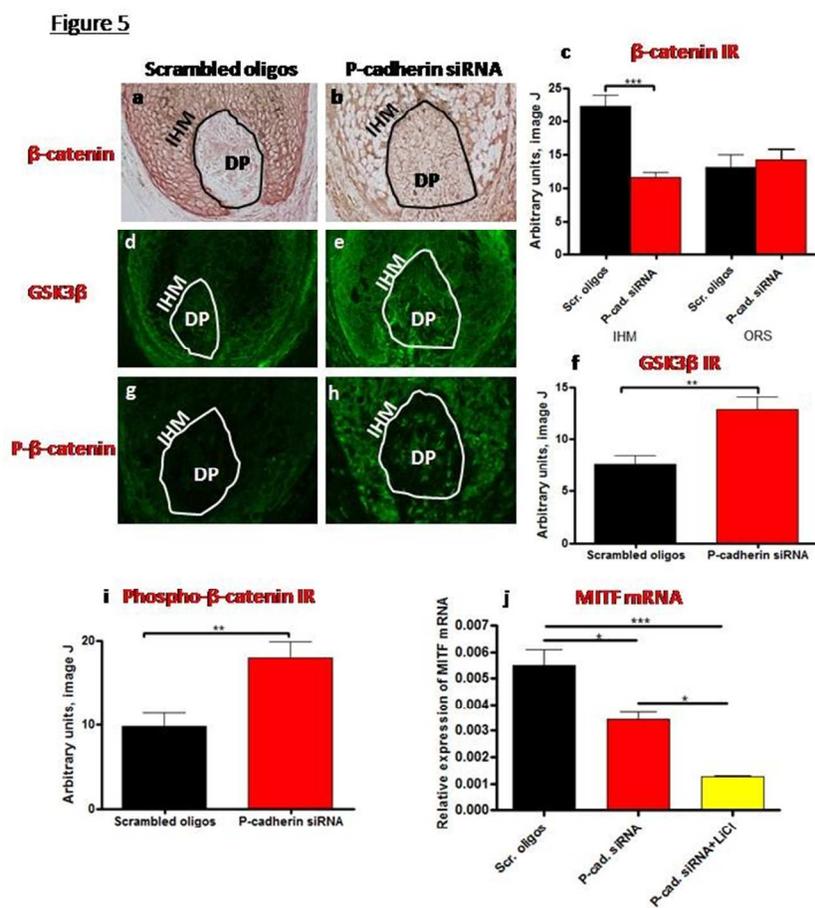
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Figure 5

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## Supplementary data

### Supplementary Text S1

Signaling by the Wnt pathway is triggered by the binding of secreted Wnt growth factor proteins to the Frizzled receptor. In the absence of Wnt ligands, cytoplasmic β-catenin is recruited into a complex that contains adenomatous polyposis coli and axins, and is amino-terminally phosphorylated by axin-bound casein kinase 1a and glycogen synthase kinase 3β (GSK3β) at Ser33/37/Thr41. Following phosphorylation, β-catenin is targeted for proteasome-dependent degradation. In the presence of Wnt ligands, the GSK3β-dependent phosphorylation of β-catenin is blocked, resulting in β-catenin stabilization and nuclear translocation, where it can heterodimerize with T-cell factor and Lymphoid enhancer-binding factor-1 transcription factors, leading to the activation of specific β-catenin target genes, such as Axin2 (Bienz, 2005; Heuberger and Birchmeier, 2010; Klaus and Birchmeier, 2008; Liu *et al.*, 2002; Tauriello and Maurice, 2010).

### Supplementary Text S2

In order to check whether the observed reduction in the number of microscopically visible dendrites per gp100+ cell resulted from increased HF melanocyte apoptosis, gp100/TUNEL double-immunofluorescence was established. This also served as an independent confirmation that only anagen VI HFs were compared with each other, as catagen development is associated with HF melanocyte apoptosis (Sharov *et al.*, 2005; Tobin *et al.*, 1998). By gp100/TUNEL double-immunofluorescence no significant differences in HF melanocyte apoptosis between test and control anagen hair bulbs were observed (Supplementary Figure 1).

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## Supplementary Text S3

We show here that P-cadherin appears dispensable for pigmentation in normal human epidermis and thus seems to be of selective importance in the HFPU, where E-cadherin expression is low or absent, while P-cadherin is prominently expressed. However, P-cadherin expression was demonstrated in retinal pigment epithelium (RPE) cells of the developing mouse retina (Xu *et al.*, 2002) and also in adult human RPE, where it is co-expressed with E-cadherin (Burke *et al.*, 1999). Interestingly, diffuse pigmentary abnormalities confined to the macula and surrounding areas are an early and characteristic ophthalmologic features of HJMD patients (Leibu *et al.*, 2006). Therefore, it deserves to be explored in future studies whether P-cadherin is not only essential in the HFPU, but also in the RPE.

## Supplementary Text S4

Normal skin keratinocytes, including those of the hair matrix, constitutively produce SCF (Longley *et al.*, 1993; Peters *et al.*, 2003). Its cognate receptor, c-Kit, is expressed on various cell types including HF melanocytes (Broudy, 1997) and hair matrix keratinocytes (Peters *et al.*, 2003). SCF/c-Kit-mediated signaling plays a key role in the control of hair pigmentation (Botchkarev *et al.*, 1999; Hachiya *et al.*, 2009; Jackson, 1997; Nishikawa *et al.*, 1991; Slominski *et al.*, 1998), melanoblast and/or melanocyte migration, melanocyte survival, proliferation and differentiation as well as the maintenance of postnatal cutaneous melanogenesis (Besmer *et al.*, 1993; Botchkareva *et al.*, 2001; Grichnik *et al.*, 1998; Hachiya *et al.*, 2009; Nishikawa *et al.*, 1991; Steel *et al.*, 1992; Williams *et al.*, 1992). Indeed, c-Kit and MITF expression are significantly lower in unpigmented than in pigmented human HFs, and, administration of a neutralizing anti-Kit antibody abolishes MITF and tyrosinase expression, resulting in depigmentation of human HFs (Hachiya *et al.*, 2009). Moreover, melanoblasts that form the P-cadherin positive HFPU during murine HF development retain c-Kit expression during their differentiation into melanin-producing HFPU melanocytes, whereas amelanotic melanocytes residing in the ORS lose their c-Kit expression

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(Peters *et al.*, 2002). SCF signaling triggers the phosphorylation of MITF (Hemesath *et al.*, 1998; Wu *et al.*, 2000) and is associated with an increased production of MITF protein via the phosphorylation of cAMP responsive element binding protein (CREB), due to the activation of RSK by mitogen-activated protein kinase (MAPK) pathway in human melanocytes (Sato-Jin *et al.*, 2008). Furthermore, SCF stimulates the expression and function of c-Kit (Imokawa *et al.*, 1996).

On this background, the reduced expression of c-Kit observed in P-cadherin-silenced human HFs may well result from a markedly diminished expression of SCF in the adjacent, P-cadherin-positive inner hair matrix keratinocytes. Mechanistically, P-cadherin silencing in both, the innermost hair matrix keratinocytes and in HFPU melanocytes likely results in reduced intracellular β-catenin levels and a subsequent downregulation of β-catenin target genes, including MITF, in both cell populations. This downregulation of canonical Wnt signaling probably causes reductions in both, SCF expression and secretion by hair matrix keratinocytes and c-Kit expression on the adjacent HFPU melanocytes. Reduced c-Kit signaling in HFPU melanocytes then may further downregulate MITF expression, tyrosinase activity, gp100 expression, and melanosome transfer, thus leading to greatly reduced intrafollicular melanogenesis (Supplementary Figure S5b).

Our results demonstrated that nuclear expression levels of MITF-M appear relatively similar in P-cadherin siRNA treated HFMs and the control in this study. However, there is evidence that MITF-M expression level in the cytoplasm is reduced after P-cadherin siRNA treatment. As MITF-M is initially synthesized in the cytoplasm, it must thereafter be translocated to the nucleus in order to function as a transcription factor for several melanocyte-specific genes. Therefore, the relative availability of MITF-M in the melanocyte (i.e., expression levels in cytoplasmic and subsequent nuclear translocation kinetics) is likely to influence the subsequent activation of the melanogenic program in melanocytes. For example, GSK3 inhibition in melanocytes promotes nuclear translocation of MITF (Bellei *et al.*, 2008). The activation of the melanogenic program by GSK3 inhibition not only stimulates MITF expression, but

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also promotes its translocation from the cytoplasm to the nuclear compartment. Thus, MITF activity may be regulated by its cellular localization, and that our short-term pulse reduction of MITF-M via P-cadherin siRNA treatment may reduce overall MITF-M associated gene transcriptional activity in HFMs.


## Supplementary Text S5

Visible hair pigmentation requires the transfer of melanin granules from differentiated melanocytes of the HFPU to keratinocytes of the precortical hair matrix (Slominski *et al.*, 2005; Tobin, 2011). For effective transfer, melanosomes must accumulate at the distal end of the melanocyte dendrites, requiring microtubule-dependent melanosome transfer along the dendrites before coupling to myosin Va within the actin-rich regions of the dendrites (Belleudi *et al.*, 2011; Singh *et al.*, 2010; Wu *et al.*, 1998). The cadherins are indirectly linked to microtubules and/or filamentous actin (via adherens junctions) by β- and α- catenin. In addition, the adherens junctions play a role in microtubule and actin organization within the cell (Harris and Tepass, 2010). Therefore, our ultrastructural results are in line with the hypothesis that P-cadherin silencing in human HFMs may result in actin and microtubule abnormalities which may block orderly melanosome transfer to adjacent keratinocytes.

P-cadherin silencing also greatly reduced the number and length of dendrites generated by human HFPU melanocytes *in situ*, as shown by confocal microscopy (Figure 2i-k), possibly as a result of attenuated MITF (Figure 4h-k) and SCF/c-Kit (Figure 4a-g) expression. This may also be a cause for disruption and reduction of melanosome transfer (Supplementary Figure S2). Melanosome transfer to keratinocytes results, at least in part, from the direct delivery of melanosomes into keratinocytes via filopodia (Scott *et al.*, 2002; Singh *et al.*, 2010; Singh *et al.*, 2008). Tubular filopodia are composed of and form actin filaments, by physical contact and fusion with the target cell, a direct cytoplasmic bridge which allows organelle transfer. In melanocytes, the filopodia extend from the dendrite tips and adhere to the surface of adjacent keratinocytes in order to facilitate melanosome transfer into the

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keratinocyte cytoplasm (Scott *et al.*, 2002; Singh *et al.*, 2010; Singh *et al.*, 2008). This mechanism of melanosome transfer may be impaired by P-cadherin function, secondary to abnormalities of the adherens junctions and the melanocyte cytoskeleton induced by P-cadherin silencing or spontaneous function-impairing mutations. In conclusion, our data, including reduced expression of gp100 (a marker antigen for studying melanosome transfer) and ultrastructural evidence that hair matrix keratinocytes in P-cadherin-silenced HFs exhibit accumulation of an excessive amount of melanosomes, raise the possibility that P-cadherin expression might be directly involved in orderly melanosome transfer within the human HFPU. However, this hypothesis would have to be further explored by formal melanosome tracking assays, which were outside of the scope of the current study

## Supplementary Materials and Methods

### Tissue collection

The same human anagen VI hair follicles (HFs) from five patients undergoing routine face-lift surgery whose growth response to P-cadherin silencing we had previously examined (Samuelov *et al.*, 2012) were re-analyzed in the current study for pigmentary phenomena [after ethics approval and informed patient consent, as described before (Samuelov *et al.*, 2012)]. Five independent HF organ culture experiments in serum-free medium were performed using the previously described protocol (Bodo *et al.*, 2005; Philpott *et al.*, 1990; Sanders *et al.*, 1994), with 60-132 HFs per culture. This was complemented with two full-thickness human skin organ cultures (Bodo *et al.*, 2010; Lu *et al.*, 2007) from two different patients, also under serum-free conditions.

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## Hair follicle and skin organ culture

Per experiment, HFs were divided into equal numbers for the two different treatments of P-cadherin siRNA and scrambled oligos groups. In order to explore the effect of GSK3β inhibition on HF pigmentation, in one culture, the culture medium was supplemented with the potent GSK3β inhibitor lithium chloride (LiCl) (Tighe *et al.*, 2007) in the concentration of 40mM (Sigma, Munich, Germany). LiCl was added only once to the culture medium (either as the only treatment or 5-7 hours (hrs) after transfection) and HFs were embedded in Shandon Cryomatrix (Pittsburgh, PA, USA) and snaped frozen in liquid nitrogen 24hrs afterwards.

Hair shaft length was measured daily using an inverted binocular microscope (Kloepper *et al.*, 2008; Philpott *et al.*, 1990) and culture medium was replaced after the transfection and then every other day. On days 2 and 4 (and also on day 3 in the culture where two transfections were performed), samples of culture medium were taken for lactate dehydrogenase (LDH) level measuring as a general cell toxicity parameter in human skin organ culture (Lu *et al.*, 2007). This showed no significant increase in the LDH release of P-cadherin-silenced HFs compared to scrambled oligo-controls after a single transfection, while a rise in LDH levels after repeated siRNA treatment was demonstrated (data not shown). This indicates minimal toxicity after a single transfection, while repeated transfection carries the expected risk of increased toxicity. Twenty-four hours after the transfection (on day 1 in the single transfection cultures and on day 4 in the double transfection culture), 12-15 HFs were frozen in liquid nitrogen for RNA extraction and mRNA analysis. The rest of the HFs were embedded in Shandon Cryomatrix (Pittsburgh, PA, USA) and snap frozen in liquid nitrogen on day 4 of culture. HFs that were treated with LiCl, were embedded 24h after the beginning of treatment.

6 µm thick cryosections of HFs were cut for immunohistological staining, and were stored at -80°C until use. The efficacy of gene silencing was confirmed by P-cadherin specific immunohistochemistry (Samuelov *et al.*, 2012) and qRT-PCR (data not shown).

In the two skin organ cultures, 2 mm full-thickness skin punches were transfected with P-cadherin siRNA and control siRNA at day 0 (6 skin punches per group) using exactly same serum-free organ culture protocol as in the HF cultures. In one culture, 24h after the transfection, skin punches were frozen in liquid nitrogen for RNA extraction and CDH3 mRNA analysis. In the second culture, skin punches were embedded in Shandon Cryomatrix (Pittsburgh, PA, USA) and snap frozen in liquid nitrogen on day 4 of culture. 7 μm thick cryosections of skin punches were cut for immunohistochemistry. The efficacy of gene silencing was confirmed by P-cadherin specific immunohistochemistry and qRT-PCR (data not shown).

**P-cadherin knockdown**

P-cadherin knockdown was performed as previously described (Samuelov *et al.*, 2012). In four HF organ culture experiments (each from a different patient), HFs were transfected with human P-cadherin siRNA (Sigma-Aldrich, Munich, Germany, SASI_Hs02_00332561, 5'- CCAAUAUCUGUCCCUGAAA-3') at day 0 for 5-7 hrs. In one additional HF organ culture, HFs were transfected twice – i.e. at days 0 and 3 of culture. Transfection with scrambled oligonucleotides (Santa Cruz, CA, USA, sc-37007) served as control. After transfection HFs were maintained in a 24-well plate with 500 μl Williams medium E (Biochrom, Cambridge, UK) supplemented with 1% L-glutamine (Invitrogen, Paisley, UK), 0.02% hydrocortisone (Sigma-Aldrich, Munich, Germany), 0.1% insulin (Sigma-Aldrich) and 1% antibiotic/antimycotic mixture (Gibco, Karlsruhe, Germany). All reagents required for transfection, except P-cadherin specific siRNA and control siRNA, were obtained from Santa Cruz Biotechnology Inc (siRNA transfection reagent, sc-29528; siRNA transfection medium, sc-36868). HF transfection was performed according to the manufacturer's protocol (Santa Cruz Biotechnology Inc, CA, USA) (Samuelov *et al.*, 2012).

In order to ensure no off-targets activation by the P-cadherin siRNA, we used two different computer softwares (Blast and siSPOTR) in which we checked the first 100

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genes that may be influenced by our siRNA. Following this, we did a MicroArray analysis of three HF organ cultures from 3 different patients (data not shown) in which we compared the panel of genes affected by P-cadherin siRNA vs. scrambled oligos. These data demonstrated that none of the 100 genes that came out in the mentioned computer softwares, were affected in those 3 HF organ cultures.

