The Peripheral Clock Regulates Human Pigmentation

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Although the regulation of pigmentation is well characterized, it remains unclear whether cell-autonomous controls regulate the cyclic on–off switching of pigmentation in the hair follicle (HF). As human HFs and epidermal melanocytes express clock genes and proteins, and given that core clock genes (PER1, BMAL1) modulate human HF cycling, we investigated whether peripheral clock activity influences human HF pigmentation. We found that silencing BMAL1 or PER1 in human HFs increased HF melanin content. Furthermore, tyrosinase expression and activity, as well as TYRP1 and TYRP2 mRNA levels, gp100 protein expression, melanocyte dendricity, and the number gp100+ HF melanocytes, were all significantly increased in BMAL1 and/or PER1-silenced HFs. BMAL1 or PER1 silencing also increased epidermal melanin content, gp100 protein expression, and tyrosinase activity in human skin. These effects reflect direct modulation of melanocytes, as BMAL1 and/or PER1 silencing in isolated melanocytes increased tyrosinase activity and TYRP1/2 expression. Mechanistically, BMAL1 knockdown reduces PER1 transcription, and PER1 silencing induces phosphorylation of the master regulator of melanogenesis, microphthalmia-associated transcription factor, thus stimulating human melanogenesis and melanocyte activity *in situ* and *in vitro*. Therefore, the molecular clock operates as a cell-autonomous modulator of human pigmentation and may be targeted for future therapeutic strategies.

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INTRODUCTION

Despite its high regeneration potential and the relative aging resistance of the hair follicle (HF), it is the HF pigmentary system that routinely shows the first signs of aging (canities or graying), long before HF melanocyte stem cells in the bulge have undergone irreversible changes (Tobin and Paus, 2001; Paus, 2011; Seiberg, 2013). There is a strong link between the clock system and age-related pathologies (Kondratov and Antoch, 2007)—for example, *Bmal1* mutant mice showed signs associated with aging, including reactive oxygen species (ROS) accumulation and reduction in life span, fat, muscle, and bone mass (Kondratov *et al.*, 2006; Geyfman and Andersen, 2010; Kondratova and Kondratov, 2012). As ROS accumulation in HF melanocytes is believed to be involved in age-related graying (Arck *et al.*, 2006; Wood *et al.*, 2009) and clock genes have been linked with ROS homeostasis (Kauser *et al.*, 2011; Geyfman *et al.*, 2012; Lai *et al.*, 2012; Lee *et al.*, 2013; Avitabile *et al.*, 2014), investigating the role of the molecular clock in HF pigmentation may therefore enhance our understanding of how canities and pigmentation disorders develop, highlight potential therapies, and translationally demonstrate how the peripheral molecular clock can influence local tissue physiology (Al-Nuaimi *et al.*, 2014).

The hair pigment (eumelanin, pheomelanin) is produced in dendritic cells, the melanocytes of the HF pigmentary unit (HFPU; Slominski *et al.*, 2005a; Tobin, 2011; Supplementary Figure S1 online), which produce melanin exclusively in the growth phase, anagen (Slominski *et al.*, 1991, 1994; Stenn *et al.*, 1998; Stenn and Paus, 2001). These melanocytes undergo apoptosis during the following regressive phase of the hair cycle, catagen (Schneider *et al.*, 2009), being subsequently replenished from melanocyte stem cells located in the bulge and melanoblasts in the outer root sheath (Tobin *et al.*, 1998; Commo and Bernard, 2000; Slominski *et al.*, 2005a; Nishimura, 2011; Tobin, 2011). Melanocytes produce melanin by converting tyrosine in a multi-step process in specialized organelles, melanosomes (Medes, 1932; Chávez-Béjar *et al.*, 2013). Once mature, melanosomes are transferred via filopodia from the melanocytes to either epidermal keratinocytes or to those hair matrix keratinocytes that will differentiate into the hair cortex and medulla (Scott *et al.*, 2002; Singh *et al.*, 2010; Wu and Hammer, 2014; Supplementary Figure S1).

Many different factors are implicated in HF melanocyte biology and melanogenesis control (Hearing, 1999; Ancans *et al.*, 2001; Slominski *et al.*, 2004; Lin and Fisher, 2007; Schallreuter *et al.*, 2008; Simon *et al.*, 2009; Hirobe, 2011;

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Abbreviations: HF, hair follicle; MITF, microphthalmia-associated transcription factor; qRT–PCR, quantitative real time reverse-transcriptase–PCR

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Singh *et al.*, 2012; Samuelov *et al.*, 2013; Smith *et al.*, 2013; see Supplementary Table S1) including the rate-limiting enzyme tyrosinase and microphthalmia-associated transcription factor (MITF) the ''master regulator'' of both melanogenesis and melanocyte biology, which leads to the transcription of tyrosinase (Du *et al.*, 2003; Vachtenheim and Borovanský, 2010; Pogenberg *et al.*, 2012; Praetorius *et al.*, 2013; Shoag *et al.*, 2013). The timing mechanisms that ultimately control the hair cycle–dependent activation and deactivation of intrafollicular melanogenesis are yet to be uncovered.

It is its cyclic growth, regression, and pigmentation activity that makes the HF an informative model system to dissect how oscillating molecular systems—that is, circadian rhythmicity controlled by clock gene activity (Schroeder and Colwell, 2013; Partch *et al.*, 2014; Robinson and Reddy, 2014)—interact with local tissue physiology (Geyfman and Andersen, 2010; Plikus *et al.*, 2013; Al-Nuaimi *et al.*, 2010, 2014). The clock and the HF were first linked when it was shown that core clock genes regulate the murine hair cycle (Lin *et al.*, 2009) and cell cycle progression *in vivo* (Plikus *et al.*, 2013).

