Topobiology of Human Pigmentation: P-Cadherin Selectively Stimulates Hair Follicle Melanogenesis

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Topobiology of human pigmentation:

P-cadherin selectively stimulates hair follicle melanogenesis

Liat Samuelov[1,2], Eli Sprecher[1,3], Koji Sugawara[2,4], Suman K. Singh[5], Desmond J. Tobin[5], Daisuke Tsuruta[6], Tamás Bíró[7], Jennifer E. Kloepper[2*], Ralf Paus[2,8*]

[1] Department of Dermatology, Tel-Aviv Sourasky Medical Center, Tel-Aviv, Israel

[2] Department of Dermatology, University of Luebeck, Luebeck, Germany

[3] Department of Human Molecular Genetics & Biochemistry, Sackler Faculty of Medicine, Tel-aviv University, Ramat Aviv, Israel

[4] Department of Dermatology, Osaka City University Graduate School of Medicine, Osaka, Japan

[5] Centre for Skin Sciences, School of Life Sciences, University of Bradford, Bradford, United Kingdom

[6] Department of Dermatology, University of Kurume, Kurume, Japan

[7] DE-MTA ''Lendulet'' Cellular Physiology Group, Department of Physiology, University of Debrecen, Debrecen, Hungary

[8] Institute of Inflammation and Repair, University of Manchester, Manchester, United Kingdom

*authors contributed equally to this work

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P-cadherin regulates hair pigmentation

Corresponding author:

Ralf Paus, MD. Dept. of Dermatology, University of Luebeck, Ratzeburger Allee 160, 23538 Luebeck, Germany, Tel.: +49 451/500-2543 Fax: +49 451/500-6595 Email: ralf.paus@uksh.de; ralf.paus@manchester.ac.uk

The work was conducted at the University of Luebeck, Luebeck, Germany

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 melanocyte subtype-specific topobiological regulator of normal human pigmentation, possibly via GSK3β-mediated canonical Wnt signaling.

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

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.

Discussion

To our knowledge, it is previously unreported 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**).

member identified here not only opens a new frontier in general pigment and cell adhesion biology, but is also clinically relevant: it suggests 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.

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 written informed patient consent with adherence to the Helsinki Guidelines) (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.

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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References

Aksan I, Goding CR (1998) Targeting the microphthalmia basic helix-loop-helix-leucine zipper transcription factor to a subset of E-box elements in vitro and in vivo. *Mol Cell Biol* 18:6930-6938.

Bauer R, Bosserhoff AK (2006) Functional implication of truncated P-cadherin expression in malignant melanoma. *Exp Mol Pathol* 81:224-230.

Bellei B, Pitisci A, Catricala C, Larue L, Picardo M (2010) Wnt/beta-catenin signaling is stimulated by alpha-melanocyte-stimulating hormone in melanoma and melanocyte cells: implication in cell differentiation. *Pigment Cell Melanoma Res* 24:309-325.

Bergman R, Sapir M, Sprecher E (2004) Histopathology of hypotrichosis with juvenile macular dystrophy. *Am J Dermatopathol* 26:205-209.

Berson JF, Theos AC, Harper DC, Tenza D, Raposo G, Marks MS (2003) Proprotein convertase cleavage liberates a fibrillogenic fragment of a resident glycoprotein to initiate melanosome biogenesis. *J Cell Biol* 161:521-533.

Bertolotto C, Abbe P, Hemesath TJ, Bille K, Fisher DE, Ortonne JP*, et al.* (1998a) Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. *J Cell Biol* 142:827-835.

Bertolotto C, Busca R, Abbe P, Bille K, Aberdam E, Ortonne JP*, et al.* (1998b) Different cis-acting elements are involved in the regulation of TRP1 and TRP2 promoter activities by cyclic AMP: pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. *Mol Cell Biol* 18:694-702.

Bodo E, Biro T, Telek A, Czifra G, Griger Z, Toth BI*, et al.* (2005) A hot new twist to hair biology: involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. *Am J Pathol* 166:985-998.

Borghi N, James Nelson W (2009) Intercellular adhesion in morphogenesis: molecular and biophysical considerations. *Curr Top Dev Biol* 89:1-32.

Botchkareva NV, Khlgatian M, Longley BJ, Botchkarev VA, Gilchrest BA (2001) SCF/c-kit signaling is required for cyclic regeneration of the hair pigmentation unit. *FASEB J* 15:645-658.