## Hair follicle melanocyte culture

Hair follicle melanocytes (HFMs) were isolated from normal human haired scalp tissue, obtained from a healthy adult Caucasian female (age 59 years) after elective plastic surgery with informed consent and with local ethics committee approval. All cell culture reagents were obtained from Invitrogen Ltd (Invitrogen Ltd, Paisley, Scotland) unless otherwise stated. Skin samples were collected in RPMI 1640 medium and were processed within five hours of surgery. HFM cultures were established as described previously (Kauser *et al.*, 2005). Briefly, EMEM supplemented with 2% FCS, 1x concentrated non-essential amino acid mixture, 100 units/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine, 5 ng/ml endothelin-1and 5 ng/ml basic fibroblast growth factor (bFGF) was combined with keratinocyte serum free medium (K-SFM) supplemented with 25 mg/ml bovine pituitary extract (BPE), 0.2 ng/ml recombinant epidermal growth factor (rEGF), 100 units/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were incubated at 37ºC in a 5% $CO_2$ atmosphere with medium replenished every third day. Contaminating fibroblasts, when present, were eliminated by treating the cultures with 150 mg/ml geneticin sulphate (G418) for cycles of 48 hrs (Halaban and Alfano, 1984) and the identity of isolated cells was confirmed by immunophenotyping at passage 1 with the melanocyte-lineage specific antibody NKI/beteb directed against glycoprotein100 (gp100) (Vennegoor *et al.*, 1988).

**Melanin histochemistry**

Briefly, cryosections and HFM cultures were air dried and fixed in ethanol-acetic acid. These were washed in Tris-buffered saline (TBS) and distilled water several times. Cryosections and HFMs were treated with ammoniacal silver solution (Fluka, Seelze, Germany) for 40 minutes (min) at 56°C in the dark. After washing in distilled water, they were treated with 5% aqueous sodium thiosulphate (Merck, Darmstadt, Germany) for 1 min. Then, they were washed in running tap water for 3 min and were counterstained with haematoxylin for 45 seconds. After washing in distilled water, sections and HFMs were dehydrated and sections mounted in Eukitt (O. Kindler, Freiburg, Germany). Quantitative melanin histochemistry was conducted by measuring melanin content in a defined reference area in the hair matrix on both sides of the dermal papilla (DP). 14-15 HFs per group were evaluated from 3 different patients (2 patients with one transfection and one patient with two transfections with P-cadherin siRNA vs. scrambled oligos).

In HFMs, melanin content was quantified as follows. After trypsinisation cells were counted using a Neubauer counting chamber, pelleted by centrifugation and solubilised in 1 M sodium hydroxide, boiled and melanin content measured spectrophotometrically at 475 nm. A standard curve of synthetic melanin (Sigma, Poole, Dorset, UK) was the basis for melanin content determination. Melanin content and cell counts were determined at least three times and average values were taken. Melanin content was measured as pg melanin per cell (i.e. HFMs) and expressed as percentage increase in melanin content above control cells.

**Immunohistochemistry** (LSAB – Peroxidase)

The cryosections were first air dried for 10 min and then fixed in acetone at -20°C for another 10 min. After air drying for 10 min the slides were washed three times for 5 min in TBS. Endogenous peroxidase blocking was achieved by putting the slides in 3% hydrogen peroxide for 15 min, followed by three washing steps with TBS, 5 min each. The application of the different primary antibodies (Supplementary Table S1)

followed (in appropriate antibody diluents) (DCS Detection Line, Hamburg, Germany) overnight at 4°C. After washing three times for 5 min with TBS, sections were stained with polylink biotinylated secondary antibody (DCS Detection Line, Hamburg, Germany) for 20 min at room temperature (RT). After three washings for 5 min with TBS, a 20 min application of Horse Radish Peroxidase label (HRP) Avidin Biotin Complex (DCS Detection Line) followed, both at RT. Finally, the slides were labelled with 3-Amino-9-Ethylcarbazole (AEC) (DCS ChromoLine, Hamburg, Germany) and counterstained with haematoxylin with interspersed washing steps (Samuelov *et al.*, 2012).

**Immunofluorescence microscopy and quantitative immunohistomorphometry**

Immunfluorescence stainings for β –catenin, phospho- β –catenin and GSK3β were performed as described before (Samuelov *et al.*, 2012).

Immunfluorescence microscopy for SCF, gp100, c-Kit, MITF, ACTH and quantitative immunohistomorphometry are described in the following lines.

For the SCF immunostaining, the slides were washed with phosphate buffered saline (PBS), cryosections were incubated overnight at 4°C with the primary antibodies in the respective

dilutions in antibody diluent (DCS Detection Line, Hamburg, Germany) (Supplementary Table S1) followed by incubation of 45 minutes (min) at room temperature (RT) with FITC-labeled goat anti-mouse (Jackson Immunoresearch Laboratories, West Grove, PA, USA), 1:200 in antibody diluent (DCS Detection Line). Incubation steps were interspersed with three washes, 5 min each.

For gp100 staining, cryosections were first fixed in acetone and after washing steps with PBS, were incubated with the primary antibody (Supplementary Table S1) diluted in Dako antibody diluent (Dako, Glostrup, Denmark) overnight at 4°C. The cryosecions were then incubated with a secondary rhodamine-labeled goat anti-mouse antibody (Jackson Immunoresearch Laboratories), 1:200 in PBS and goat normal serum, for 45 min at RT, after washing steps.

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For <u>MITF</u> staining, after fixation with acetone at -20°C and washing with TBS, cryosections were incubated overnight at $4^o$C with the primary antibody (Supplementary Table S1) in the respective dilution in Dako antibody diluent (Dako), followed by incubation of 45 min at RT with FITC-labeled Rabbit anti-mouse IgG (Jackson Immunoresearch Laboratories) diluted 1:200 in TBS and normal rabbit serum.

For <u>ACTH</u> staining, cryosections were first fixed in acetone and after washing steps with PBS, were incubated with the primary antibody (Supplementary Table S1) diluted in Dako antibody diluent (Dako) for one hour at $37^o$C. The cryosecions were then incubated with a secondary FITC-labeled goat anti-rabbit antibody (Jackson Immunoresearch Laboratories), 1:200 in PBS and goat normal serum, for 45 min at RT, after washing steps.

Since <u>c-Kit</u> *is* expressed on various cell types including melanocytes and the SCF-c-Kit signaling play a key role in the control of hair pigmentation (Botchkarev *et al.*, 1999; Hachiya *et al.*, 2009; Slominski *et al.*, 1998), <u>c-Kit</u> immunoreactivity was investigated by using the highly sensitive immunofluorescent TSA technique (Perkin Elmer, Boston, MA, USA) (Roth *et al.*, 1999). Briefly, cryosections of human HFs were incubated with the primary antibody (Supplementary Table S1) diluted in antibody diluent (DCS Detection Line), overnight at $4^o$C, followed by incubation with a biotinylated antibody against rabbit IgG (Jackson immunoresearch Laboratories) (1:200 in antibody diluent, 45 min, RT). Next, streptavidin–horseradish peroxidase was administered (1:100 in Tris-NaCl-Tween Buffer, 30 min, RT). Finally, the reaction was amplified by tetramethylrhodamine.

In addition, <u>α-MSH</u> immunoreactivity was also investigated by using the highly sensitive immunofluorescent TSA technique. Briefly, cryosections of human HFs were incubated with the primary antibody (Supplementary Table S1) diluted in TNB, overnight at $4^o$C, followed by incubation with a biotinylated antibody against rabbit IgG (Jackson immunoresearch Laboratories) (1:200 in TNB, 45 min, RT). Next, streptavidin–horseradish peroxidase was administered (1:100 in Tris-NaCl-Tween Buffer, 30 min, RT). Finally, the reaction was amplified by tetramethylrhodamine.

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The immunostaining intensity levels for the examined antigens were compared by quantitative immunohistochemistry as previously described (Ito *et al.*, 2005a; Ito *et al.*, 2005b; Peters *et al.*, 2005), using NIH image software (NIH, Bethesda, MD, USA). Three reference areas were defined (1, innermost hair matrix; 2, precortical hair matrix; 3, ORS) and analysis was performed in the specific area according to the antigen expression.

**Tyrosinase activity and melanocyte apoptosis *in situ***

The tyramide-based tyrosinase assay was used for measuring tyrosinase activity *in situ* as previously described (Han *et al.*, 2002; Kloepper *et al.*, 2010). In this procedure, tyrosinase reacts with biotinyl tyramide, causing the substrate to deposit near the enzyme. These biotinylated deposits are then visualized with streptavidin conjugated to a fluorescent dye. This assay is highly specific and serves as a sensitive indicator of tyrosinase activity, which is restricted to melanogenically active melanocytes of the HF pigmentary unit (Tobin, 2011).

In order to exclude confounding influences of HF cycling on pigmentation [note that HF melanogenesis is strictly coupled to the anagen stage of the hair cycle and is switched-off during catagen (Schneider *et al.*, 2009; Slominski *et al.*, 2005; Tobin, 2011)], and in view of the fact that P-cadherin silencing prematurely induces catagen (Samuelov *et al.*, 2012), we exclusively analyzed melanogenically fully active anagen VI HFs. To reliably determine the hair cycle stage of the examined HFs, the stringent, standardized hair cycle assessment criteria were employed including the evaluation of hair matrix proliferation and apoptosis a in the two treatment groups by Ki-67/TUNEL double immunolabeling see (Kloepper *et al.*, 2010).

**gp100/TUNEL double-IF**

Briefly, after fixation in 1% paraformaldehyde [in phosphate buffered saline (PBS)] and post-fixation in ethanol-acetic acid (2:1) (with washing steps in between), the sections were incubated first with anti TDT-Enzyme (TUNEL; ApopTag Fluorescein In

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Situ Apoptosisdetection kit; Millipore, Berlin, Germany) as described previously (Bodo *et al.*, 2004; Kloepper *et al.*, 2008; Peters *et al.*, 2006), and after washing steps and pre-incubation with goat serum and PBS, were incubated with mouse anti-human NKI/beteb (Monosan, Uden, Netherlands), diluted 1:20 in PBS and goat serum, overnight at 4°C. As secondary antibodies, we used first anti-digoxigenin according to the manufacture's protocol (ApopTag Fluorescein In Situ Apoptosisdetection kit; Millipore), and secondly rhodamine-coupled goat anti-mouse IgG (Jackson immunoresearch Laboratories), diluted 1:200 in PBS and goat serum, 45min at RT.

In all immunofluorescence staining methods, sections were counterstained with 4'-6-Diamidino-2-phenylindole (DAPI) in a concentration of 1µg/ml (1 min at RT) for identification of cell nuclei.

**Quantitative Real-Time PCR (qRT-PCR)**

Total RNA was extracted from whole organ cultured HFs using TRIreagent and digested with recombinant RNase-free DNase-1 (both from Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. After isolation, one µg of total RNA was reverse-transcribed into cDNA by using High Capacity cDNA kit (Applied Biosystems) following the manufacturer's protocol. PCR amplification was performed by using specific TaqMan primers and probes (Applied Biosystems, assay IDs: Hs00165976_m1 for human tyrosinase, Hs00241497_m1 for human SCF, Hs00173854_m1 for human gp100, Hs01117294_m1 for human MITF, Hs00174947_m1 for human proopiomelanocortin (POMC) and Hs00610344_m1 for human axin2). As internal housekeeping gene controls, transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA) or β-actin were determined (Assay IDs: Hs99999903 for human ACTB, Hs99999904 for human PPIA and Hs99999905_m1 for human GAPDH). The amount of the above mentioned transcripts was normalized to those of the control genes using the ΔCT method.

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## Statistical analysis

Quantitative immunohistochemistry data were expressed as mean ± SEM and analyzed by Mann–Whitney test (GRAPHPAD PRISM version 4.00 for Windows; GraphPad Software, San Diego, CA, USA). All qRT-PCR results were analysed by *Student's t test for unpaired samples*. PCR results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA) or β-actin (Samuelov *et al.*, 2012).

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## Supplementary Figures

**Supplementary Figure S1**
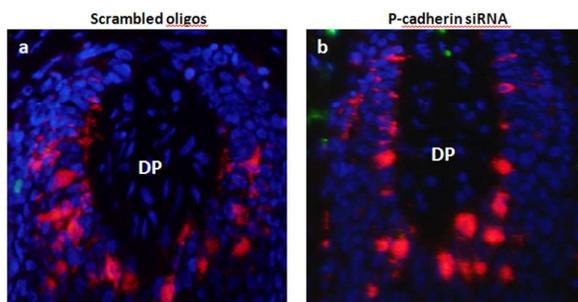


**Supplementary Figure S1. gp100/TUNEL double-immunofluorescence**

No evidence of hair follicle melanocyte apoptosis in both P-cadherin siRNA (b) and scrambled oligos (a) treated anagen hair bulbs. Red immunofluorescence (IF) is for gp100 and green IF is for TUNEL staining.  DP – dermal papilla.

**Supplementary Figure S2**

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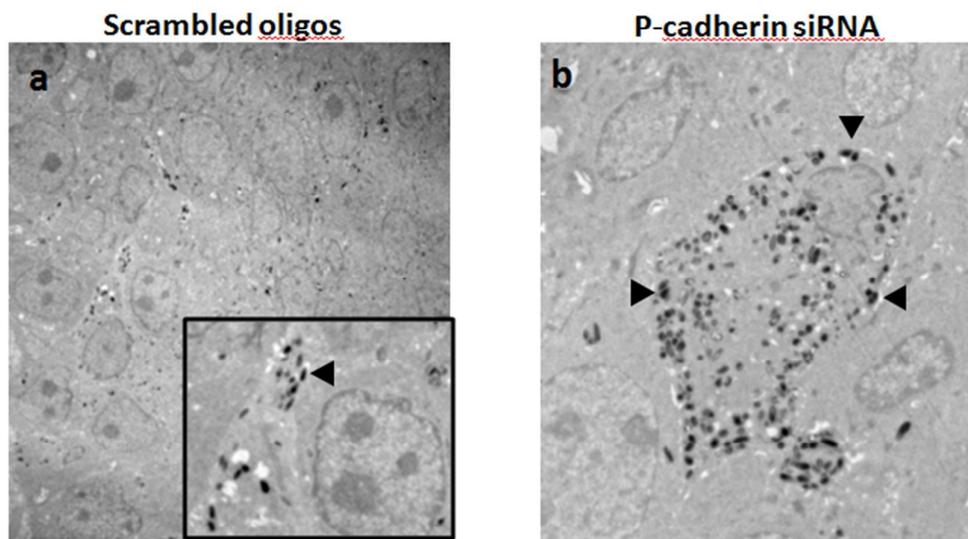


**Supplementary Figure S2. Effects of P-cadherin silencing on melanosome transfer by Transmission Electron Microscopy**

(a) The hair follicle (HF) pigmentary unit (HFPU) in a scrambled oligos-treated HF. The melanosomes are efficiently transferred to the adjacent keratinocytes with no evidence of blockade in melanosome transfer. The inserted image demonstrates in higher magnification the presence of melanosomes inside the keratinocytes (arrow head).

(b) The HFPU in a P-cadherin siRNA-treated HF. The melanocytes contain a huge number of melanosomes with significantly reduced melanosomes in the adjacent keratinocytes. Arrow heads represent melanosomes inside a melanocyte. Note the normally appearing melanocyte.

**Supplementary Figure S3**
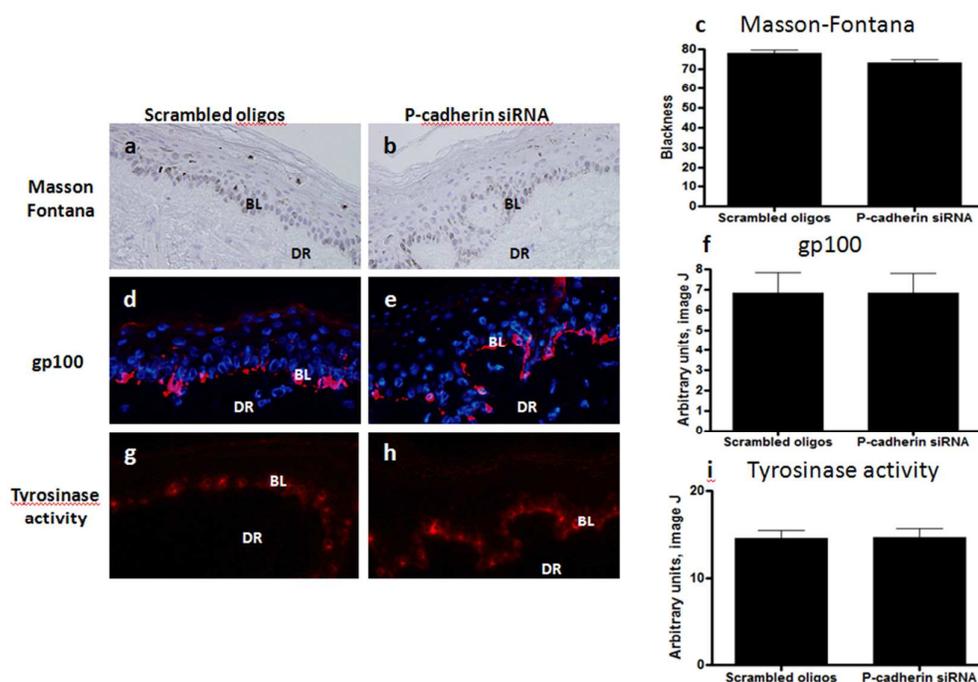


**Supplementary Figure S3. The effects of P-cadherin knockdown on epidermal pigmentation**

2mm skin punches were transfected with P-cadherin siRNA vs. scrambled oligos. P-cadherin knock-down was confirmed both at the mRNA and protein level (Samuelov *et al.*, 2012). All measurements were done by comparing the expression in the basal layer of the epidermis, in both treatment groups of skin punches.