Although previous reports have demonstrated that both isolated human epidermal and HF melanocytes rhythmically express clock genes/proteins in culture (Zanello *et al.*, 2000; Sandu *et al.*, 2012), it remains unknown whether the molecular clock impacts on melanogenesis or melanocyte functions. Recently, we have shown that clock genes directly regulate the anagen-to-catagen transition in isolated, organ-cultured human scalp HFs in the absence of central clock inputs (Al-Nuaimi *et al.*, 2014), suggesting that the molecular clock is an integral component of the ''hair cycle clock'' (Paus and Foitzik, 2004). As melanogenesis is coupled to anagen, this highlights the peripheral molecular clock (Supplementary Figure S2 online) as a plausible candidate in timing the on/off switching of HF pigmentation. Moreover, serotonin and melatonin are produced and metabolized in human skin and HFs and are integral components of circadian rhythms, with melatonin being a master hormonal regulator of this system (Borjigin *et al.*, 2011; Dardente, 2012), are produced and metabolized in human skin and are implicated in the control of the human hair cycle and pigmentation (Slominski *et al.*, 2005b, 2008; Kobayashi *et al.*, 2005; Paus *et al.*, 2014). In addition, pro-opiomelanocortin and corticotropin-releasing hormone, key regulators of melanogenesis that lead to the production of α-melanocyte-stimulating hormone and ACTH, are expressed systemically in a cyclic manner (Slominski *et al.*, 2000, 2013).

We have therefore investigated the hypothesis that the peripheral molecular clock regulates human HF melanogenesis by studying how BMAL1 and PER1 gene knockdown impacts on the HFPU in micro-dissected, organ-cultured human scalp HFs, using key pigmentation research read-out parameters. This was followed-up in human skin organ culture and isolated human epidermal melanocytes, so as to evaluate the HF specificity of any pigmentation regulatory effects of reducing peripheral clock activity by gene silencing and to obtain mechanistic insights.

RESULTS

Silencing of core clock components PER1 or BMAL1 increases melanin content in human anagen VI HFs in a hair cycle–independent manner

Previously, we had observed that clock gene silencing appeared to increase the melanin content of human HFs (Al-Nuaimi *et al.*, 2014). Therefore, utilizing Masson-Fontana histochemistry for visualizing melanin in *BMAL1* or *PER1* knock-down HFs we analyzed whether the increase in pigmentation simply reflects the anagen-prolonging effects of gene silencing (Kloepper *et al.*, 2010; Gáspár *et al.*, 2011; Samuelov *et al.*, 2013; Al-Nuaimi *et al.*, 2014).

This was not the case, since in both *BMAL1*- and *PER1*-silenced human HFs (anagen VI) the melanin content was increased compared to control HFs in the same anagen stage ($P = 0.014$ and $P = 0.015$, respectively; Figure 1a–c). This was further investigated by high-resolution light microscopy (Supplementary Figure S6a), which independently confirmed a significant increase in melanosome-associated melanin content in multiple HFs (small interfering RNA (siRNA) group = 12 HFs, control group = 10 HFs, each derived from three different patients). These data document that the activity of the molecular clock is involved in modulating melanin content in a hair cycle–independent manner and suggested that the peripheral molecular clock may be involved in modulating HF melanogenesis.

Knockdown of core clock proteins increases melanosome biogenesis in HF melanocytes and their transfer to HF keratinocytes

Next, by counting melanosome number in a specific reference area in melanocytes and keratinocytes, we asked whether the melanin increase observed histochemically was influenced by increased melanosome biogenesis and/or the transfer of melanosomes from HFPU melanocytes to precortical hair matrix keratinocytes (Supplementary Figure S6b).

This demonstrated that both knockdown groups had significantly more melanosomes in both HFPU melanocytes and in precortical hair matrix keratinocytes when compared with a scrambled-oligo control 24 hours post knockdown (Figure 1d and f; $P < 0.001$ for both). After 4 days, a significantly higher melanosome number was still observed in PER1 silenced HFs ($P < 0.002$); however, this was no longer the case for *BMAL1* knockdown HFs, which showed no difference from the control (Figure 1e and g). However, in both clock-silenced groups, the number of melanosomes in HF keratinocytes was significantly higher when compared with the control HFs (Figure 1e and g; $P < 0.001$). Thus, peripheral clock disruption increased both intrafollicular melanosome biogenesis and melanosome transfer within human anagen VI HFs *in situ*.

Silencing core molecular clock members increases gp100 immunoreactivity and the number of melanocyte dendrites

Subsequently, we examined the impact of core clock silencing on the expression of the premelanosomal marker gp100, which also permits one to assess melanocyte dendricity (Singh *et al.*, 2010; Samuelov *et al.*, 2013). Quantitative