Cavallaro U, Dejana E (2011) Adhesion molecule signalling: not always a sticky business. *Nat Rev Mol Cell Biol* 12:189-197.

Dann CE, Hsieh JC, Rattner A, Sharma D, Nathans J, Leahy DJ (2001) Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. *Nature* 412:86-90.

Dobrosi N, Toth BI, Nagy G, Dozsa A, Geczy T, Nagy L*, et al.* (2008) Endocannabinoids enhance lipid synthesis and apoptosis of human sebocytes via cannabinoid receptor-2-mediated signaling. *FASEB J* 22:3685-3695.

Du J, Miller AJ, Widlund HR, Horstmann MA, Ramaswamy S, Fisher DE (2003) MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. *Am J Pathol* 163:333-343.

Edelman GM (1989) Topobiology. *Sci Am* 260:76-82, 84-76, 88.

Engler AJ, Humbert PO, Wehrle-Haller B, Weaver VM (2 (009Multiscale modeling of form and function. *Science* 324:208-212.

Fanelli MA, Montt-Guevara M, Diblasi AM, Gago FE, Tello O, Cuello-Carrion FD*, et al.* (2008) P-cadherin and beta-catenin are useful prognostic markers in breast cancer patients; beta-catenin interacts with heat shock protein Hsp27. *Cell Stress Chaperones* 13:207-220.

Fujita M, Furukawa F, Fujii K, Horiguchi Y, Takeichi M, Imamura S (1992) Expression of cadherin cell adhesion molecules during human skin development: morphogenesis of epidermis, hair follicles and eccrine sweat ducts. *Arch Dermatol Res* 284:159-166.

Furukawa F, Fujii K, Horiguchi Y, Matsuyoshi N, Fujita M, Toda K*, et al.* (1997) Roles of E- and P-cadherin in the human skin. *Microsc Res Tech* 38:343-352.

Gaspar E, Nguyen-Thi KT, Hardenbicker C, Tiede S, Plate C, Bodo E*, et al.* (2010) Thyrotropin-releasing hormone selectively stimulates human hair follicle pigmentation. *J Invest Dermatol* 131:2368-2377.

Goodwin M, Yap AS (2004) Classical cadherin adhesion molecules: coordinating cell adhesion, signaling and the cytoskeleton. *J Mol Histol* 35:839-844.

Gruss C, Herlyn M (2001) Role of cadherins and matrixins in melanoma. *Curr Opin Oncol* 13:117-123.

Haass NK, Smalley KS, Herlyn M (2004) The role of altered cell-cell communication in melanoma progression. *J Mol Histol* 35:309-318.

Haass NK, Smalley KS, Li L, Herlyn M (2005) Adhesion, migration and communication in melanocytes and melanoma. *Pigment Cell Res* 18:150-159.

Han R, Baden HP, Brissette JL, Weiner L (2002) Redefining the skin's pigmentary system with a novel tyrosinase assay. *Pigment Cell Res* 15:290-297.

Hirai Y, Nose A, Kobayashi S, Takeichi M (1989) Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. II. Skin morphogenesis. *Development* 105:271-277.

Hoashi T, Tamaki K, Hearing VJ (2010) The secreted form of a melanocyte membrane-bound glycoprotein (Pmel17/gp100) is released by ectodomain shedding. *FASEB J* 24:916-930.

Horiguchi Y, Furukawa F, Fujita M, Imamura S (1994) Ultrastructural localization of E-cadherin cell adhesion molecule on the cytoplasmic membrane of keratinocytes in vivo and in vitro. *J Histochem Cytochem* 42:1333-1340.

Ikeya M, Lee SM, Johnson JE, McMahon AP, Takada S (1997) Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature* 389:966-970.

Indelman M, Hamel CP, Bergman R, Nischal KK, Thompson D, Surget MO*, et al.* (2003) Phenotypic diversity and mutation spectrum in hypotrichosis with juvenile macular dystrophy. *J Invest Dermatol* 121:1217-1.220

Kauser S, Thody AJ, Schallreuter KU, Gummer CL, Tobin DJ (2005) A fully functional proopiomelanocortin/melanocortin-1 receptor system regulates the differentiation of human scalp hair follicle melanocytes. *Endocrinology* 146:532-543.

Kawakami A, Sakane F, Imai S, Yasuda S, Kai M, Kanoh H*, et al.* (2008) Rab7 regulates maturation of melanosomal matrix protein gp100/Pmel17/Silv. *J Invest Dermatol* 128:143-150.