(a-c) No significant difference was found in melanin content by Masson-Fontana.

(d-f) No significant difference was found in gp100 expression.

(g-i) No significant difference was found in tyrosinase activity *in situ*.

BL – basal layer; DR – dermis.

**Supplementary Figure S4.**

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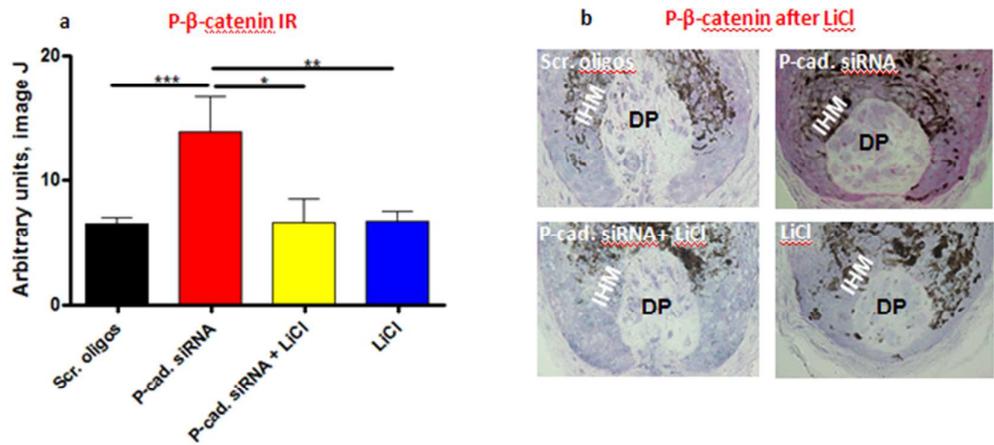


**Supplementary Figure S4. The effects of P-cadherin knockdown on Phospho-β-catenin expression**

(a,b) Phospho-β-catenin immunoreactivity in scrambled oligos, P-cadherin siRNA, P-cadherin siRNA+lithium chloride (LiCl) and LiCl treated hair follicles (HFs). Note the significant reduction in immunoreactivity by simultaneous treatment with LiCl and P-cadherin siRNA.

*p<0.05; **p<0.01; ***p<0.001, *Mann Whitney* test, n=4-9 HFs.

Cad. – cadherin; IR – immunoreactivity; LiCl – lithium chloride; P-β-catenin - phospho-β-catenin; Scr. – scrambled.

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**Supplementary Figure S5**

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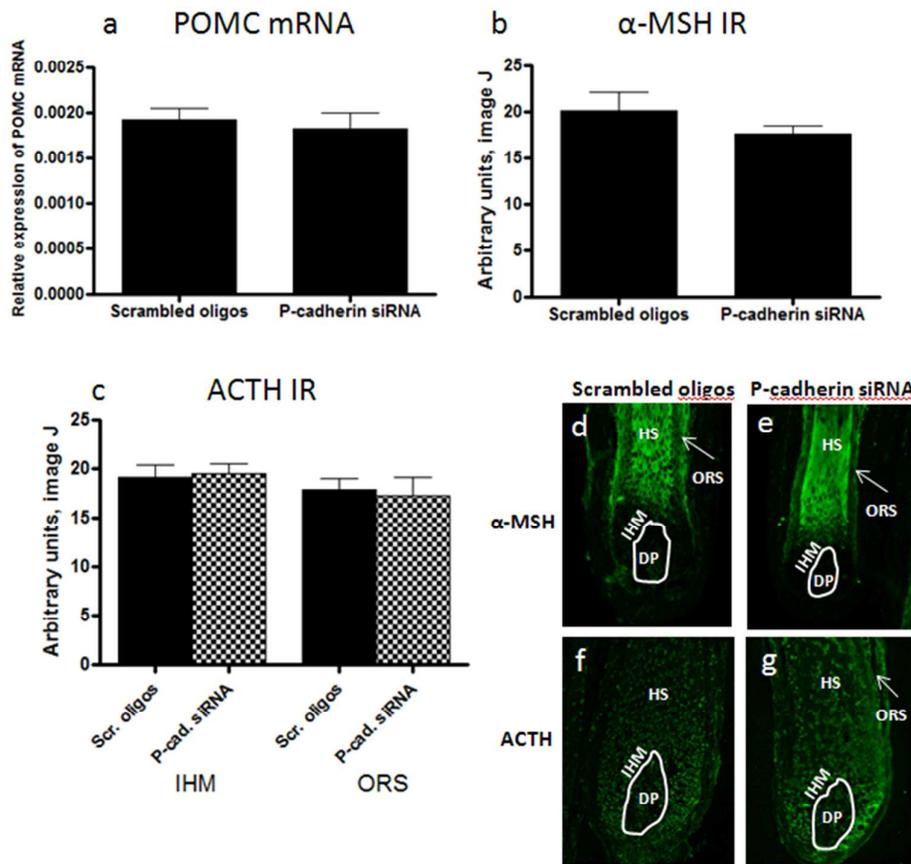
**Supplementary Figure S5. Effect of P-cadherin silencing on expression of proopiomelanocortin, alpha-melanocyte stimulating hormone and adrenocorticotropic hormone**

(a) No significant difference between the P-cadherin siRNA and scrambled oligos treated hair follicles (HFs) was demonstrated in proopiomelanocortin (POMC) mRNA level.

(b,d-e) No significant difference between the P-cadherin siRNA and scrambled oligos treated HFs was demonstrated in alpha-melanocyte stimulating hormone (α-MSH) protein level. α-MSH expression was evaluated in the outer root sheath (ORS) keratinocytes

(c,f-g) No significant difference between the P-cadherin siRNA and scrambled oligos treated HFs was demonstrated in adrenocorticotropic hormone (ACTH) protein level. ACTH immunoreactivity was measured both at the ORS and the innermost hair matrix keratinocytes. The white lines encircle the dermal papilla.

α-MSH – alpha-melanocyte stimulating hormone; ACTH – adrenocorticotropic hormone; Cad. – cadherin; DP – dermal papilla; HS – hair shaft; IHM – innermost hair matrix; IR – immunoreactivity; ORS – outer root sheath; POMC – proopiomelanocortin; Scr. – scrambled.

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**Supplementary Figure S6.**

**(a)** Intact P-cadherin: HF melanogenesis is switched-on during anagen VI

↑ β-catenin

P-cad

Melanogenesis, ↑ Tyrosinase activity, gp100

β-catenin Target Genes (e.g. axin2, MITF)

↑ MITF

P-cad.

↑ β-catenin Target Genes (e.g. axin2)

↑ P-CREB

↑ β-catenin

↑ c-Kit

↑ c-Kit

↑ c-Kit

ME

P-cad.

↑ SCF

↑ SCF

**Melanocyte**

↑ SCF

**Keratinocyte**
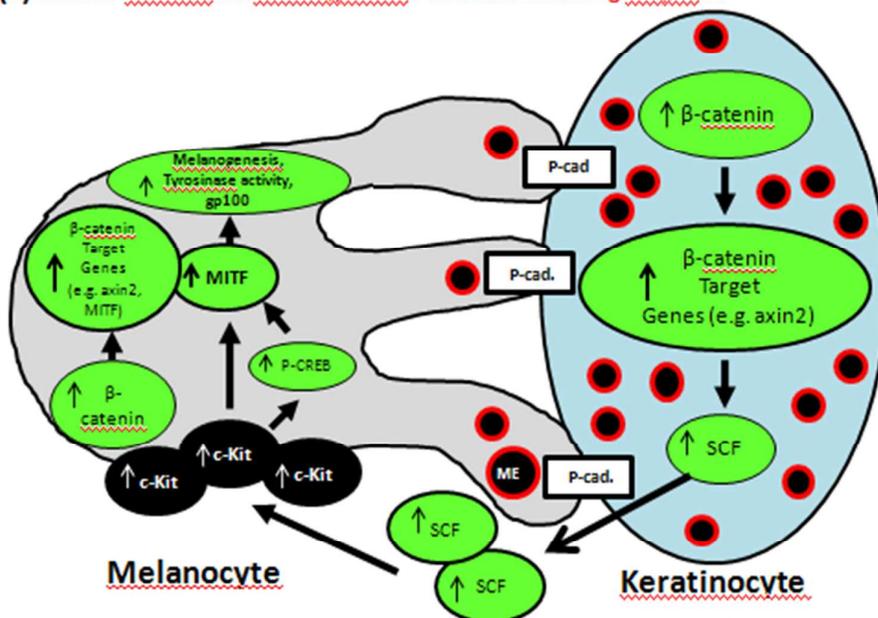
**(b)** P-cadherin absence: melanosome transfer is blocked
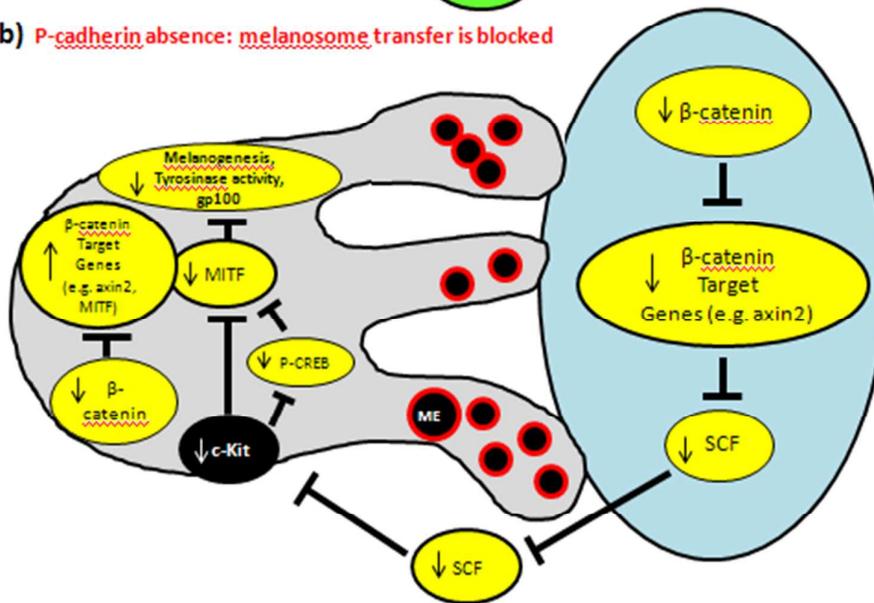
↓ β-catenin

Melanogenesis, ↓ Tyrosinase activity, gp100

β-catenin ↑ Target Genes (e.g. axin2, MITF)

↓ MITF

↓ β-catenin Target Genes (e.g. axin2)

↓ P-CREB

↓ β-catenin

ME

↓ c-Kit

↓ SCF

↓ SCF

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**Supplementary Figure S6. A hypothetical scenario: How P-cadherin may impact on melanocytes within the human hair follicle pigmentary unit**

(**a**) A normal state with intact P-cadherin. P-cadherin connects the melanocytes and the keratinocytes in the area of the follicular pigmentary unit. In a normal state, the Wnt signaling pathway is active both in the melanocytes and the keratinocytes, resulting in normal stem cell factor (SCF) (in the keratinocytes) and microphthalmia-associated transcription factor (MITF) (in the melanocytes) expression and a corresponding upregulation of c-Kit, tyrosinase activity, gp100, follicular melanogenesis and normal transfer of melanosomes to the adjacent keratinocytes.

(**b**) A state of P-cadherin absence (due to silencing, down regulation or mutation as in HJMD patients). P-cadherin knockdown in both melanocytes and keratinocytes results in increased degradation of β-catenin and reduced β-catenin target genes expression. This causes reduced MITF expression in the melanocytes, while in the keratinocytes probably reduced SCF is expressed and secreted with the correspondence down regulation of c-Kit on the surface of adjacent melanocytes. Reduction of c-Kit expression and signaling reduces MITF expression and phosphorylation of cAMP responsive element binding protein (CREB), another cause for MITF down regulation, unrelated to the canonical Wnt signaling pathway. Reduction in MITF expression results in down regulation of tyrosinase activity, gp100 expression and hair follicle (HF) melanogenesis. In addition, P-cadherin slencing results in reduced melanosome transfer to the adjacent keratinocytes in the HF pigmentary unit.

ME – melanosome; MITF - microphthalmia-associated transcription factor; P-CREB – phosphorylated cAMP responsive element binding protein; P-cad. – P-cadherin; SCF – stem cell factor.

**Supplementary Table S1**

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| Primary antibody | clone | Origin | References | Ig class | Dilution | Company | Secondary detection system |
|---|---|---|---|---|---|---|---|
| P-cadherin | 56 | Mouse | (Kovacs and Walker, 2003; Paredes *et al.*, 2005) | IgG1 | 1:50 | BD Transduction Laboratories, Heidelberg, Germany | LSAB-PR |
| β-catenin | 14 | Mouse | (Tsuji *et al.*, 2001) | IgG1 | 1:50 | BD Transduction Laboratories, Heidelberg, Germany | LSAB-PR, IF-FITC |
| GSK3β | 7 | Mouse | (Bijur and Jope, 2001; Lucas *et al.*, 2001) | IgG1 | 1:20 | BD Transduction Laboratories, Heidelberg, Germany | IF-FITC |
| Phospho-β-catenin (Ser33/37/Thr41) | | Rabbit | (Kielhorn *et al.*, 2003; Nakopoulou *et al.*, 2006) | PA | 1:50 | Cell Signaling, Danvers, MA, USA | LSAB-PR, IF-FITC |
| SCF | hKL12 | Mouse | (Puputti *et al.*, 2010; Sihto *et al.*, 2007) | IgG1 | 1:20 | Acris antibodies, Hiddenhausen, Germany | IF-FITC |
| gp100 | NKI/beteb | Mouse | (Dhaene *et al.*, 2000; Kloepper *et al.*, 2010) | IgG2b | 1:30 | Monosan, Uden, Netherlands | IF - Rh |
| MITF | D5 | Mouse | (Zhuang *et al.*, 2007) | IgG1 | 1:50 | Dako, Glostrup, Denmark | IF-FITC |
| ACTH | | Rabbit | (Sakai *et al.*, 2003; Slominski *et al.*, 1993) | PA | 1:30 | Chemicon, Temecula, CA, USA | IF-FITC |
| c-Kit | YR145 | Rabbit | (Vila *et al.*, 2009) | IgG | 1:1000 | Cell Marque, Rocklin, CA, USA | TSA |
| α-MSH | | Rabbit | (Kono *et al.*, 2001) | PA | 1:500 | Sigma, Munich, Germany | TSA |

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**Supplementary Table S1.** Antibodies used for immunohistological stainings are listed and described in detail. α-MSH - α-Melanocyte-stimulating hormone; ACTH - Adrenocorticotropic hormone; FITC - fluorescein isothiocyanate; GSK3β - Glycogen synthase kinase 3 beta; IF - immunofluorescence; LSAB-PR - Labeled Streptavidin Biotin Peroxidase; MITF - microphthalmia-associated transcription factor; PA – polyclonal antibody; Rh - rhodamine; SCF - stem cell factor; TSA - tyramide signal amplification conjugated.

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# P-Cadherin Regulates Human Hair Growth and Cycling via Canonical Wnt Signaling and Transforming Growth Factor-β2

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P-cadherin is a key component of epithelial adherens junctions, and it is prominently expressed in the hair follicle (HF) matrix. Loss-of-function mutations in *CDH3*, which encodes P-cadherin, result in hypotrichosis with juvenile macular dystrophy (HJMD), an autosomal recessive disorder featuring sparse and short hair. Here, we attempted to recapitulate some aspects of HJMD *in vitro* by transfecting normal, organ-cultured human scalp HFs with lipofectamine and *CDH3*-specific or scrambled control siRNAs. As in HJMD patients, P-cadherin silencing inhibited hair shaft growth, prematurely induced HF regression (catagen), and inhibited hair matrix keratinocyte proliferation. *In situ*, membrane β-catenin expression and transcription of the β-catenin target gene, axin2, were significantly reduced, whereas glycogen synthase kinase 3 β (GSK3β) and phospho-β-catenin immunoreactivity were increased. These effects were partially reversed by inhibiting GSK3β. P-cadherin silencing reduced the expression of the anagen-promoting growth factor, IGF-1, whereas that of transforming growth factor β 2 (TGFβ2; catagen promoter) was enhanced. Neutralizing TGFβ antagonized the catagen-promoting effects of P-cadherin silencing. In summary, we introduce human HFs as an attractive preclinical model for studying the functions of P-cadherin in human epithelial biology and pathology. This model demonstrates that cadherins can be successfully knocked down in an intact human organ *in vitro*, and shows that P-cadherin is needed for anagen maintenance by regulating canonical Wnt signaling and suppressing TGFβ2.