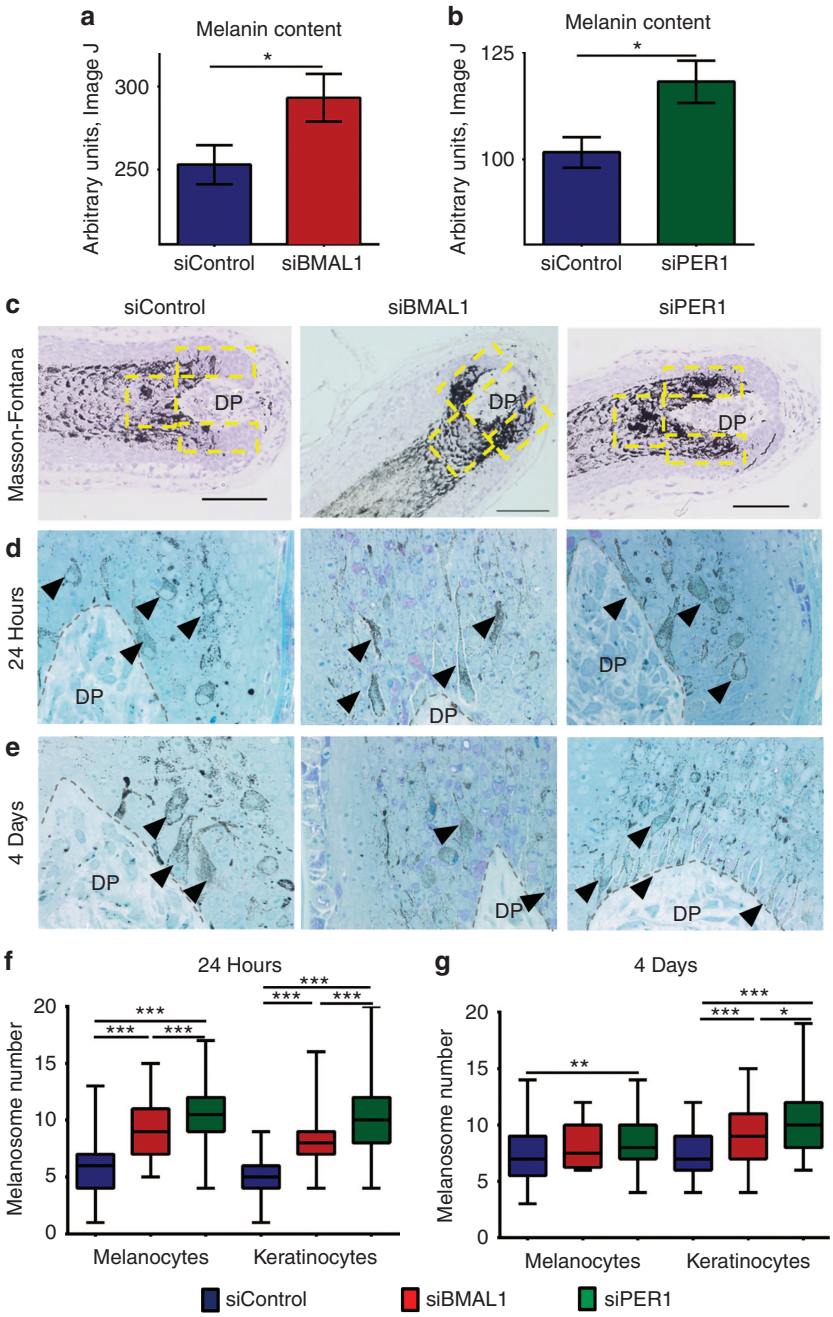


Figure 1. Partial silencing of BMAL1 or PER1 increases melanin content of human hair follicle (HF) and melanosome number. In both the PER1 (**a**) and BMAL1 (**b**) knockdown groups, there was a higher melanin content in HFs (**c**; scale = 50 μm; $n = 22$). Subsequently, the number of melanosomes in melanocytes and keratinocytes was determined from high-resolution light microscopy (**d**, **e**; × 1,000 magnification; $n = 9$, three patients; **f**, **g**; $n = 9$, three patients) finding that PER1 silencing increased the melanosome number in melanocytes and keratinocytes at both time points. BMAL1 silencing increased the melanosome number in both melanocytes and keratinocytes at 24 hours (Student's *t*-test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, error bars are ± SEM). DP, dermal papilla.

immunohistomorphometry showed that silencing *PER1* increased both gp100 immunoreactivity in individual HF melanocytes ($P < 0.001$; Figure 2a and e) and their dendricity (Figure 2d and e; Supplementary Figure S3). Silencing either member of the core molecular clock increased the number of dendrites per HF melanocyte at 24 hours, with *BMAL1* siRNA–treated HFs still showing a significant increase in melanocyte dendricity at day 4 (Figure 2d). Although the *PER1* siRNA group also showed a tendency to have more dendrites compared with *BMAL1* siRNA–treated group, this was not statistically significant at day 4.

These data demonstrate that the silencing-induced reduction in intracellular *BMAL1* or *PER1* activity increases the dendricity of HFPU melanocytes and therefore their capacity to transfer melanosomes to the surrounding precortical matrix cells. This may in part explain the increased number of