Kloepper JE, Hendrix S, Bodo E, Tiede S, Humphries MJ, Philpott MP*, et al.* (2008) Functional role of beta 1 integrin-mediated signalling in the human hair follicle. *Exp Cell Res* 314:498-508.

Kloepper JE, Sugawara K, Al-Nuaimi Y, Gaspar E, van Beek N, Paus R (2010) Methods in hair research: how to objectively distinguish between anagen and catagen in human hair follicle organ culture. *Exp Dermatol* 19:305-312.

Kuphal S, Bosserhoff AK (2006) Influence of the cytoplasmic domain of E-cadherin on endogenous N-cadherin expression in malignant melanoma. *Oncogene* 25:248-259.

Kuphal S, Bosserhoff AK (20 (11E-cadherin cell-cell communication in melanogenesis and during development of malignant melanoma. *Arch Biochem Biophys*.

Kushimoto T, Basrur V, Valencia J, Matsunaga J, Vieira WD, Ferrans VJ*, et al.* (2001) A model for melanosome biogenesis based on the purification and analysis of early melanosomes. *Proc Natl Acad Sci U S A* 98:10698-10703.

Linker C, Lesbros C, Gros J, Burrus LW, Rawls A, Marcelle C (2005) beta-Catenin-dependent Wnt signalling controls the epithelial organisation of somites through the activation of paraxis. *Development* 132:3895-3905.

Lovatt M, Bijlmakers MJ (2010) Stabilisation of beta-catenin downstream of T cell receptor signalling. *PLoS One* 5.

Magerl M, Tobin DJ, Muller-Rover S, Hagen E, Lindner G, McKay IA*, et al.* (2001) Patterns of proliferation and apoptosis during murine hair follicle morphogenesis. *J Invest Dermatol* 116:947-955.

McGill GG, Haq R, Nishimura EK, Fisher DE (2006) c-Met expression is regulated by Mitf in the melanocyte lineage. *J Biol Chem* 281:10365-10373.

Muller-Rover S, Tokura Y, Welker P, Furukawa F, Wakita H, Takigawa M*, et al.* (1999) E- and P-cadherin expression during murine hair follicle morphogenesis and cycling. *Exp Dermatol* 8:237-246.

Nagafuchi A, Shirayoshi Y, Okazaki K, Yasuda K, Takeichi M (1987 (Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature* 329:341-343.

Nakajima H, Fukazawa K, Wakabayashi Y, Wakamatsu K, Imokawa G (2012) Withania somnifera extract attenuates stem cell factor-stimulated pigmentation in human epidermal equivalents through interruption of ERK phosphorylation within melanocytes. *J Nat Med* 66:435-446.

Nelson WJ, Nusse R (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 303:1483-1487.

Nishimura EK, Granter SR, Fisher DE (2005) Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. *Science* 307:720-724.

Paredes J, Correia AL, Ribeiro AS, Albergaria A, Milanezi F, Schmitt FC (2007) P-cadherin expression in breast cancer: a review. *Breast Cancer Res* 9:214.

Paus R (2011) A neuroendocrinological perspective on human hair follicle pigmentation. *Pigment Cell Melanoma Res* 24:89-106.

Peters EM, Tobin DJ, Botchkareva N, Maurer M, Paus R (2002) Migration of melanoblasts into the developing murine hair follicle is accompanied by transient c-Kit expression. *J Histochem Cytochem* 50:751-766.

Philpott MP, Green MR, Kealey T (1990) Human hair growth in vitro. *J Cell Sci* 97 (Pt 3):463-471.

Pla P, Moore R, Morali OG, Grille S, Martinozzi S, Delmas V*, et al.* (2001) Cadherins in neural crest cell development and transformation. *J Cell Physiol* 189:121-132.

Plonka PM, Passeron T, Brenner M, Tobin DJ, Shibahara S, Thomas A*, et al.* (2009) What are melanocytes really doing all day long...? *Exp Dermatol*.18:799-819

Samuelov L, Sprecher E, Tsuruta D, Bíro T, Kloepper JE, Paus R (2012) P-cadherin regulates human hair growth and cycling via canonical Wnt signaling and transforming growth factor-beta2. *Journal Invest Dermatol*.