## INTRODUCTION

P-cadherin is a member of the classical cadherin family and a key component of adherens junctions in various epithelia, including the epidermis and hair follicles (HFs; Shimoyama *et al.*, 1989; Andl *et al.*, 2002; Paredes *et al.*, 2007). It has major roles in cell recognition and signaling, morphogenesis, and tumor development (Goodwin and Yap, 2004; Paredes *et al.*, 2007; Fanelli *et al.*, 2008). It comprises five extracellular domains, a transmembrane domain, and a small

intracellular domain that interacts with β-catenin. The latter is a crucial factor in the canonical Wnt signaling pathway (Nelson and Nusse, 2004; Fanelli *et al.*, 2008; Heuberger and Birchmeier, 2010; for details, see Figure 1a). As p63 directly interacts with the promoter for *CDH3*, the gene that encodes P-cadherin, the latter is a p63 target gene (Shimomura *et al.*, 2008).

*CDH3* mutations cause two autosomal recessive disorders, hypotrichosis with juvenile macular dystrophy (HJMD;OMIM 601553) and ectodermal dysplasia, ectrodactyly and macular dystrophy (EEM;OMIM 225280), which are associated with hair abnormalities. Dermatologically, these patients show sparse and short hair throughout life, an increased percentage of HFs in the regression (catagen) or resting stage (telogen) of the hair cycle, and abnormal hair shafts (Supplementary Figure S1a–d online; Sprecher *et al.*, 2001; Indelman *et al.*, 2002, 2007; Bergman *et al.*, 2004; Kjaer *et al.*, 2005; Leibu *et al.*, 2006; Shimomura *et al.*, 2008, 2010; Jelani *et al.*, 2009). Although this suggests that P-cadherin is required for normal hair growth, it remains unknown how P-cadherin impacts the human HFs. Unfortunately, P-cadherin-deficient mice fail to recapitulate the HJMD phenotype (Radice *et al.*, 1997). This suggests that the role of P-cadherin-mediated signaling in human epithelial biology and pathology is best studied directly in human HFs.

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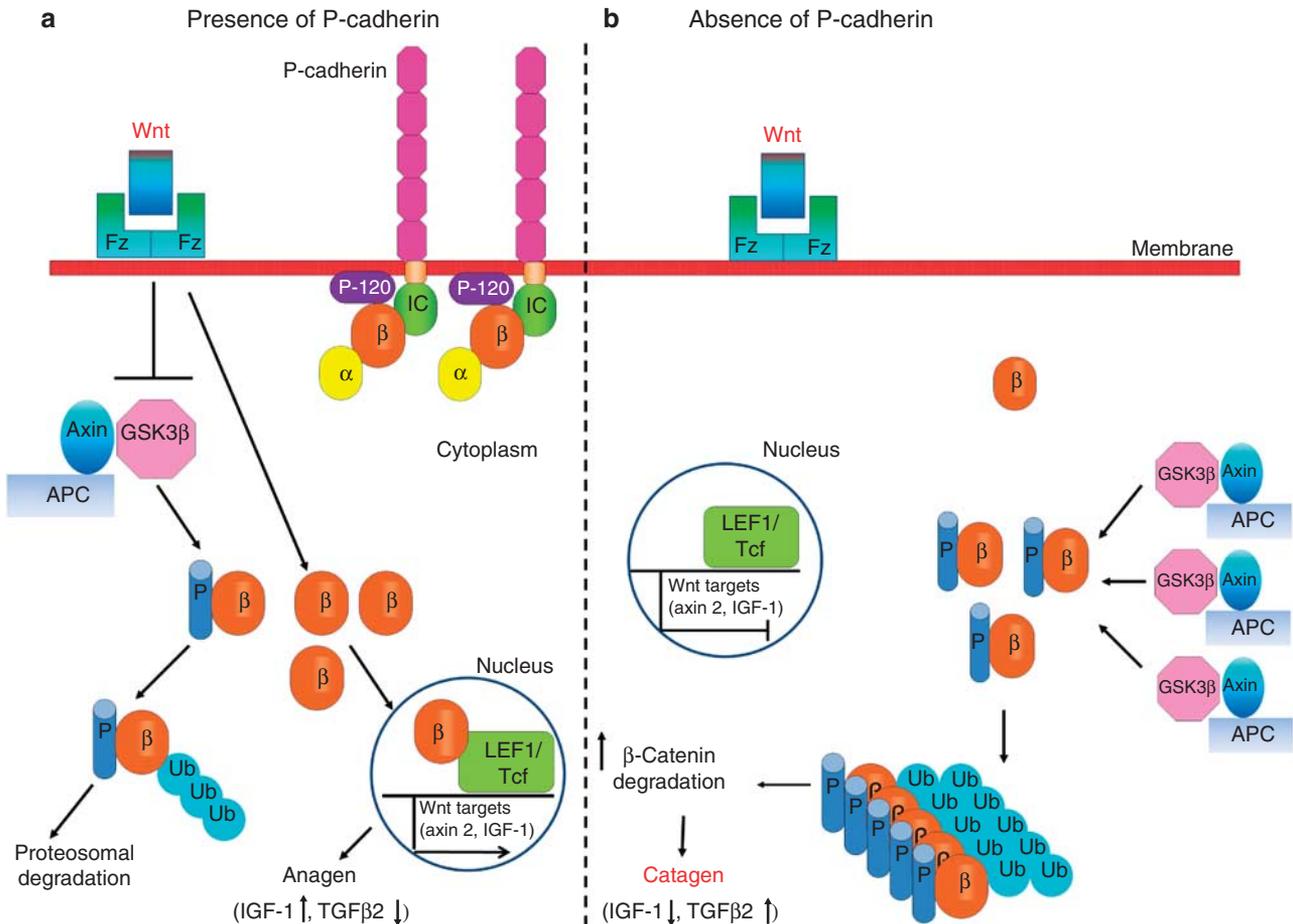
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**Figure 1. Working hypothesis: P-cadherin regulates human hair growth via the canonical Wnt signaling pathway through β-catenin.** (**a**) β-Catenin is found in epithelial cells either bound to the cytoplasmic domain of classical cadherins or free in the cytoplasm. In the absence of Wnt ligands, excess cytoplasmic β-catenin is recruited to a destruction complex that contains adenomatous polyposis coli (APC), axins, and glycogen synthase kinase 3 β (GSK3β). β-Catenin gets phosphorylated by this complex and is targeted for proteosomal degradation. In the presence of Wnt ligands, the destruction complex is inactivated. This leads to an increase of cytoplasmic β-catenin and its translocation into the nucleus, where it binds lymphoid enhancer–binding factor 1 (LEF1) and T-cell factor (Tcf). β-Catenin/LEF/Tcf complexes control the expression of genes involved in the control of hair morphogenesis and cycling, including axin2 and IGF1. For greater simplicity, the reported regulation of P-cadherin by p63 (Shimomura *et al.*, 2008) is not shown here. (**b**) The absence of P-cadherin. Canonical Wnt signaling and cadherin-mediated cell adhesion depend on the same cytoplasmic pool of β-catenin (Gottardi and Gumbiner, 2001). P-cadherin absence (P-cadhein knockdown or in hypotrichosis with juvenile macular dystrophy patients) results in over-degradation of cytoplasmic β-catenin and reduced β-catenin-dependent Wnt target gene expression. α, α-catenin; β, β-catenin; FZ, Frizzled; IC, intracytoplasmic domain of P-cadherin; P-phospho; TGFβ, transforming growth factor β; Ub, ubiquitin.

To do so, we have generated a previously unreported, widely accessible *in vitro*-surrogate model of HJMD, based on the use of organ-cultured human scalp HFs, a prototypic ectodermal–mesodermal interaction unit (Paus and Cotsarelis, 1999; Schneider *et al.*, 2009). Although, evidently, a complex human genetic disorder cannot be expected to be fully recreated *in vitro*, we hoped that such an *in vitro* model could shed new light on how the hair phenotype develops in HJMD patients and on the as yet obscure role of P-cadherin signaling in human epithelial biology. Specifically, we asked how P-cadherin silencing in organ-cultured human HFs affects hair growth, cycling, and hair shaft formation. In addition, we aimed to obtain indications as to which signaling pathway(s) P-cadherin may use to mediate its HF effects.

## RESULTS

### P-cadherin expression is strongest in the innermost hair matrix of human anagen HFs

First, we established the protein expression pattern of P-cadherin in fully growing human HFs (i.e., in the anagen VI stage of the hair cycle). Immunohistology showed that, just as in mice (Muller-Rover *et al.*, 1999), P-cadherin protein is most prominently expressed in the innermost cell layer of the human hair matrix (Figure 2a). This confirms the intrafollicular P-cadherin expression pattern previously described by Fujita *et al.* (1992). However, prominent P-cadherin immunoreactivity was also seen throughout the human epidermis, with sharply reduced expression in its proliferation compartment, i.e., the basal layer of the epidermis (Supplementary Figure S2a online). This is in contrast with previous
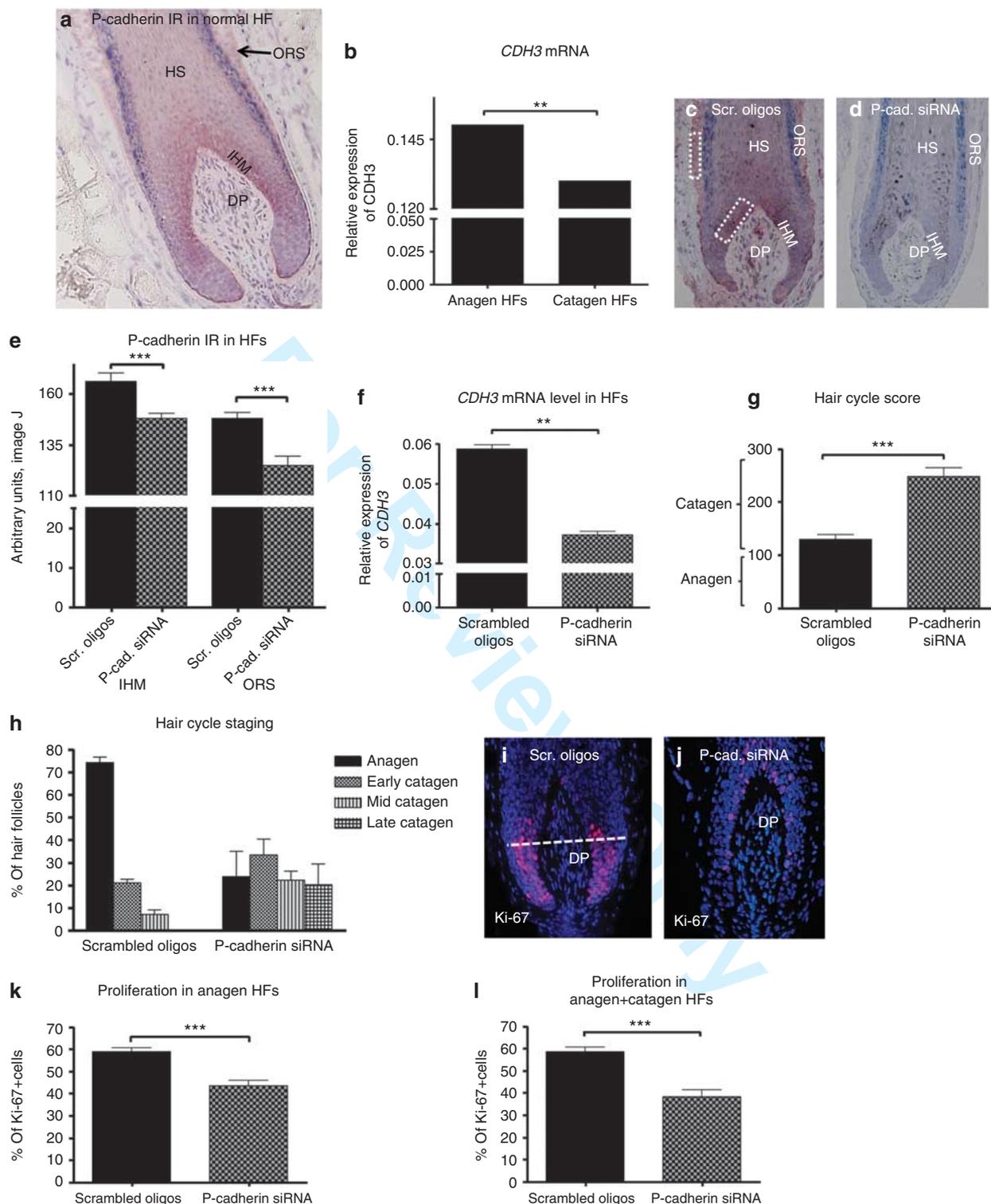
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**Figure 2. Constitutive P-cadherin expression and P-cadherin knockdown in normal human hair follicles (HFs).** (**a**) P-cadherin immunoreactivity (IR; red) in anagen VI human HF. (**b**) Significantly reduced *CDH3* transcript steady-state levels in catagen HFs (RNA from anagen and catagen HFs, cultured for 8 days). **$P < 0.01$, Student's *t* test for unpaired samples. (**c–e**) P-cadherin knockdown: IR is reduced in the innermost hair matrix (IHM) and outer root sheath (ORS) of P-cadherin siRNA-treated HFs. (**e**) Data were pooled from two highly comparable, independent experiments. (**c**) Dotted lines represent reference areas of measurement. ***$P < 0.001$, Mann–Whitney test, $n = 17$–23. (**f**) Reduced *CDH3* mRNA level in P-cadherin siRNA-treated HFs. **$P < 0.01$, Student's *t* test for unpaired samples. (**g**, **h**) Hair cycle staging was assessed by Ki-67 staining and quantitative Masson–Fontana histochemistry. Data are presented as hair cycle score (HCS; score represents the mean hair cycle stage of all HFs per treatment group). ***$P < 0.0001$, Mann–Whitney test. (**i–l**) Significantly higher keratinocyte proliferation in HFs treated with (**i**, **k**, and **l**) scrambled oligos, compared with (**j**, **k**, and **l**) P-cadherin siRNA-treated HFs. Only anagen HFs ($n = 11$–18) and also anagen and catagen HFs ($n = 20$–21) were counted below Auber's line (dotted line). ***$P < 0.001$, Mann–Whitney test. DP, dermal papilla; HS, hair shaft.

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reports in both mice and humans that state that P-cadherin expression is largely confined to the epidermal basal layer (Hirai *et al.*, 1989; Fujita *et al.*, 1992).

### P-cadherin expression declines during catagen

As hair cycle–dependent changes in the expression level of molecules during the HF transformation from growth (anagen) to apoptosis-driven HF regression (catagen) frequently indicate a functional role of the molecule under investigation during this organ transformation process (Stenn and Paus, 2001; Schneider *et al.*, 2009), we also examined whether constitutive P-cadherin expression differs between human anagen VI and catagen HFs. Indeed, P-cadherin expression sharply declines during spontaneous, apoptosis-driven HF regression (catagen), both at the protein (Supplementary Figure S2b–c online) and transcript steady state (Figure 2b) levels. This regulated, hair cycle–dependent intrafollicular P-cadherin expression pattern suggests a functional role for P-cadherin during the anagen–catagen transformation, the clinically most important phase in HF cycling (Paus and Foitzik, 2004; Schneider *et al.*, 2009).

### P-cadherin knockdown is possible in human HFs and human skin organ culture

Next, we attempted to silence P-cadherin expression in organ-cultured human anagen scalp HFs (Kloepper *et al.*, 2010) and full-thickness human scalp skin fragments (Lu *et al.*, 2007). P-cadherin gene knockdown was performed by transfection with *CDH3*-specific or scrambled control siRNAs, using lipofectamine. Successful *in situ* knockdown of P-cadherin was documented at the gene and protein levels in both organ-cultured human scalp HFs (Figure 2c–f) and the epidermis (Supplementary Figure S2d–g online). No significant difference in E-cadherin expression was documented following P-cadherin knockdown, indicating the specificity of silencing (Supplementary Figure S2h–j).

To the best of our knowledge, successful knockdown of a cadherin family member in an intact human organ has not previously been reported. This invites exploitation of organ-cultured human HFs and skin as attractive preclinical models for dissecting the molecular and cellular consequences of P-cadherin loss of function *in situ*. Availability of such an experimental model constitutes a significant methodological advance, as the functions of P-cadherin mediated signaling in human epithelial physiology are as yet ill-understood (Faraldo *et al.*, 2007; Shimomura *et al.*, 2008; Bandara *et al.*, 2010; Jacobs *et al.*, 2011).

### P-cadherin silencing results in premature catagen induction and hair growth inhibition

As the prominent clinical and histological features of HJMD patients are short and sparse hair since birth, associated with miniaturization and telogen HFs (Supplementary Figure S1a–d), we next examined whether P-cadherin knockdown results in any changes of human hair growth and/or cycling. As assessed by quantitative hair cycle histomorphometry and Ki-67 immunohistomorphometry, this was the case: *in vitro* P-cadherin knockdown prematurely induced catagen

development in microdissected organ-cultured human anagen VI scalp HFs (Figure 2g–l). This was independently confirmed by quantitative Masson–Fontana histochemistry (Kloepper *et al.*, 2010), which revealed the expected sharp reduction in the HF melanin content (data not shown) as a result of the catagen-associated switch-off of intrafollicular melanogenesis (Slominski *et al.*, 2005). Furthermore, P-cadherin silencing *in vitro* resulted in significant inhibition of hair shaft growth (Figure 3).