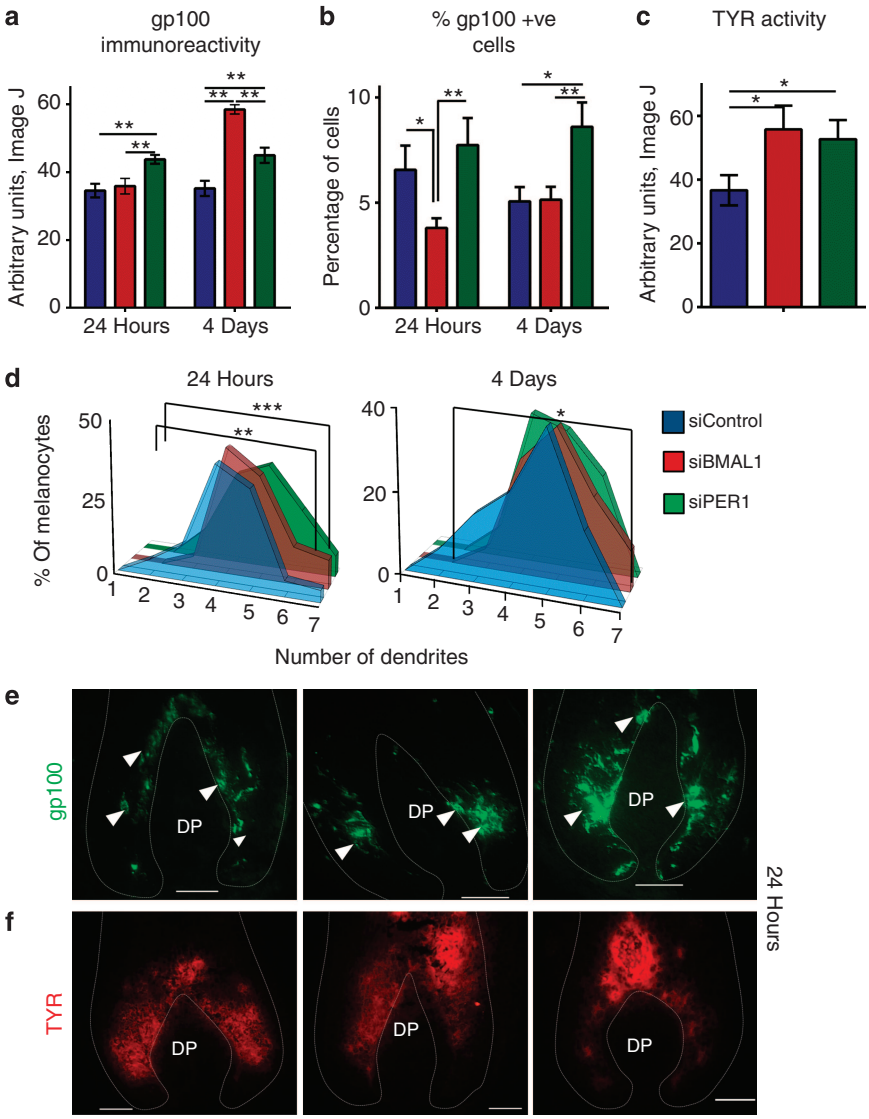


Figure 2. Silencing core clock members increases gp100 immunoreactivity, melanocyte dendricity, and tyrosinase activity in human HFs. (**a**, **e**) The melanocyte marker gp100 was enriched in the siPER1 group at 24 hours and both knockdown groups 4 days post knockdown. (**b**, **e**) gp100 analysis demonstrated that silencing PER1 increases melanocyte number at both time points, yet BMAL1 silencing decreased melanocyte number at 24 hours, recovering by day 4, and increases dendricity for both knockdown groups when compared to the scrambled oligo control at 24 hours, and at day 4 for the BMAL1 knockdown group (**d**). (**c**, **f**) Finally, tyrosinase activity *in situ* was significantly increased in both knockdown groups ($n=27$; Student's *t*-test, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, error bars and ± SEM from three patients). Arrowheads depict immunoreactive melanocytes.

melanosomes that had been detected in these cells by high-resolution light microscopy (Figure 1d and e).

Disrupting the molecular core clock alters the number of intrafollicular melanocytes

This led on to the question whether disrupting the molecular core clock also impacts on the number of HF melanocytes *in situ*, using gp100 immunoreactivity as a marker for HF melanocytes (Du *et al.*, 2003; Tobin, 2011; Ulmer *et al.*, 2014). Silencing *PER1* significantly increased the number of gp100+ HF melanocytes after 4 days ($P=0.019$; Figure 2b and e) when compared with control HFs. Notably, 24 h after *BMAL1* silencing, the number of gp100+ HF melanocytes decreased ($P=0.019$).

In order to determine whether the observed increase in melanocyte number is due to melanocyte proliferation, gp100/Ki67 double-immunostaining was performed. This showed that the number of gp100/Ki67+ double-positive cells in *BMAL1*- or *PER1*-silenced HFs is not significantly different from that in control HFs at the time points chosen (Supplementary Figure S5). Therefore, this suggests that the increase in melanocyte number by *PER1* knockdown was not due to melanocyte proliferation.

BMAL1 or PER1 silencing increases intrafollicular tyrosinase activity

To elucidate whether there was also an increase in the enzymatic machinery of melanogenesis after peripheral clock knockdown, the activity of the rate-limiting enzyme of

melanogenesis, tyrosinase, was assessed using an *in situ* enzyme activity assay (Han *et al.*, 2002; Gáspár *et al.*, 2011; Samuelov *et al.*, 2013). Quantitative assessment revealed a significantly higher level of intrafollicular tyrosinase activity *in situ* 24 hours post knockdown for both *BMAL1* siRNA– or *PER1* siRNA–treated human organ-cultured HFs (Figure 2c and f; $P=0.0065$ and $P=0.0045$, respectively). This suggests that the observed increase in intrafollicular melanin and melanosome synthesis was indeed caused by a clock-regulated increase in tyrosinase-driven melanogenesis.

Silencing BMAL1 or PER1 increases intrafollicular tyrosinase transcription

To elucidate the mechanism by which clock proteins BMAL1 or PER1 may influence intrafollicular melanogenesis, the transcription of key melanogenesis-associated genes (Slominski *et al.*, 2004; Vachtenheim and Borovanský, 2010; Tobin, 2011; Otręba *et al.*, 2012; Choi *et al.*, 2014) was investigated by quantitative real-time reverse-transcriptase–PCR. There was no significant increase in *MITF* or *gp100* transcript steady-state levels after silencing of either clock gene, even though *gp100* mRNA levels tended to be (nonsignificantly) increased in both clock knockdown groups.