Schepsky A, Bruser K, Gunnarsson GJ, Goodall J, Hallsson JH, Goding CR*, et al.* (2006) The microphthalmia-associated transcription factor Mitf interacts with beta-catenin to determine target gene expression. *Mol Cell Biol* 26:8914-8927.

Schneider MR, Schmidt-Ullrich R, Paus R (2009 (The hair follicle as a dynamic miniorgan. *Curr Biol* 19:R132-142.

Shimomura Y, Wajid M, Shapiro L, Christiano AM (2008) P-cadherin is a p63 target gene with a crucial role in the developing human limb bud and hair follicle. *Development* 135:743-753.

Shimoyama Y, Hirohashi S, Hirano S, Noguchi M, Shimosato Y, Takeichi M*, et al.* (1989a) Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. *Cancer Res* 49:2128-2133.

Shimoyama Y, Yoshida T, Terada M, Shimosato Y, Abe O, Hirohashi S (1989b) Molecular cloning of a human Ca2+-dependent cell-cell adhesion molecule homologous to mouse placental cadherin: its low expression in human placental tissues. *J Cell Biol* 109:1787-1794.

Singh SK, Kurfurst R, Nizard C, Schnebert S, Perrier E, Tobin DJ (2010) Melanin transfer in human skin cells is mediated by filopodia--a model for homotypic and heterotypic lysosome-related organelle transfer. *FASEB J* 24:3756-3769.

Singh SK, Nizard C, Kurfurst R, Bonte F, Schnebert S, Tobin DJ (2008) The silver locus product (Silv/gp100/Pmel17) as a new tool for the analysis of melanosome transfer in human melanocyte-keratinocyte co-culture. *Exp Dermatol* 17:418-426.

Slominski A, Wortsman J, Plonka PM, Schallreuter KU, Paus R, Tobin DJ (2005) Hair follicle pigmentation. *J Invest Dermatol* 124:13-21.

Sprecher E, Bergman R, Richard G, Lurie R, Shalev S, Petronius D*, et al.* (2001) Hypotrichosis with juvenile macular dystrophy is caused by a mutation in CDH3, encoding P-cadherin. *Nat Genet* 29:134-136.

Takeda K, Yasumoto K, Takada R, Takada S, Watanabe K, Udono T*, et al.* (2000) Induction of melanocyte-specific microphthalmia-associated transcription factor by Wnt-3a. *J Biol Chem* 275:14013-14016.

Takeichi M (1991) Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 251:1451-1455.

Takeichi M, Hatta K, Nose A, Nagafuchi A (1988) Identification of a gene family of cadherin cell adhesion molecules. *Cell Differ Dev* 25 Suppl:91-94.

Taneyhill LA (2008) To adhere or not to adhere: the role of Cadherins in neural crest development. *Cell Adh Migr* 2:223-230.

Tobin DJ (2011) The cell biology of human hair follicle pigmentation. *Pigment Cell Melanoma Res* 24:75-88.

Toth BI, Benko S, Szollosi AG, Kovacs L, Rajnavolgyi E, Biro T (2009) Transient receptor potential vanilloid-1 signaling inhibits differentiation and activation of human dendritic cells. *FEBS Lett* 583:1619-1624.

Vachtenheim J, Borovansky J (2010) "Transcription physiology" of pigment formation in melanocytes: central role of MITF. *Exp Dermatol* 19:617.627-

Valencia JC, Hoashi T, Pawelek JM, Solano F, Hearing VJ (2006) Pmel17: controversial indeed but critical to melanocyte function. *Pigment Cell Res* 19:250-252; author reply 253-257.

Van Marck V, Stove C, Van Den Bossche K, Stove V, Paredes J, Vander Haeghen Y*, et al.* (2005) P-cadherin promotes cell-cell adhesion and counteracts invasion in human melanoma. *Cancer Res* 65:8774-8783.

Yamaguchi Y, Morita A, Maeda A, Hearing VJ (2009) Regulation of skin pigmentation and thickness by Dickkopf 1 (DKK1). *J Investig Dermatol Symp Proc* 14:73-75.

Yamaguchi Y, Passeron T, Watabe H, Yasumoto K, Rouzaud F, Hoashi T*, et al.* (2007) The effects of dickkopf 1 on gene expression and Wnt signaling by melanocytes: mechanisms underlying its suppression of melanocyte function and proliferation. *J Invest Dermatol* 127:1217-1225.