Therefore, P-cadherin knockdown in healthy human scalp HFs *in vitro* partially recapitulates the hair growth inhibition that is seen in HJMD patients with mutated *CDH3 in vivo* (Sprecher *et al.*, 2001). This was associated with a significant inhibition of hair matrix keratinocyte proliferation in catagen and anagen HFs (see Figure 2k–l), which explains the reduced rate of hair shaft formation and is in line with the observed premature catagen development in P-cadherin-silenced human HFs.

### Ultrastructurally, P-cadherin silencing causes HF dystrophy

The hair growth inhibitory effect of P-cadherin silencing *in situ* was followed up at the ultrastructural level. Transmission electron microscopy showed that, compared with scrambled-control-treated HFs, P-cadherin-silenced HFs contain large, abnormal aggregates of keratin clumps inside the cytoplasm of precortical hair matrix keratinocytes (Supplementary Figure S3a–b online), whereas no ultrastructural abnormalities were detected in the hair shaft cortex and medulla, except for narrower intercellular spaces (Supplementary Figure S3c–f online).

Quantitative TUNEL immunohistomorphometry showed that the number of apoptotic keratinocytes in the precortical hair matrix is not significantly higher than that in control HFs treated with scrambled oligos (data not shown). Therefore, the observed keratin clumping phenomenon suggests a defect in the correct intracellular transport and arrangement of keratin filaments in the absence of adequate P-cadherin levels/signaling, and is unlikely to result indirectly from enhanced, catagen-associated hair matrix keratinocyte apoptosis. The concept of a defect in keratin transport and arrangement is also in line with the observed HF dystrophy and reduced hair shaft formation.

### P-cadherin silencing impairs normal hair keratin expression

To obtain additional pointers to how P-cadherin silencing may impair hair shaft formation and may cause HF dystrophy, gene expression profiling was performed. Q-PCR analysis revealed that three different hair keratins (KRT36, KRT37, and KRT84) are significantly upregulated by P-cadherin silencing (Supplementary Figure S4a–c online). Although this remains to be functionally tested, this abnormal hair keratin expression profile may relate to the observed hair shaft inhibition *in vitro* (Figure 3) and/or to the abnormal hair phenotype seen in HJMD patients *in vivo* (Supplementary Figure S1a–b online; Sprecher *et al.*, 2001; Bergman *et al.*, 2004). These transcriptional observations, which need to be confirmed on the protein level, make it conceivable that, in the absence of functional P-cadherin signaling, overexpression and/or
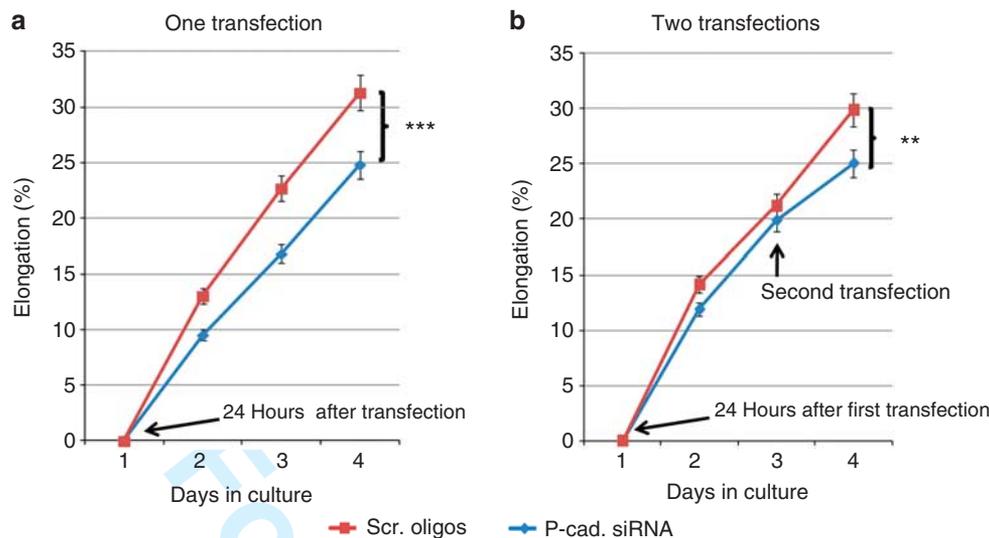
**Figure 3. P-cadherin (P-cad.) knockdown inhibits hair shaft production in organ culture *in situ*.** (**a**) Reduced hair shaft elongation after P-cad. silencing. Data were pooled from four highly comparable, independent experiments, with one transfection at day 0; $n = 107–110$ in each group. (**b**) No additive effect of repeated knockdown on hair shaft elongation. Data were pooled from two highly comparable, independent experiments, with two transfections with P-cad. siRNA or scrambled (Scr.) oligos at days 0 and 3 of culture; $n = 63–66$ in each group. Note the separation of graphs after the second transfection. Growth rates are expressed as percent difference over day 1. Significance refers to the growth rate of hair follicles (HFs) on day 4, which had been treated with Scr. oligos. ***$P < 0.0001$; **$P < 0.01$, HFs treated with P-cad. siRNA versus Scr. oligos-treated HFs, Mann–Whitney test.

miscombination of selected hair shaft keratins can impair hair shaft structure and resilience.

### P-cadherin silencing affects the expression of prominent hair cycle regulators

Subsequently, we explored whether the P-cadherin silencing–induced hair cycle abnormality goes along with changes in two key hair cycle regulatory growth factors, i.e., insulin-like growth factor-1(IGF-1) and transforming growth factor β 2 (TGFβ2; Philpott *et al.*, 1994; Stenn and Paus, 2001; Soma *et al.*, 2002; Hibino and Nishiyama, 2004; Weger and Schlake, 2005; Schneider *et al.*, 2009). The steady-state mRNA level of IGF-1, the key growth factor that maintains HFs in anagen (Philpott *et al.*, 1994; Ben Amitai *et al.*, 2006; Kwack *et al.*, 2009; Zhao *et al.*, 2011), significantly declines after P-cadherin knockdown (Figure 4a).

In contrast, the immunoreactivity of TGFβ2, the key catagen-inducing growth factor (Hibino and Nishiyama, 2004), significantly increases in P-cadherin-silenced HFs (Figure 4b–d). This TGFβ upregulation is functionally important, as the effects of P-cadherin knockdown on HF growth and cycling can be partially reversed by culturing P-cadherin-silenced HFs in the presence of TGFβ-neutralizing antibody (Supplementary Figure S5a–d online). Thus, P-cadherin-mediated signaling may be required to maintain human HFs in anagen, at least in part, via suppressing TGFβ2 expression and by maintaining sufficient IGF-1 levels.

### P-cadherin silencing regulates canonical Wnt signaling

P-cadherin and the Wnt signaling pathway rely on the same pool of cytoplasmic β-catenin (Gottardi and Gumbiner, 2001;

Kuphal and Behrens, 2006; Heuberger and Birchmeier, 2010; Figure 1a), and adequate Wnt signaling is thought to be important for anagen maintenance in murine HFs (Van Mater *et al.*, 2003; Shimizu and Morgan, 2004; Schneider *et al.*, 2009; Li *et al.*, 2011). Therefore, we finally investigated whether P-cadherin silencing affects β-catenin stabilization and Wnt target gene expression in human keratinocytes of the innermost hair matrix, i.e., the site of maximal intrafollicular P-cadherin expression (see Figure 2a). To this end, β-catenin and glycogen synthase kinase 3 β (GSK3β) expression was assessed immunohistologically.

After intrafollicular P-cadherin silencing, membrane β-catenin immunoreactivity is significantly reduced, whereas GSK3β and phospho-β-catenin protein expression is significantly increased *in situ* (Figure 5a–g). Therefore, P-cadherin silencing in keratinocyes of the innermost hair marix significantly upregulates β-catenin degradation, likely through GSK3b. This is expected to result in reduced active β-catenin available for the Wnt signaling pathway (Figure 1b). Further evidence of reduced β-catenin activity by P-cadherin knockdown was obtained by demonstrating that transcription of a key β-catenin target gene, axin 2 (Lovatt and Bijlmakers, 2010), is reduced in silenced HFs (Figure 5h). These effects (i.e., axin2 downregulation and phospho-β-catenin upregulation following P-cadherin knockdown) are partially reversed by culturing P-cadherin-silenced HFs in the presence of the potent GSK3β inhibitor, lithium chloride (Tighe *et al.*, 2007; Figure 5i–k).

Taken together, these experiments demonstrate P-cadherin to be a key regulator of canonical Wnt signaling in human HFs, whose maintenance appears to be critical for sustaining HFs in their growth phase (anagen).
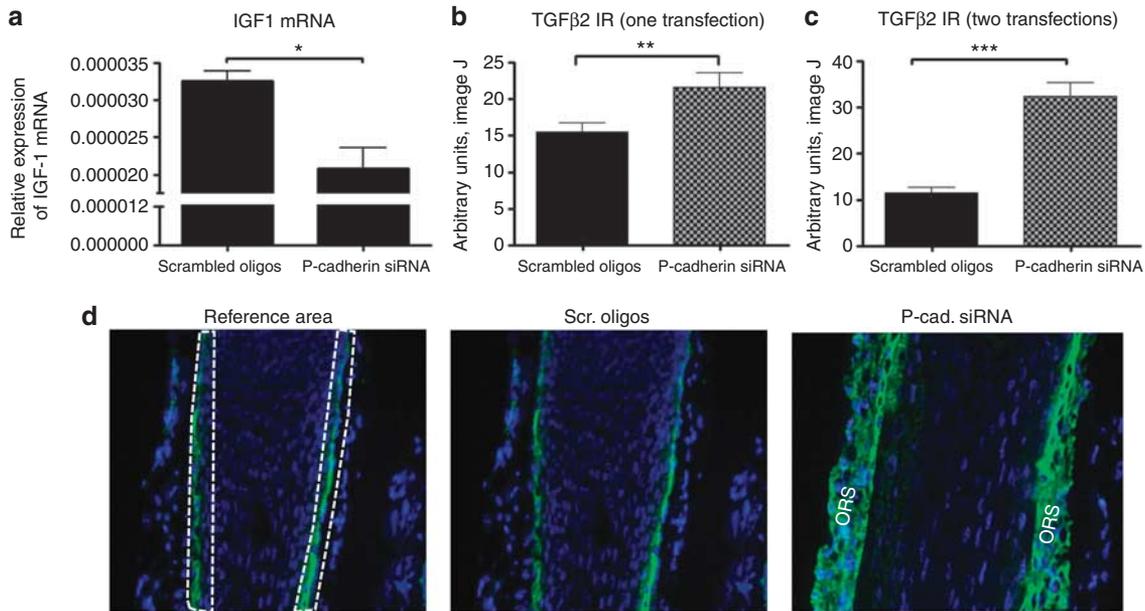
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**Figure 4. P-cadherin knockdown reduces IGF-1 and increases transforming growth factor β2 (TGFβ2) expression.** (**a**) Reduced intrafollicular IGF-1 transcript level in the P-cadherin siRNA-treated hair follicles (HFs) compared with scrambled oligo-treated HFs. *$P<0.05$, Student's *t* test for unpaired samples. (**b**) Increased intrafollicular TGFβ2 expression after P-cadherin silencing. Pooled data from three highly comparable experiments with one transfection on day 0. **$P<0.01$, Mann–Whitney test, $n=25–35$. (**c**) Increased intrafollicular TGFβ2 expression after P-cadherin silencing. The graph represents data obtained from one experiment in which HFs were transfected twice, at days 0 and 3 of culture. ***$P<0.0001$, Mann–Whitney test, $n=8–10$. (**d**) Higher TGFβ2 protein immunoreactivity (IR) in the outer root sheath of the P-cadherin siRNA-treated HFs. The dotted lines in the left picture represent the area of measurement and evaluation. ORS, outer root sheath.

## DISCUSSION

Here we provide early evidence that P-cadherin protein expression and its presence in adherens junctions is essential for normal epithelial growth in an intact human organ. Specifically, we document that P-cadherin operates as a potent regulator of human hair shaft formation and hair growth. It has not been shown directly before that a member of the cadherin family has an essential regulatory role in the control of normal human HF growth and cycling.

Our data suggest that P-cadherin maintains human HFs in anagen through activation of canonical Wnt signaling and operates as a major molecular brake on HF regression (catagen) by inhibiting GSK3β-mediated β-catenin degradation, thereby ensuring active canonical Wnt signaling (Figure 1a). This is well in line with the increasing recognition that canonical Wnt signaling is crucial for murine hair growth (Huelsken *et al.*, 2001; Andl *et al.*, 2002; Millar, 2002; Van Mater *et al.*, 2003; Shimizu and Morgan, 2004; Schneider *et al.*, 2009; Baker *et al.*, 2010; Li *et al.*, 2011). This concept is supported by our findings that P-cadherin maintains expression of the anagen-promoting growth factor IGF-1, whereas it suppresses the chief catagen-promoting growth factor TGFβ2.

The current experiments suggest that if normal P-cadherin-mediated signaling is antagonized by gene silencing, or becomes dysfunctional owing to *CDH3* gene mutations (HJMD), this results in a decrease of membrane-bound β-catenin. The latter is likely released from the cadherin–catenin complex in hair matrix keratinocytes and is targeted for ubiquitination and degradation, as attested by the observed upregulation of GSK3β and phospho-β-catenin after P-cadherin silencing. This, in turn, is expected to sharply reduce the amount of cytoplasmic β-catenin available for canonical Wnt signaling, thus resulting in the downregulation of key β-catenin target genes required for anagen maintenance (for details, see Figure 1b) (Huelsken *et al.*, 2001; Andl *et al.*, 2002; Schneider *et al.*, 2009; Baker *et al.*, 2010).

P-cadherin knockdown did not reveal any significant change of intraepidermal β-catenin expression, compared with human skin fragments treated with scrambled oligos (Supplementary Figure S6 online). This suggests that the effects of P-cadherin silencing on β-catenin (and Wnt signaling) are HF specific and do not necessarily apply to other compartments of the human skin epithelium that normally express P-cadherin (Supplementary Figure S2a online). The principle that cadherin family members can impact Wnt activity is further supported by recent studies that demonstrate that *in vivo* knockdown of N-cadherin reduces β-catenin signaling and that E-cadherin depletion diminishes Wnt-related transcriptional activity (Casagolda *et al.*, 2010; Zhang *et al.*, 2010; Howard *et al.*, 2011).

Although it is extremely difficult to obtain sufficient amounts of human HFs to permit western blot analyses of inner hair matrix proteins, the latter would be desirable to independently confirm the quantitative immunohistomorphometry *in situ* data on phospho-β-catenin presented here by quantitative biochemical evidence.
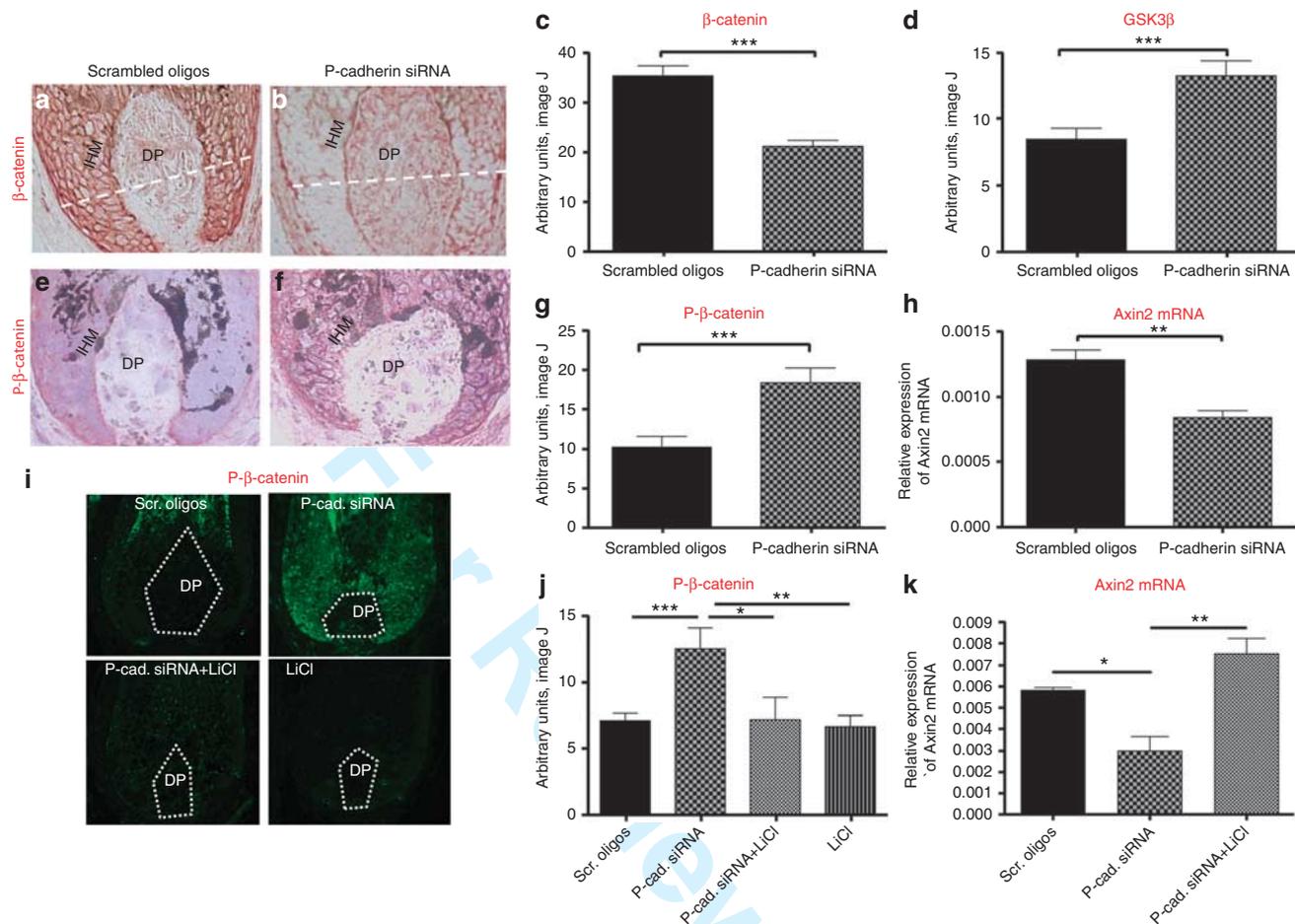
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**Figure 5. P-cadherin (P-cad.) knockdown increases degradation of β-catenin and reduces the expression of β-catenin target genes.** (a–c) Significantly reduced β-catenin immunoreactivity (IR) below Auber's line (dotted line) following P-cad. silencing. Pooled data from two experiments. ***$P < 0.0001$, Mann–Whitney test, $n = 19$–23. (d–g) Significantly increased glycogen synthase kinase 3 β (GSK3β) and phospho-β-catenin (P-β-catenin) IR in the innermost hair matrix (IHM). For GSK3β and P-β-catenin IR, the graphs represent data taken from two highly comparable experiments in which hair follicles (HFs) were transfected once. Evaluation was carried out by quantification of IR staining in the IHM with either an anti-P-β-catenin or an anti-GSK3β antibody (for P-β-catenin, ***$P < 0.001$, $n = 17$–18; for GSK3β ***$P < 0.001$, $n = 16$–21; Mann–Whitney test). All data include anagen and catagen HFs. (h, k) *Axin2* mRNA levels are significantly downregulated in HFs treated with P-cad. siRNAs (h). Culturing siRNA-treated HFs with lithium chloride (LiCl) significantly reversed the effect of P-cad. silencing on *Axin2* transcript level (k). **$P < 0.01$, *$P < 0.05$, Student's *t* test for unpaired samples. (i–j) P-β-catenin IR in HFs treated with either scrambled (Scr.) oligos, P-cad. siRNA, P-cad. siRNA + LiCl, or LiCl (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Mann–Whitney test, $n = 8$–11). DP, dermal papilla.