However, a significant increase in the steady-state tyrosinase mRNA was observed in both the *BMAL1*- and the *PER1*-silenced groups (Figure 3a), in line with the observed increase in tyrosinase activity *in situ* (Figure 2c and f). The melanogenesis-promoting enzyme, tyrosinase-related protein 1 (*TYRP1*; Cheli *et al.*, 2010; Ghanem and Fabrice, 2011), a key MITF-target gene (see Supplementary Figure S1), was also significantly increased when compared with the oligo control group, yet only in the *PER1*-silenced HFs; TYRP2 transcription was tendentially increased (not significant) (Figure 3a). Together with the tyrosinase activity assay, this suggests that an upregulation in enzymatic machinery of melanogenesis is at least in part responsible for the increased HF pigmentation that is induced by dampening of peripheral clock activity.

Silencing core clock members increases melanin content, gp100 protein expression, and tyrosinase activity in epidermal melanocytes *in situ*

To probe whether the observed regulation of human pigmentation by peripheral core clock genes is restricted to HF melanocytes, *BMAL1* and *PER1* were also silenced in human skin biopsies that were organ cultured for 24 hours. Quantitative Masson-Fontana histochemistry and gp100 immunohistomorphometry demonstrated a significant increase in the intraepidermal melanin content and gp100 immunoreactivity in both groups (si*BMAL1* $P<0.001$, si*PER1* $P=0.004$), yet with higher gp100 immunoreactivity in *BMAL1*-silenced group (Figure 4a and b). Furthermore, tyrosinase activity was significantly increased in both *BMAL1*- and *PER1*-silenced HFs (Figure 4c). However, no differences in melanocyte dendricity was noted (Supplementary Figure S3d).

PER1 silencing increases the levels of phosphorylated MITF

Given that MITF is described as the master regulator of melanogenesis that controls the transcription of tyrosinase, *TYRP1* and *TYRP2* (Xu *et al.*, 2007; Cheli *et al.*, 2010; Vachtenheim and Borovanský, 2010; Wan *et al.*, 2011; Chen *et al.*, 2014) MITF immunoreactivity and MITF activation/phosphorylation were assessed *in situ* as changes in the intrafollicular levels of phosphorylated MITF, which regulates MITF target genes (Cheli *et al.*, 2010; Vachtenheim and Borovanský, 2010), might explain the pigmentary effects of clock gene silencing in the absence of changes in MITF transcription.

Immunohistomorphometric analysis of total MITF immunoreactivity (Figure 3c) mirrored the transcription data, showing no change between each knockdown group and oligo-treated control HFs (Figure 3b). In contrast, *PER1* silencing increased MITF phosphorylation when compared with both the oligo control and *BMAL1*-silenced HFs ($P=0.016/0.019$; Figure 3b and c). This suggests that the increase in pigmentation by dampening clock gene activity observed is caused by an increase in MITF activity as opposed to the MITF protein level.

Effects of BMAL1 silencing on melanogenesis may be mediated by a reduction in PER1

Interestingly, many of the reported melanogenesis-related read-out parameters were upregulated to a greater degree following *PER1* silencing than after *BMAL1* knockdown, namely, gp100 immunoreactivity, melanocyte, and melanosome number, as well as tyrosinase and *TYRP1* expression (see Figures 1f, g, 2b and 3a). As BMAL1 directly stimulates the transcription of PER1 (Supplementary Figure S2), we also assessed how *BMAL1* silencing affected *PER1* gene and protein expression. qRT–PCR demonstrated that silencing *BMAL1* strongly reduced *PER1* transcript levels (Figure 3a) and protein expression (Figure 3d and e). This suggests that the pigmentary events observed in the *BMAL1* siRNA–treated HFs group are caused at least in part by a significant reduction in PER1 activity. As other investigated clock genes (*CRY1*) were not affected by *BMAL1* silencing (Figure 3a), this highlights a key role for PER1 in melanocyte control.

Human melanocytes directly respond to clock gene silencing

Finally, we asked whether the complex pigmentary effects induced by clock gene silencing in human skin and HFs are mediated indirectly (for example, via keratinocytes) or operate directly within human melanocytes. To answer this question, clock genes were silenced in isolated, cultured primary human epidermal melanocytes—that is, in the absence of epithelial cell input.

This showed that both tyrosinase activity (Figure 4e) and MITF phosphorylation (Figure 4f) were significantly increased in siRNA—treated melanocytes compared with control melanocytes (both $P<0.001$), with only very few nuclei showing MITF-P immunoreactivity in the scrambled-oligo control group. Also, by qRT–PCR *TYRP2* mRNA steady-state level was increased in *BMAL1*-silenced melanocytes, whereas in the PER1-silenced melanocytes both TYRP1 and TYRP2 increased (Figure 3a). However, gp100 immunoreactivity and dendricity did not change significantly after clock silencing in cultured human epidermal melanocytes (Figure 4d; Supplementary Figure S3).

DISCUSSION

Here, to our knowledge, we present previously unreported evidence that the peripheral molecular clock operates as a cell-autonomous modulator of human pigmentation in both HF and epidermal melanocytes. The organ culture models used here allow one to investigate the influence of gene knockdown in a human organ that maintains an *in vivo*-like functional state (Philpott *et al.*, 1990; Sugawara *et al.*, 2012; Samuelov *et al.*, 2013; Al-Nuaimi *et al.*, 2014). Importantly, skin and HF organ culture permits one to study the role of peripheral clock silencing on local tissue physiology in the absence of any input from the central clock pacemaker (Al-Nuaimi *et al.*, 2014).