Yasumoto K, Watabe H, Valencia JC, Kushimoto T, Kobayashi T, Appella E*, et al.* (2004) Epitope mapping of the melanosomal matrix protein gp100 (PMEL17): rapid processing in the endoplasmic reticulum and glycosylation in the early Golgi compartment. *J Biol Chem* 279:28330-28338.

Yasumoto K, Yokoyama K, Shibata K, Tomita Y, Shibahara S (1994) Microphthalmia-associated transcription factor as a regulator for melanocyte-specific transcription of the human tyrosinase gene. *Mol Cell Biol* 14:8058-8070.

Figure legends

Figure 1. P-cadherin expression in normal human anagen VI hair follicles overlaps the hair follicle pigmentary unit and reduced melanin content in HJMD patients and in P-cadherin silenced anagen VI hair follicles

(a) Hematoxylin Eosin (H&E) staining of HJMD patient's scalp biopsy. A vellus hair follicle (HF) with no melanin content in the hair shaft (HS) and precortical hair matrix (HM). Arrowhead points to melanin granules in the dermal papilla (melanin incontinence).

(b) H&E staining of normal scalp HF. Arrowheads indicate melanin in the HM and HS.

(c,d) NKIbeteb (gp100) (c) and P-cadherin (d) immunoreactivity (IR) in sequential sections of an anagen VI HF. Dotted lines represent the area of P-cadherin and NKIbeteb expression.

(e) Reduced melanin content by Masson-Fontana staining in P-cadherin siRNA-treated HFs. **p<0.01, n=12, *Mann Whitney test.*

(f,g) Reduced melanin content in P-cadherin siRNA-treated anagen VI HFs. The corresponding Ki-67 staining demonstrates the proliferation activity of the HM keratinocytes below Auber's line (dotted line), a marker of anagen stage (Kloepper *et al.*, 2010).

Scale bars=50μm (a,c,d,f,g) and 100μm (b).

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). **p<0.01, *Mann Whitney test,* n=10-13.

(d) Reduced mRNA level of TYR in P-cadherin siRNA-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 HFs.

(g) Histomorphometric analysis of gp100 IR. *p<0.05, *Mann Whitney test,* n=13-16.

(h) Reduced gp100+ cells in P-cadherin siRNA-treated HFs. *p<0.05, *Mann Whitney test,* n=13-16.

(i-k) Reduced number per gp100+ cells and shorter dendrites (yellow arrow heads) in P-cadherin siRNA-treated HFs by confocal microscopy. **p<0.01, *Mann Whitney test,* n=96-119.

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

White dotted lines represent reference areas of measurements. Yellow dotted lines represent the basement membrane. Scale bars=50μm.

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.

(d-g) Reduced melanin content by Masson-Fontana staining in P-cadherin silenced HFMs.

(h-k) Reduced tyrosinase activity in P-cadherin silenced HFMs. 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.

C, f and n represent negative controls with retrieving the primary antibody (c and n) or without silver nitrate (f). Scale bars= 10uM.

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

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

(a-c) Reduced stem cell factor (SCF) expression in P-cadherin silenced HFs. *** p<0.0001, *Mann Whitney test*, n=15-17.

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

(e-g) Reduced c-Kit expression in P-cadherin silenced HFs ** p<0.01, *Mann Whitney test*, n=11-14.

(h-j) Reduced number of microphthalmia-associated transcription factor (MITF) positive cells around the dermal papilla (DP) (arrows) in P-cadherin silenced HFs. **p<0.01, *Mann Whitney test*, n=11-16.

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

Dotted lines represent reference areas of measurements. White lines encircle the DP. Scale bars=50μm.

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) specifically in the innermost hair matrix (IHM) of anagen hair follicles (HFs) following P-cadherin silencing. ***p<0.0001, *Mann Whitney test*, n=14-22.

(d-f) Upregulation of glycogen synthase kinase 3 beta (GSK3β) IR in the IHM following P-cadherin silencing. **p<0.01, *Mann Whitney test*, n=9-15.

(g-i) Increased phospho-β-catenin IR in the IHM of anagen HFs following P-cadherin silencing. **p<0.01, *Mann Whitney test*, n=14-16.

(j) Reduced Microphthalmia-associated transcription factor (MITF) mRNA level in P-cadherin silenced HFs. Culturing P-cadherin silenced HFs with lithium chloride did not reverse the effect of P-cadherin silencing in terms of MITF transcript level. *p<0.05; ***p<0.001, *Student's t test for unpaired samples.*

Scale bars=30μm.

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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