The observed keratin clumping (Supplementary Figure S3a online) may result from defective intracellular transport and arrangement of keratin filaments in the absence of adequate P-cadherin levels/signaling. This fits well to the observed HF dystrophy and reduced hair shaft formation, and is in line with the concept that P-cadherin is a direct p63 target gene (Shimomura *et al.*, 2008), as p63 constitutes a crucial control of normal keratinization (Kim *et al.*, 2009; Mikkola *et al.*, 2010). Perhaps this p63 connection also explains the abnormal hair keratin gene expression seen after P-cadherin silencing (Supplementary Figure S4a–c online).

Just as in murine HFs (Muller-Rover *et al.*, 1998), P-cadherin is the only classical cadherin that is physiologically expressed in the innermost hair matrix, i.e., at the key epithelial interface with the inductive HF mesenchyme (Figure 2a, Supplementary Figure S2h–j online). Therefore, it is conceivable that this special epithelial HF compartment depends on adequate P-cadherin signaling for normal epithelial cell function and anagen maintenance. This may render hair matrix keratinocyte proliferation, terminal keratinocyte differentiation in the precortical hair matrix, and consequently hair shaft formation from this cell pool very sensitive to P-cadherin silencing and P-cadherin genetic dysfunction.

Our data also suggest that the innermost cell layer of the matrix has the capacity to regulate the human hair cycle independently of epithelial stem cells in the—much more distally located—HF bulge (which is absent under the current HF organ culture conditions). Therefore, this region of the hair matrix must generate as yet unknown P-cadherin-dependent signals that impact on whether a HF stays in anagen or enters into catagen and which translate into changes in the intrafollicular balance between anagen-maintaining and catagen-promoting growth factors (e.g., IGF-1 vs. TGFβ2).

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To elucidate these P-cadherin-dependent signals that have an impact on and/or emanate from the innermost hair matrix, certainly, is an important future research target. Moreover, the present study paves way for the development of a potential new class of human hair growth inhibitors, namely P-cadherin inhibitory agents, as a previously unreported strategy for the management of hirsutism.

P-cadherin expression was also demonstrated in retinal pigment epithelium of mice (Xu et al., 2002) and humans (Burke et al., 1999). Therefore, it is interesting to note that a key feature of HJMD patients is progressive macular degeneration leading to blindness in the third decade of life. This raises the intriguing, speculative question whether P-cadherin may have an important role in the—insufficiently understood—pathogenesis of age-related macular degeneration, the leading cause of blindness in the adult population in the Western world (Gragoudas et al., 2004).

The effects of P-cadherin silencing in normal adult human scalp HFs *in vitro* correspond well to the hair phenotype of patients with HJMD and EEM syndromes, who characteristically exhibit an abnormally high percentage of catagen HFs and show retarded/reduced hair shaft formation (Sprecher et al., 2001; Bergman et al., 2004). Therefore, our study also provides a clinically relevant *in vitro*-surrogate model for studying (a) the general consequences of P-cadherin loss of function in human epithelial biology *in situ* and (b) the pathomechanisms that underlie the complex and as yet ill-understood hair phenotypes seen in HJMD and EEM patients.

## MATERIALS AND METHODS

### Tissue collection

Human anagen VI HFs were isolated from scalp skin obtained from six patients undergoing routine face-lift surgery (one man and five women aged 42–59 years; mean age 50 years) after written, informed consent and adherence to the Declaration of Helsinki Principles.

### P-cadherin knockdown

In four HF cultures, HFs were transfected once with human P-cadherin siRNA (Sigma-Aldrich, Munich, Germany, SA-SI_Hs02_00332561, 5′-CCAAUAUCUGUCCCUGAAA-3′) at day 0, whereas in two additional cultures HFs were transfected twice, i.e., at days 0 and 3. HFs treated with control siRNA (scrambled oligos; Santa Cruz Biotechnology, Santa Cruz, CA, sc-37007) served as control. After transfection (and between the two transfections in the two additional cultures mentioned above), HFs were maintained in a 24-well plate with 500 μl William's E medium (Biochrom, Cambridge, UK) supplemented with 1% L-glutamine (Invitrogen, Paisley, UK), 0.02% hydrocortisone (Sigma-Aldrich), 0.1% insulin (Sigma-Aldrich), and 1% antibiotic/antimycotic mixture (Gibco, Karlsruhe, Germany; Philpott et al., 1990). All reagents required for transfection were obtained from Santa Cruz Biotechnology (siRNA transfection reagent, sc-29528; siRNA transfection medium, sc-36868). HF transfection was performed according to the manufacturer's protocol (Santa Cruz Biotechnology).

### Human HF and skin organ culture

Normally pigmented anagen VI HFs were microdissected and organ-cultured under serum-free conditions in insulin- and hydrocortisone-supplemented William's E medium (Philpott et al., 1990; Kloepper et al., 2010). Six HF organ cultures were prepared (each derived from a separate patient; total HF number in each culture: 52–132). Two full-thickness human skin organ cultures from two different patients (aged 58 and 77 years) were also prepared as described elsewhere (Lu et al., 2007; Bodo et al., 2010).

For each experiment, HFs were equally divided between those treated with P-cadherin siRNA (test) or scrambled oligos (control). In one culture, culture medium was also supplemented with 40 mM lithium chloride (Sigma, Munich, Germany; either as the only treatment, or 5–7 hours after transfection) or 5 μg ml$^{-1}$ TGFβ-neutralizing antibody (monoclonal anti-TGFβ1-3 antibody; R&D systems, Wiesbaden, Germany; either as the only treatment or simultaneously with the transfection). Lithium chloride was added only once to the culture medium (5–7 hours after transfection), and HFs were embedded 24 hours afterward. TGFβ-neutralizing antibody was added first, 5–7 hours after transfection, and a concentration of 5 μg ml$^{-1}$ was added every day in a 4-day culture period.

Hair shaft length was measured daily using an inverted binocular microscope (Philpott et al., 1990), and culture medium was replaced immediately after the transfection and then every other day. On days 2 and 4 (and also on day 3 in the two transfection cultures), samples of culture medium were taken for measuring the lactate dehydrogenase (LDH) level as a general cell toxicity parameter in human skin organ culture (Lu et al., 2007). This showed no significant increase in the LDH release of P-cadherin-silenced HFs compared with HFs treated with scrambled oligo controls after a single transfection, but demonstrated the expected rise in LDH levels after repeated siRNA treatment (data not shown). This indicates minimal toxicity after a single transfection, whereas repeated transfection carries the expected risk of increased toxicity. At 24 hours after the transfection (on day 1 in the one transfection cultures and on day 4 in the two transfection cultures), 12–25 HFs from each culture were frozen in liquid nitrogen for RNA extraction and mRNA analysis. The rest of the HFs in each culture were embedded in Shandon Cryomatrix (Pittsburgh, PA) and snap-frozen in liquid nitrogen on day 4 of culture. HFs that were treated with lithium chloride were embedded 24 hours after the beginning of treatment.

Five to seven 6-μm-thick cryosections of organ-cultured human HFs were prepared for subsequent immunohistology, and were stored at −80 °C until use.

In the two skin organ cultures, 2-mm full-thickness skin punches were transfected with P-cadherin siRNA and control siRNA at day 0 (six pieces per group) using a similar protocol as that used for HF organ culture. In one culture, 24 hours after transfection, skin punches were frozen in liquid nitrogen for RNA extraction and mRNA analysis. In the second culture, skin punches were embedded in Shandon Cryomatrix and snap-frozen in liquid nitrogen on day 4 of culture. Cryosections (7 μm thick) of skin punches were cut for immunohistological staining.

The efficacy of gene silencing was evaluated using P-cadherin-specific immunohistochemistry and Q-PCR, both in the HF and skin organ cultures.

### Materials and Methods for supplementary data

Immunohistochemistry, apoptosis/proliferation assay, melanin histochemistry, hair cycle staging, quantitative (immuno)histomorphometry, HJMD patient histology, quantitative real-time PCR

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(Q-PCR), transmission electron microscopy, and statistical analysis can be found in the supplementary materials, online.

## SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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## Supplementary data


## Supplementary Materials and Methods


### Immunohistochemistry


#### LSAB – peroxidase immunohistology

The cryosections were first air-dried for 10 min and then fixed in acetone at -20$^{o}$C for another 10 min. After air drying, the slides were washed three times for 5 min in Tris-buffered saline (TBS). Endogenous peroxidase blocking was achieved by putting the slides in 3% hydrogen peroxide for 15 min, followed by three washings with TBS, 5 min each. In the following step, the mouse anti-human P-cadherin monoclonal antibody and mouse anti-human β-catenin monoclonal antibody (both from BD transduction laboratories, Heidelberg, Germany, clones 56 and 14 respectively), rabbit anti-human phospho-β-catenin (Ser33/37/Thr41) (Cell Signaling Technology, Danvers, MA, USA) in 1:50 dilution with antibody diluent (DCS Detection Line, Hamburg, Germany) and mouse anti-human E-cadherin monoclonal antibody (Invitrogen, Darmstadt, Germany, clone HECD1, predilute antibody) were put on the cryosections at 4 $^{o}$C overnight. After washing three times for 5 min with TBS, sections were stained with polylink biotinylated secondary antibody (DCS Detection Line) for 20 min at room temperature (RT). After three washings for 5 min with TBS, a 20 min application of horse radish peroxidase labeled (HRP) avidin biotin complex (DCS Detection Line) followed, both at RT. Finally, the slides were labelled with 3-amino-9-ethylcarbazole (DCS ChromoLine, Hamburg, Germany) and counterstained with haematoxylin with washing steps in between.


For the P-cadherin staining, normal breast tissue was used as negative and positive control (Figure S7a). As positive controls we further used normal breast tissue (for E-cadherin) (Figure S7b) and normal epidermis (for GSK3β) as previously published (Kovacs *et al.*, 2003; Yamaguchi *et al.*, 2007). Positive staining of β-catenin in the human hair follicle (HF) by the same primary antibody employed here was previously demonstrated (Tsuji *et al.*, 2001). Positive staining of phospho-β-catenin by the same antibody and with a similar

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immunoreactivity pattern as seen here was previously published (Kielhorn *et al.*, 2003; Nakopoulou *et al.*, 2006). Negative controls were performed by deleting the primary antibodies, and by demonstrating negative immunoreactivity in cell compartments/tissues in accordance with the literature.

## **Immunofluorescence**

In order to better localize β-catenin and phospho-β-catenin expression in the HF and also in order to demonsrate GSK3β IR, we used also immunofluorescence techniques. For β-catenin staining, after fixation with acetone at -20$^o$C and washing with TBS, cryosections were incubated overnight at 4$^o$C with the mouse anti-human β-catenin monoclonal antibody (BD transduction laboratories) in 1:50 dilution with TBS, followed by incubation of 45 min at RT with FITC-labeled goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) diluted 1:200 in TBS and normal goat serum. For phospho-β-catenin and GSK3β staining, after washing the slides with phosphate buffered saline (PBS), cryosections were incubated overnight at 4$^o$C with the rabbit anti-human phospho-β-catenin (Ser33/37/Thr41) (Cell Signaling Technology) and mouse anti-human GSK3β (BD Transduction Laboratories) in 1:50 and 1:20 dilutions respectively with antibody diluents (DCS Detection Line). This was followed by incubation with FITC-labeled goat anti-mouse and goat anti-rabbit IgG (Jackson Immunoresearch Laboratories), respectively, for 45 min at RT in 1:200 in antibody diluent (DCS Detection Line). Incubation steps were interspersed with three washes, 5 min each. Then sections were stained with DAPI in a concentration of 1μg/ml (1 min at RT) for identification of cell nuclei.

Since TGFβ2 is a recognized key inducer of catagen in human HFs (Soma *et al.*, 2002), TGFβ2 expression in HFs treated with P-cadherin siRNA and control siRNA was investigated by using the highly sensitive immunofluorescent TSA technique (Perkin Elmer, Boston, MA, USA) (Roth *et al.*, 1999). Briefly, cryosections of human HFs were incubated with rabbit anti-human TGFβ2 (Santa Cruz Biotechnology Inc) as the primary antibody, diluted 1:4000 in Tris-NaCl-Tween Buffer (TNT), overnight at 4$^o$C, followed by incubation with a biotinylated antibody against rabbit IgG (Jackson immunoresearch Laboratories) (1:400 in TNT, 45 min,

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RT). Next, streptavidin–horseradish peroxidase was administered (1:100 in TNT, 30 min, RT).

Finally, the reaction was amplified by tetramethylrhodamine-tyramide amplification reagent at

RT for 5 min (1: 50 in amplification diluent provided in the kit).

**Apoptosis/proliferation assay**

In order to compare degree of proliferation and apoptosis of HFs in the two treatment groups,

double immunolabelling of Ki-67 mouse anti-Ki-67 antiserum (DAKO, Hamburg, Germany)

and TUNEL (ApopTag Fluorescein In Situ Apoptosisdetection kit; Millipore, Berlin, Germany)

was performed as described previously (Bodo *et al.*, 2004; Foitzik *et al.*, 2000; Kloepper *et al.*,

2008; Peters *et al.*, 2006).

**Melanin histochemistry**

In order to highlight the characteristic, catagen-associated switch-off of follicular

melanogenesis (Stenn and Paus, 2001; Tobin *et al.*, 1999), Masson–Fontana histochemistry

was employed (Kloepper *et al.*, 2010). Briefly, cryosections were air dried and fixed in

ethanol-acetic acid. The sections were washed in TBS and distilled water several times.

Cryosections were treated with ammoniacal silver solution (Fluka, Seelze, Germany) for 40

min at $56^{\circ}$C in the dark. After washing in distilled water, the sections were treated with 5%

aqueous sodium thiosulphate (Merck, Darmstadt, Germany) for 1 min. Then, the sections

were washed in running tap water for 3 min and were counterstained with haematoxylin for 45

seconds. After washing in distilled water, sections were dehydrated and mounted in Eukitt (O.

Kindler, Freiburg, Germany).

**Hair cycle staging**

Based on proliferation and apoptosis indices in combination with HF morphology and degree

of pigmentation by Masson–Fontana staining, we determined the stage of each HF (anagen,

early catagen, mid catagen, late catagen) and conducted the hair cycle score (HCS) of the

two treatment groups, as described previously (Foitzik *et al.*, 2006; Kloepper *et al.*, 2010). For

statistical analysis, anagen VI HFs were arbitrarily attributed a score of 100, HFs in early

catagen a score of 200, in mid-catagen of 300 and in late catagen of 400. The sum of scores

per group was than divided by the number of investigated HFs (Foitzik *et al.*, 2006). The mean value of these scores therefore is a reliable indicator of the mean HF stage that had been reached on average by a large population of HFs during organ culture.