Our demonstration that silencing peripheral clock activity promotes human HF pigmentation in a hair cycle–independent manner (Figure 1a–c) demonstrates that clock genes, whose expression had been reported previously in human epidermal melanocytes and malignant melanomas (Zanello *et al.*, 2000; Sandu *et al.*, 2012; Lengyel *et al.*, 2013b), are functionally important for normal melanocyte

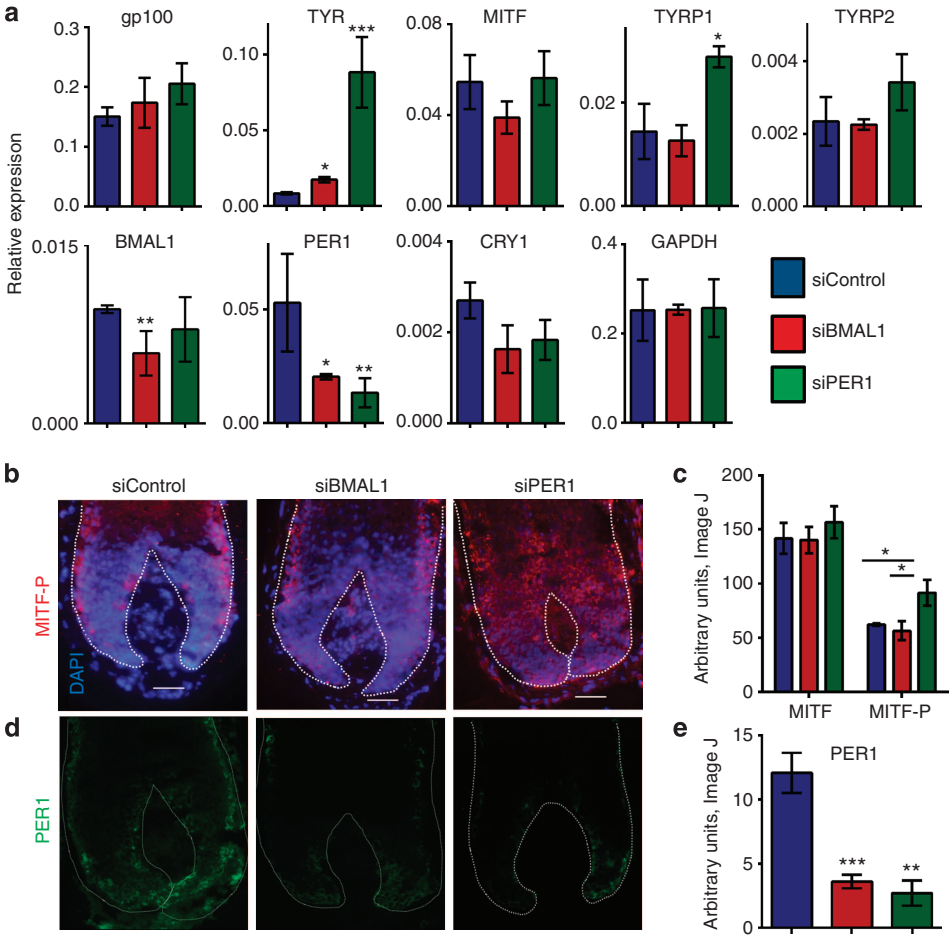
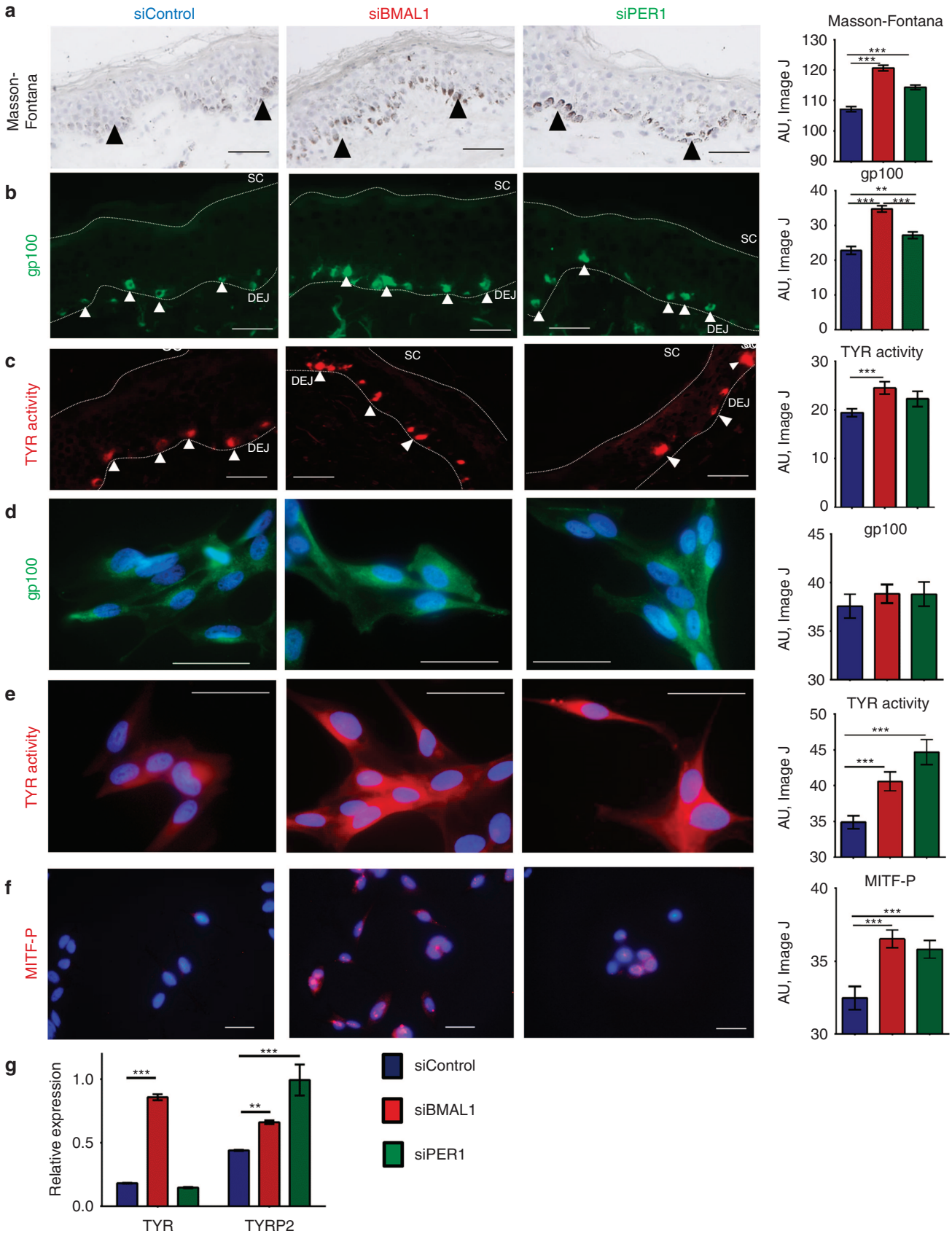