## Quantitative (immuno)histomorphometry

The immunoreactivity of the different immunohistology protocols and the relative blackness of Masson-Fontana stained sections was compared between test and control sections in well-defined reference areas by quantitative histomorphometry, as previously described (Peters *et al.*, 2005) using NIH IMAGE software (NIH, Bethesda, MD, USA). For this, the HF was divided anatomically into 4 reference compartments (outer root sheath (ORS); pre-cortical hair matrix (HM); innermost hair matrix (IHM); HM below Auber's line).

## HJMD patient histology

Scalp skin sections stained with Hematoxylin Eosin (H&E) and hair shaft samples of one 5 year old female HJMD patient, obtained for diagnostic purposes after informed consent in the context of an earlier study (Sprecher *et al.*, 2001), were evaluated by light microscopy. Due to the extreme rarity of this syndrome and the difficulty to justify biopsy taking in these pediatric patients, this was the only histological sample available to us for analysis.

## Quantitative Real-Time PCR *(Q-PCR)*

Q-PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems/Life Technologies, Foster City, CA, USA) using the 5' nuclease assay as detailed in previous reports (Bodo *et al.*, 2005; Dobrosi *et al.*, 2008; Toth *et al.*, 2009). Total RNA was extracted from whole organ cultured HFs using TRIreagent (Applied Biosystems/Life Technologies) and digested with recombinant RNase-free DNase-1 (Applied Biosystems) according to the manufacturer's protocol. After isolation, 1µg of total RNA was reverse-transcribed into cDNA by using High Capacity cDNA kit (Applied Biosystems) following the manufacturer's protocol. PCR amplification was performed by using specific TaqMan primers and probes (Applied Biosystems, assay IDs: Hs00999915_m1 for human CDH3, Hs00610344_m1 for human axin2, Hs01547656_m1 for human IGF-1, Hs00427788_m1 for

KRT36, Hs01596219_g1 for KRT37 and Hs00263692_m1 for KRT84). As internal housekeeping gene controls, transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA) or β-actin were determined (Assay IDs: Hs99999903 for human ACTB, Hs99999904 for human PPIA and Hs99999905_m1 for human GAPDH). The amount of the above mentioned transcripts was normalized to those of the control genes using the ΔCT method.

### Transmission Electron Microscopy (TEM)

Representative P-cadherin siRNA and scrambled oligos treated HFs were fixed in Karnovsky's fixative overnight at 4 ºC as previously described (Tobin *et al.*, 1998), post-fixed in 1% OsO4 (0.1M phosphate buffer, pH 7.4) for 1 h, dehydrated in an ethanol series and embedded in Polybed (Polyscience, Warrington, Pa, USA). Semi-thin sections (0.5µm) were stained with toluidine blue and ultra-thin sections (0.1µm) were stained with saturated uranyl acetate and lead citrate and were examined using a Hitach H-7650 (Tokyo, Japan) electron microscope.

### Statistical analysis

 All quantitative immunohistochemistry and HCS data were analyzed by *Mann–Whitney test* (GRAPHPAD PRISM version 4.00 for Windows; GraphPad Software, San Diego, CA, USA). Results are expressed as mean ± SEM. The results from only different highly comparable experiments using samples from different donors were pooled and statistical differences between groups were also determined by the *Mann–Whitney test*.

All Q-PCR results were analysed by *Student's t test for unpaired samples*. PCR results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA) or β-actin.

**Supplementary data for Figure legends**

**Figure 2. Constitutive P-cadherin expression and P-cadherin knockdown in normal human hair follicles**

**(g-h)** Catagen induction after treatment with P-cadherin siRNA. The graphs represent data pooled from 3 independent experiments after one transfection; n=46-47 hair follicles (HFs) in each group. Hair cycle staging was assessed by Ki-67 staining, as well as by quantitative Masson-Fontana histochemistry. Data are presented as hair cycle score (HCS) (g) or as percentage of HFs in anagen VI, early, mid or late catagen (h). To calculate the HCS, each anagen VI HF was ascribed to an arbitrary value of 100, early catagen to 200, mid catagen to 300 and late catagen to 400. Values were added per group and divided by the number of HFs. Thus, the score represents the mean hair cycle stage of all HFs per treatment group. Significance refers to the HCS of control HFs (i.e. HFs treated with scrambled oligos after 4 days of culture).

**Figure 5. P-cadherin knockdown increases degradation of β-catenin and reduces expression of β-catenin target genes**

Since P-cadherin silencing was conducted in microdissected hair follicles (HFs) which were isolated from the scalp skin prior to transfection, and not in skin/extrafollicular cell populations, the effect on β-catenin is restricted to cell population in the HF which expresses P-cadherin (innermost hair matrix).

## Supplementary Figures

**Supplementary Figure S1. Clinical and histological characteristics of HJMD patients**

**(a)** Both hypotrichosis with juvenile macular dystrophy (HJMD) and ectrodactyly and macular dystrophy (EEM) syndromes result from the same or similar mutations in the *CDH3* gene. The patients are suffering from sparse and short hair as well as progressive macular degeneration leading to blindness during the second decade of life (Shimomura *et al.*, 2008; Sprecher *et al.*, 2001). In EEM, patients have an additional feature of ectrodactyly with split hand and foot malformation, while HJMD patients show normal limb development (Shimomura *et al.*, 2010; Shimomura *et al.*, 2008). The reasons for phenotypic disparity between HJMD and EEM syndromes are not known, although co-inheritance of a mutation in a neighboring gene such as *CDH1* (encoding E- cadherin) or in modifier genes has been postulated (Kjaer *et al.*, 2005; Shimomura *et al.*, 2008).

**(b)** Abnormal hair shaft of HJMD patient with pseudomonilethrix appearance and disorganized hair shaft cuticula. Pseudomonilethrix, pili torti, longitudinal ridging, and abnormal hair shaft scaling and folding are recognized in HJMD patients (Bergman *et al.*, 2004).

**(c)** Scalp skin biopsy of HJMD patient (H&E) shows catagen (arrow) and vellus (arrow head) hair follicles (HFs) (H&E, magnification = x40). Insert demonstrates absence of terminal HFs in the subcutaneous tissue.

**(d)** Mid catagen HF of HJMD patient at high magnification (H&E, magnification = x400). The arrows point to karyorhexis, indicating HF dystrophy.

DP – dermal papilla; SC - subcutaneous tissue; DR – dermis.
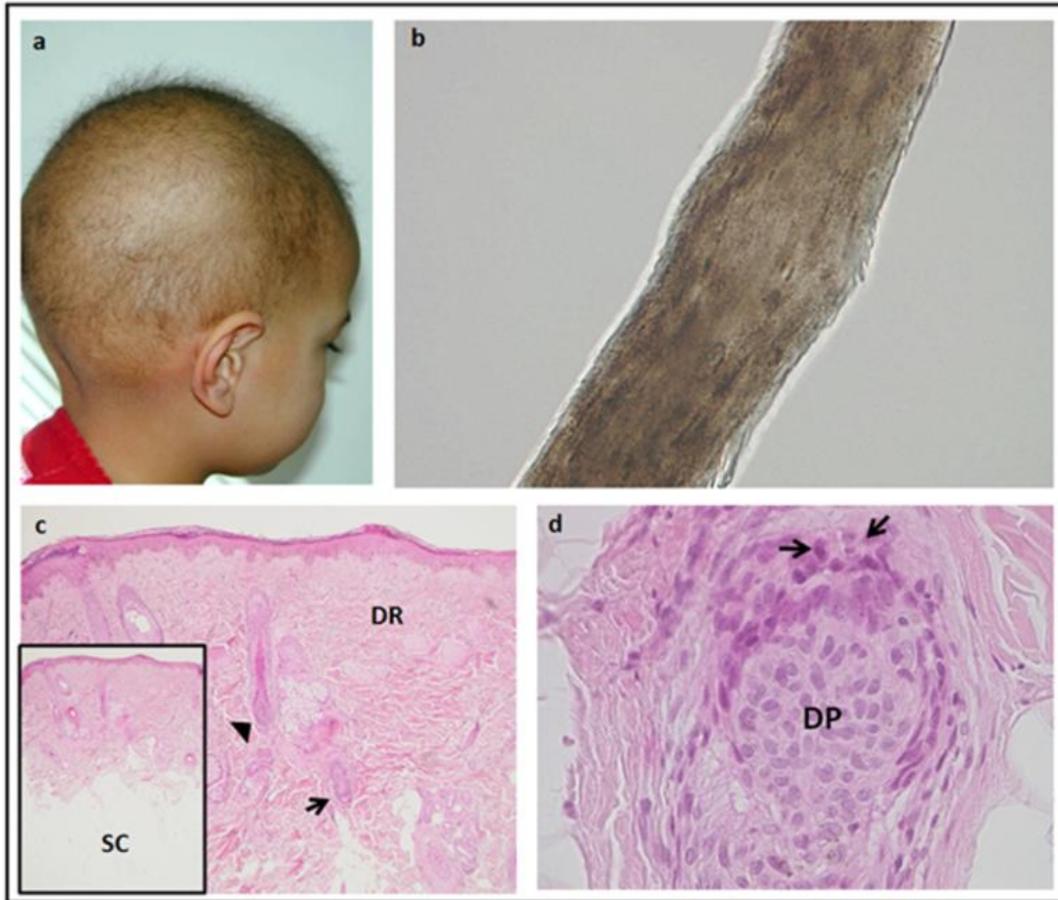
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## Supplementary Figure S1

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**Supplementary Figure S2.** Normal P-cadherin expression in human epidermis and P- and E-cadherin expression in human skin punches and hair follicles after P-cadherin knockdown

**(a)** P-cadherin expression in normal human epidermis. The dotted line represents the dermal-epidermal junction.

**(b-c)** Reduced P-cadherin expression in the innermost hair matrix (IHM) of early catagen hair follicle (HF) (c) compared to normal anagen VI HF (b) which were directly microdissected from human intact scalp without any manipulation. Note the relative reduced melanin content in the HF pigmentary unit of the early catagen HF (c).

**(d-f)** Reduced P-cadherin immunoreactivity (IR) in the epidermis of 2mm skin punches treated with P-cadherin siRNA (e), compared to scrambled oligos treated skin punches (d). **p<0.001, *Mann Whitney* test, n=6 (f). Note some remnant P-cadherin expression in the spinous layer of the epidermis following P-cadherin knockdown (e). Dotted lines encircle all epidermal layers, taken as reference area.

**(g)** Reduced CDH3 mRNA level was demonstrated in P-cadherin siRNA treated skin punches. *p<0.05, *Student's t test for unpaired samples.*

**(h-j)** No significant change in E-cadherin IR was found in transfected HFs (h). Dotted lines represent reference areas of measurement. Note the diffuse expression of E-cadherin in the human follicular keratinocytes, except in the IHM (j).

IHM – innermost hair matrix; DP – dermal papilla; ORS – outer root sheath; HS – hair shaft; HM – hair matrix; IR – immunoreactivity; HFs – hair follicles; DEJ – dermal-epidermal junction; BL – basal layer; Scr – scrambled oligos; P-cad – P-cadherin.

## Supplementary Figure S2
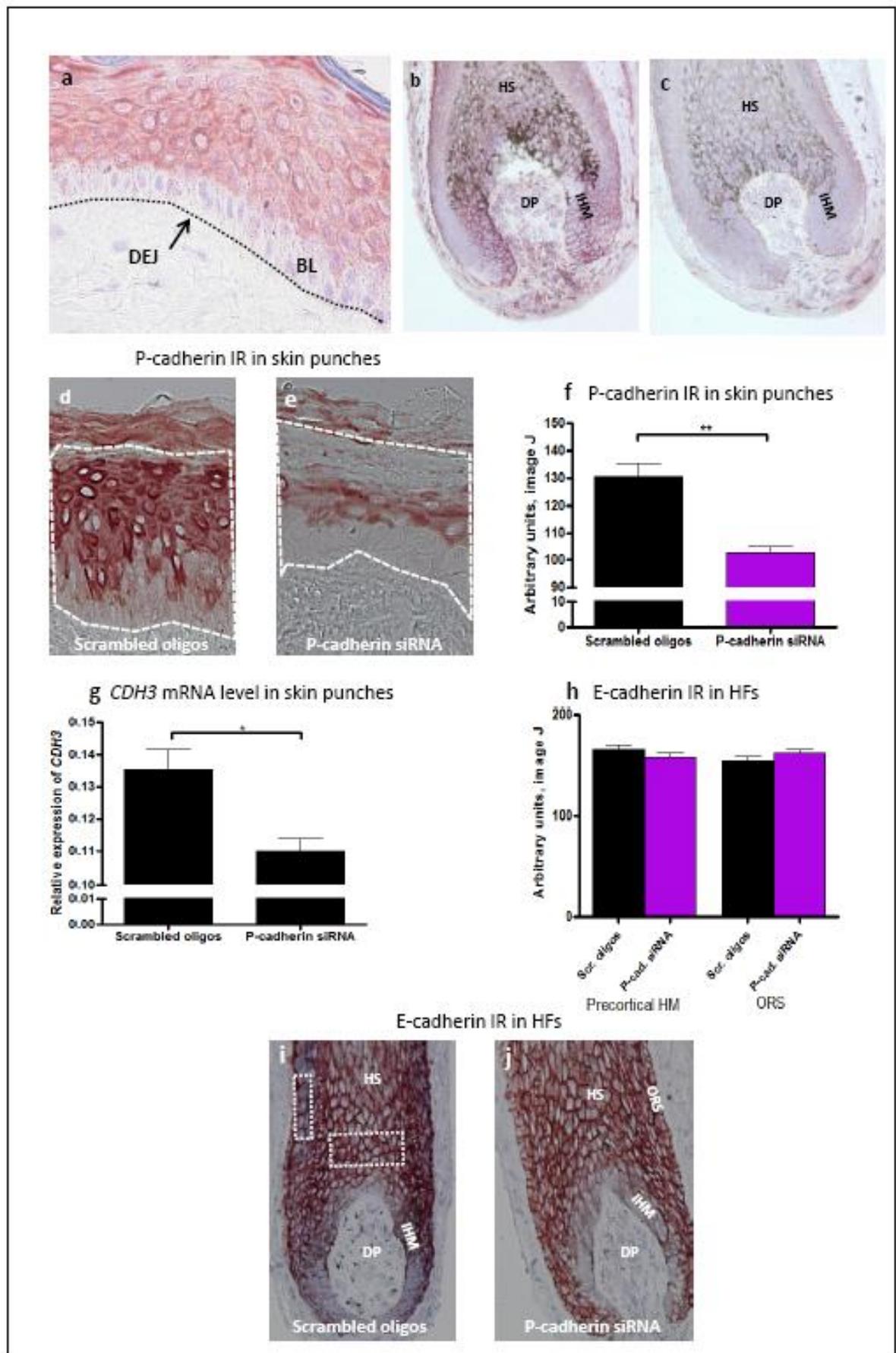
**Supplementary Figure S3.** **P-cadherin knockdown results in keratin clumps inside pre-cortical hair matrix keratinocytes**

Ultrastructural analyses by Transmission Electron Microscopy of P-cadherin siRNA treated hair follicle (HF) (a, c, e) compared with scrambled oligos treated one (b, d, f).

**(a)** Accumulation of massive keratin clumps inside the cytoplasm of the keratinocytes in the precortical hair matrix (white arrows). The white arrowheads indicate melanin granules inside a melanocyte in the HF pigmentary unit.

**(b)** No evidence of keratin clumps in the precortical hair matrix keratinocytes of a scrambled oligos treated HF. White arrowhead indicates melanin granules inside a keratinocyte.

**(c-f)** Demonstration of the precortical hair matrix region (c-d) in P-cadherin siRNA treated HF (c) and scrambled oligos treated HF (d), and of the proximal hair shaft (e-f) in P-cadherin siRNA treated HF (e) and scrambled oligos treated HF (f). The black arrowheads indicate the borderline between the cortex and the medulla of the hair shaft. Note the narrower intercellular space in P-cadherin siRNA treated HF compared to scrambled oligos treated HF. However, no difference was found in the appearance of both the cortex and the medulla.

c – cortex; m – medulla.

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**Supplementary Figure S3**
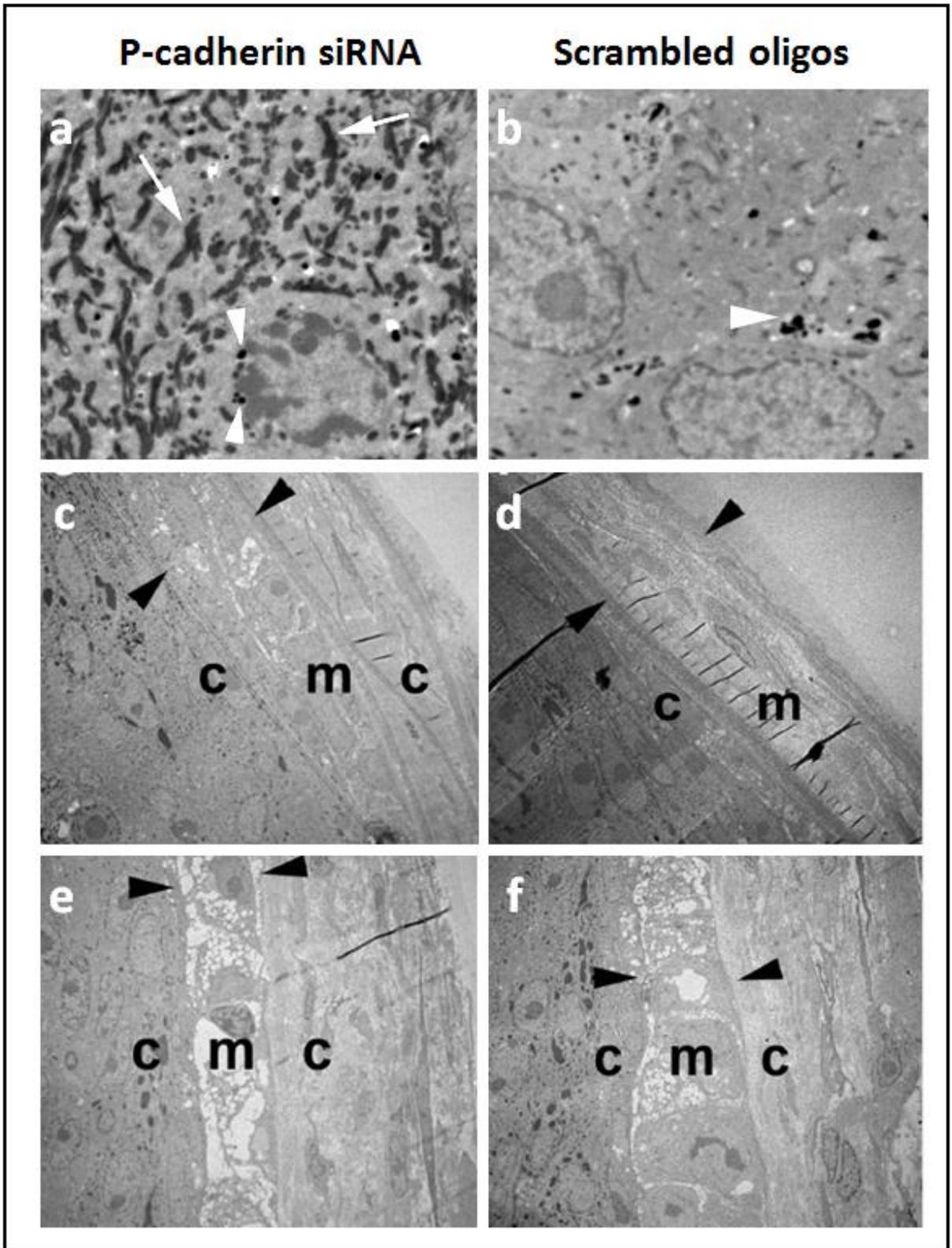
**Supplementary Figure S4.** Several hair keratins are upregulated by P-cadherin silencing

Q-PCR analysis revealed upregulation of three specific hair keratins (KRT36/37/84) steady state transcript levels in P-cadherin silenced hair follicles (HFs) compared to scrambled oligos treated HFs, in three different patients, both after one and two transfections with P-cadherin siRNA vs. scrambled oligos. The graphs represent the results in one representative patient for each hair keratin mRNA level. All the three keratins were statistically significant upregulated in the three patients, except one patient that did not show significant upregulation of KRT36. ***$p<0.001$; **$p<0.01$, *Student's t test for unpaired samples*.

**Supplementary Figure S4**
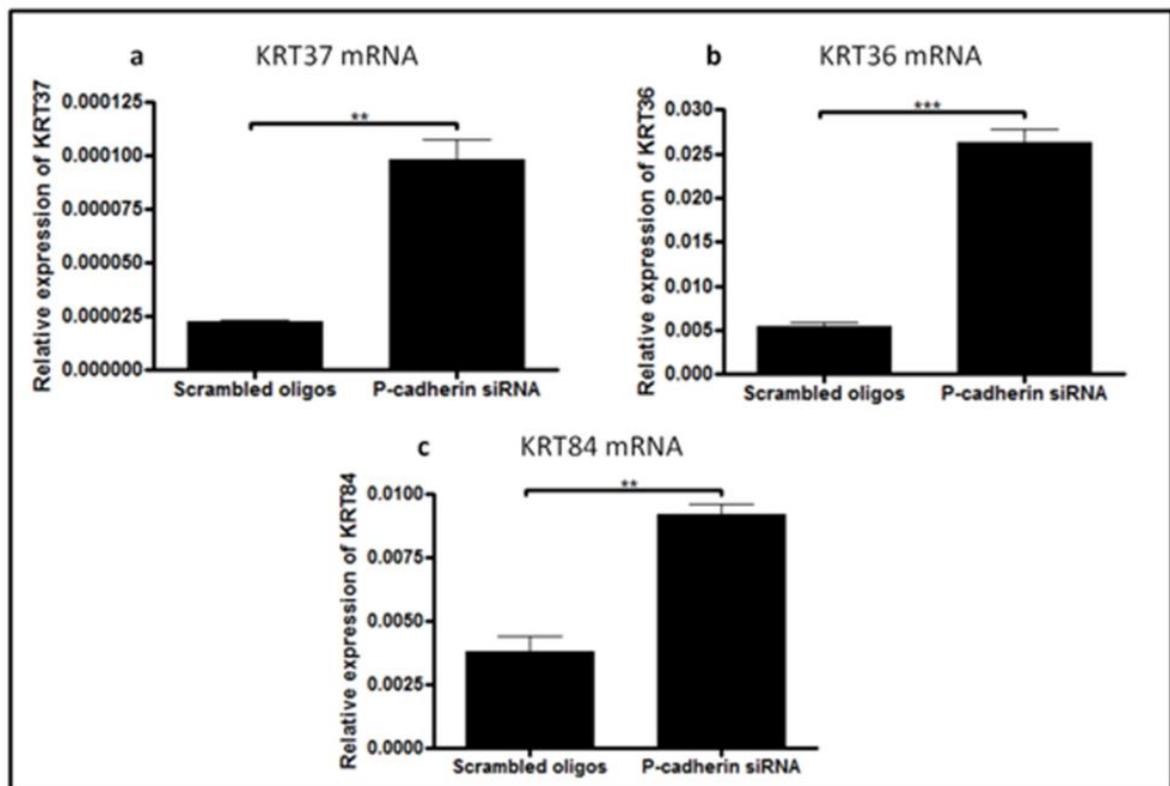
**Supplementary Figure S5.** Transforming growth factor beta (TGFβ)-neutralizing antibody partially restores the effects of P-cadherin silencing on human hair growth

**(a)** Hair shaft elongation measurements of P-cadherin siRNA, P-cadherin siRNA + transforming growth factor β (TGFβ) neutralizing antibody (NA), and hair follicles (HFs) treated only with TGFβ NA; compared to scrambled (Scr) oligo-treated HFs. ***p<0.001, **p<0.01, *Mann–Whitney* test, n=12-21.
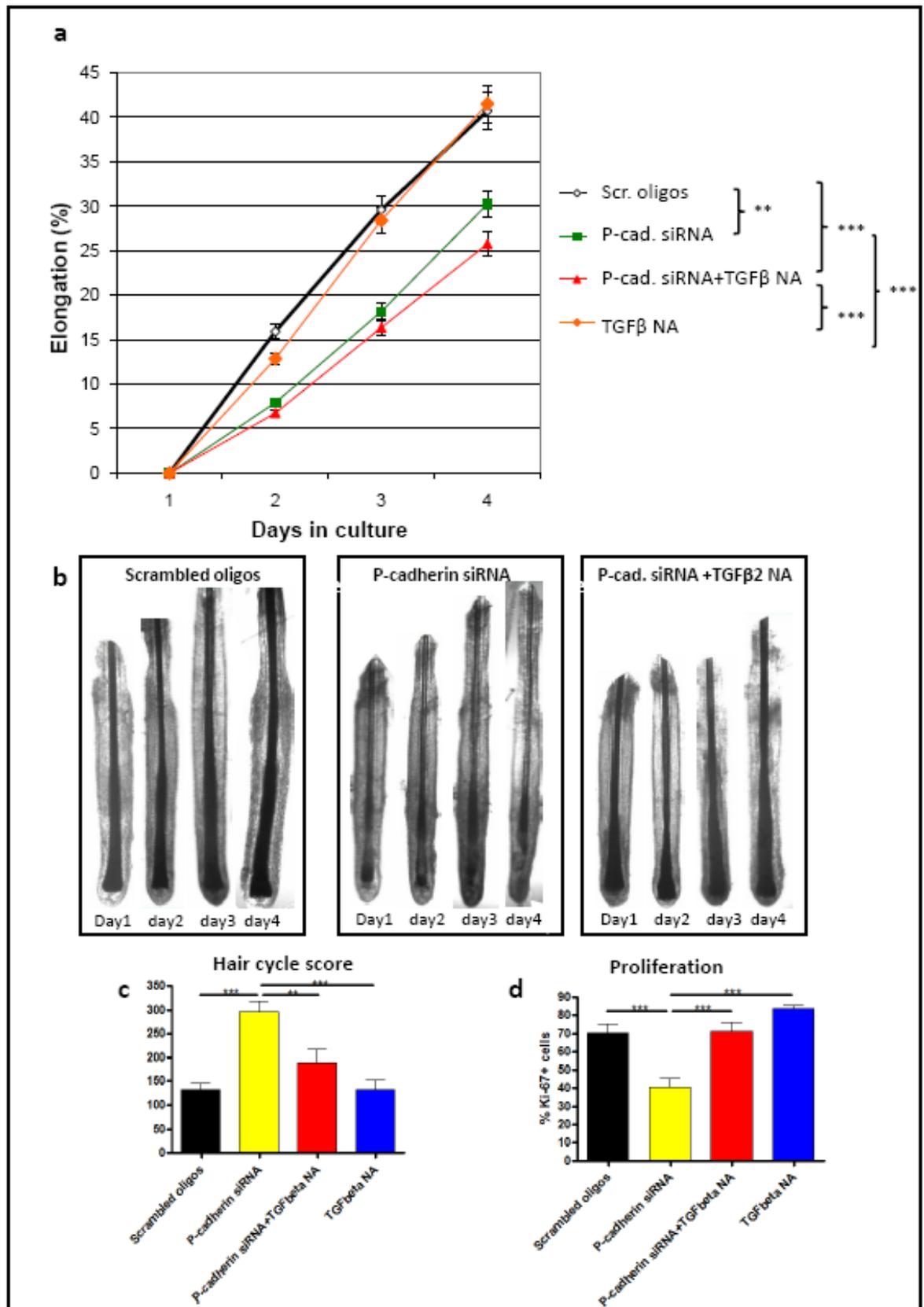
**(b)** Photodocumentation of one characteristic HF per group over a culture period of 4 days.

**(c)** Hair cycle score corresponding to (b). ***p<0.001, **p>0.01, *Mann–Whitney* test, n=12-21.

**(d)** Ki-67 analysis of hair matrix corresponding to (b). ***p<0.001, *Mann–Whitney* test, n=12-21.

NA – neutralizing antibody; TGFβ2 - transforming growth factor beta 2; Scr – scrambled; P-cad – P-cadherin.

## Supplementary Figure S5

**Supplementary Figure S6. No significant change in intraepidermal β-catenin expression following P-cadherin silencing**
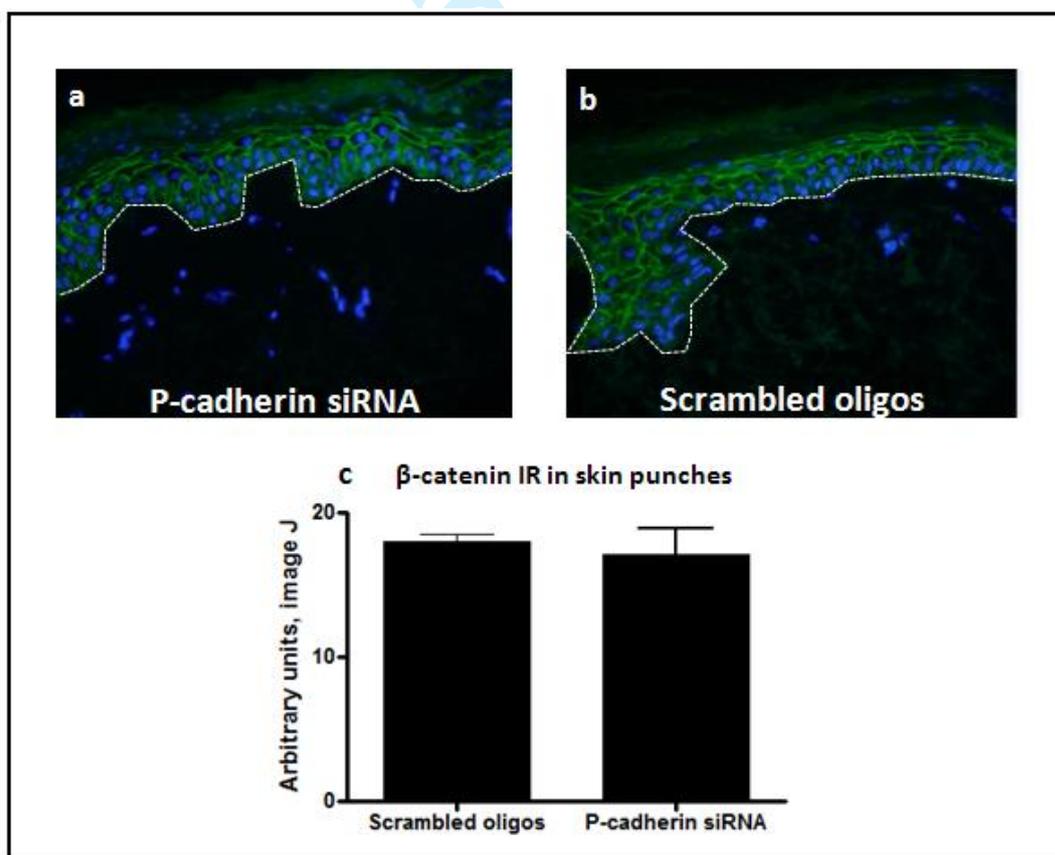
**(a)** β-catenin immunoreactivity (IR) in P-cadherin siRNA treated 2mm skin punches. Dotted line represents the dermal-epidermal junction.

**(b)** β-catenin IR in scrambled oligos treated 2mm skin punches. Dotted line represents the dermal-epidermal junction.

**(c)** No significant change was observed in intraepidermal β-catenin expression of 2mm skin punches treated with P-cadherin siRNA compared to scrambled oligos treated ones.

IR – immunoreactivity.
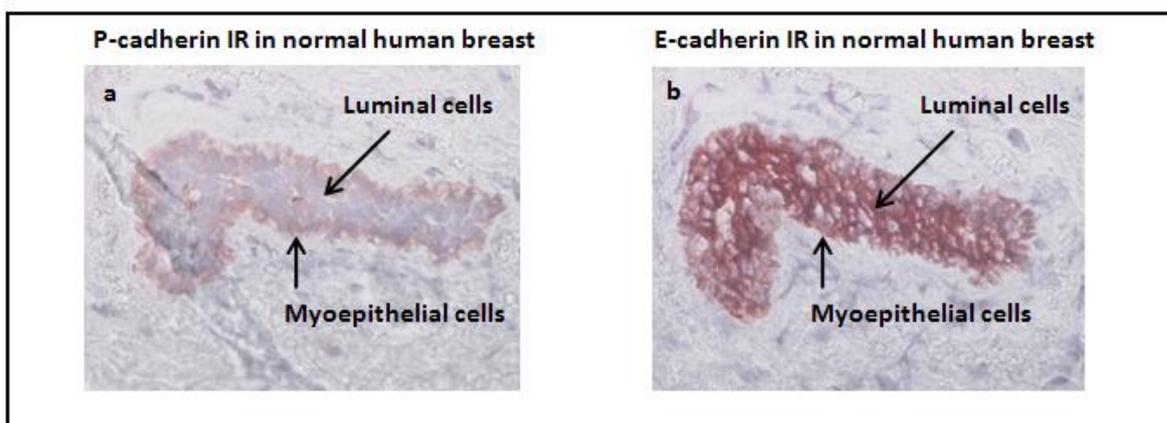
## Supplementary Figure S6

**Supplementary Figure S7.** Normal P- and E-cadherin expression in human breast tissue

**(a)** P-cadherin positive immunoreactivity (IR) in the myoepithelial cells and negative IR in the luminal epithelium, in normal breast tissue as positive and negative control.

**(b)** E-cadherin positive IR in both the myoepithelial and the luminal cells in normal breast tissue as a positive control.

<u>**Supplementary Figure S7**</u>



P-cadherin IR in normal human breast — Luminal cells, Myoepithelial cells

E-cadherin IR in normal human breast — Luminal cells, Myoepithelial cells

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