Figure 3. Silencing of core clock genes increases tyrosinase transcription and MITF phosphorylation. Quantitative real-time reverse-transcriptase–PCR was performed on clock members (PER1, BMAL1, and CRY1) and key genes involved in melanogenesis (gp100, MITF, tyrosinase (TYR), TYRP1,2). Significant transcript level changes were observed for tyrosinase in both knockdown groups (**a**), with PER1 silencing also increasing TYRP1. Although MITF transcript levels did not change, the levels of phosphorylated MITF (MITF-p) increased in the PER1 knockdown group (**b**, **c**). As BMAL1 leads to the transcription of PER1, the role of BMAL1 silencing on PER1 protein and mRNA was assessed finding that both were reduced (**d**, **e**; Student's *t*-test, $*P<0.05$, $**P<0.01$, $***P<0.001$, error bars are ± SEM, $n=15$ from three patients).

Figure 4. Knockdown of clock proteins affects human skin pigmentation and cultured primary epidermal melanocytes. To identify whether these observations are specific to HF melanocytes, clock members PER1 and BMAL1 were silenced in skin biopsies and cultured primary melanocytes. There was a significant increase in melanin content in the skin (**a**) and gp100 immunoreactivity in both knockdown groups (**b**); however, there was no change in primary melanocytes (**e**). Tyrosinase activity was increased in both the skin (**c**) and primary melanocytes (**e**) in the knockdown. Similarly, MITF-P was significantly increased (**f**). (**g**) After knockdown in primary melanocytes, there was also an increase of TYRP1 in BMAL1-silenced HFs with TYRP1,2 increasing in the PER1 knockdown (Student's *t*-test, $**P<0.01$, $***P<0.001$, error bars are ± SEM, $n=6$). Arrowheads depict melanocytes *in situ*. AU, arbitrary units; DEJ, dermal epidermal junction; SC, stratum corneum.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biology and pigmentation. We demonstrate that partial clock gene silencing increases not only melanin synthesis, *tyrosinase* and *TRP1* gene expression, and tyrosinase activity in the anagen HFs (Figure 3a) but also the melanosome number in both melanocytes and keratinocytes (Figure 1d–g). This suggests that clock silencing also increases melanosome transfer. This hypothesis is further supported by our data on the premelanosomal marker gp100 (Du *et al.*, 2003; Singh *et al.*, 2008, 2010) whose protein expression is increased (Figure 2a). Moreover, phosphorylation of MITF, the ''master regulator'' of melanogenesis biology (Vachtenheim and Borovanský, 2010), is significantly upregulated after *PER1* knockdown (Figure 3b and c).

Taken together, this shows that the peripheral clock system impacts on normal human melanocytes *in situ* at multiple different levels of melanocyte biology. Both the number of gp100+ cells and total gp100 immunoreactivity significantly changes after clock gene silencing, yet in a differential manner; melanocytes decrease after *BMAL1* knockdown (Figure 2a and b) but recover in number and show increased gp100 protein expression at day 4; in contrast, *PER1* silencing rapidly and lastingly upregulates both the HF melanocyte number and gp100 protein expression. As the proliferation of HF melanocytes *in situ* did not appear to be affected, it is conceivable that this clock-dependent change in the number of HF melanocytes reflects an impact of peripheral clock activity on the differentiation of HF melanoblasts and/or suvival of HF melanocytes in organ culture. This possibility is supported by the fact that both BMAL1 (Elshazley *et al.*, 2012; Geyfman *et al.*, 2012; Bouchard-Cannon *et al.*, 2013) and PER1 (Fu and Lee, 2003; Lengyel *et al.*, 2013a, 2013b) control the cell cycle and the apoptotic machineries in multiple different cell systems. The HF melanocyte reduction shortly after BMAL1 knockdown may thus be due to the more substantial overall effect of BMAL1 reduction, as BMAL1 is essential for clock activity (Bunger *et al.*, 2000). As such, losing BMAL1 may be more detrimental to cell survival (Elshazley *et al.*, 2012; Musiek *et al.*, 2013), thus explaining the observed temporary reduction in the HF melanocyte number.

That both *PER1* and *BMAL1* silencing increased gp100 and tyrosinase activity levels in the epidermis of organ-cultured human skin demonstrates that the clock regulation of human pigmentation is not restricted to HF melanocytes (Figure 4b and c). The molecular clock is involved in the mediation of cellular metabolism, replication, and DNA damage responses in response to UV light (Oklejewicz *et al.*, 2008; Sancar *et al.*, 2010; Geyfman *et al.*, 2012) and can also relay light cues (Magalhães Moraes *et al.*, 2014). In addition, melanocytes respond to DNA-damaging UVR stimulation by increasing dendricity, hormone production and pigmentation, as well as by acting as ''sensory'' cells within the epidermis (Slominski *et al.*, 2004; Plonka *et al.*, 2009; Singh *et al.*, 2010; Iyengar, 2013). Therefore, from a skin biology perspective, it is plausible that the biological clock and pigmentation are linked.

Our melanocyte cell culture studies demonstrate that *BMAL1* and *PER1* activity regulates human melanocyte activities in the absence of surrounding epithelial inputs and is essentially cell autonomous. This was demonstrated for tyrosinase and MITF activity and *TYRP2* transcript levels, which increased after clock silencing, suggesting that clock activity directly regulates the enzymatic production of melanin (Supplementary Figure 1; Slominski *et al.*, 2004). However, gp100 expression and melanocyte dendricity were not affected, suggesting that they may require the presence of an intact keratinocyte–melanocyte unit (Seiberg, 2001; Belleudi *et al.*, 2011), for example, in the HFPU (Tobin, 2011), whose keratinocyte component has already been documented to be clock regulated (Plikus *et al.*, 2013; Al-Nuaimi *et al.*, 2014).

Although intrafollicular BMAL1 activity may be important for HF melanocyte survival, PER1 activity appears to exert stronger effects on almost every investigated read-out parameter. As BMAL1 is directly responsible for PER1 transcription (Sato *et al.*, 2013; Robinson and Reddy, 2014; Supplementary Figure S2) and BMAL1 knockdown significantly decreased *PER1* mRNA and protein levels (Figure 3a, d and e), our results invite the speculation that the hyperpigmentation observed is primarily mediated by a reduction in PER1 levels. However, this hypothesis does not preclude that BMAL1 may also regulate additional component of the pigmentation process. For example, BMAL1 silencing might exert a similar effect as Clock knockout mice, which show upregulated tyrosine hydroxylase activity (McClung *et al.*, 2005), another important enzyme of tyrosine metabolism (Slominski *et al.*, 2004) that can promote tyrosinase activation (Videira *et al.*, 2013).

The observation that both BMAL1 and PER1 silencing essentially show the same stimulatory effect on human melanocyte functions may appear counterintuitive in that a reduced PER1 activity is expected to reduce BMAL1 expression (Bellet and Sassone-Corsi, 2010; Bass, 2012). It will be intriguing to dissect to which extent the pigmentary effects of PER1 activity reflect intracellular changes in classical circadian clock activity in human melanocytes as opposed to non-circadian PER1 activities (Franken *et al.*, 2007). It would also be interesting to now determine whether or not there is circadian rhythmicity of key HF and/or epidermal pigmentation parameters in human skin that may have escaped the notice of previous investigators.

Interestingly, melanin is a major ROS scavenger and both BMAL1 and PER1 are linked with ROS homeostasis. Indeed, BMAL1 is activated by and is protective against near-lethal doses of ROS (Tamaru *et al.*, 2013), and BMAL1 deletion leads to increased oxidative damage in mice (Khapre *et al.*, 2011; Musiek *et al.*, 2013). Conversely, PER1 can exert both a ROS-protective role (Stacy *et al.*, 1999) and can increase ROS damage (Wang *et al.*, 2013). Therefore, it will be interesting to explore the hypothesis in subsequent studies whether at least some of the increase in melanin production seen after clock silencing is a compensatory mechanism to counterbalance an intrafollicular increase in ROS production induced by the loss of clock activity.

Mechanistically, the observed upregulation in activated MITF by PER1 silencing appears to have a key role in mediating the observed pigmentary phenomena. Unfortunately,

this concept cannot be convincingly tested (for example, by silencing or antagonizing MITF in the presence of *BMAL1* or *PER1* siRNA since blocking MITF activity will inevitably inhibit pigmentation on multiple levels (Du *et al.*, 2003; Vachtenheim and Borovanský, 2010; Praetorius *et al.*, 2013). Also, it remains unclear how MITF phosphorylation is increased (Figure 3b and c) by dampening clock gene activity. One plausible possibility is that this operates via the MAPK pathway—namely, ERK1/2 activation—which subsequently activates MITF (Yanase *et al.*, 2001; Jin *et al.*, 2014). That ERK1/2 is regulated by the circadian clock (in *Neurospora*; Bennett *et al.*, 2013) further supports the hypothesis that reducing BMAL1 or PER1 activity may upregulate ERK1/2 activity and thus stimulate pigmentation. As active/phosphorylated MITF is rapidly degraded in normal melanocytes (Su *et al.*, 2013; Jin *et al.*, 2014), this may explain why no increase in intrafollicular MITF protein levels was observed in clock-silenced HFs.

Our study shows that investigating human HF and skin pigmentation in organ culture offers instructive and translationally relevant model systems for dissecting the role of the biological clock in human pigmentation. These models can now be exploited to probe whether targeting BMAL1 and/or PER1 activity—for example, via the topical application of small molecule modulators of clock proteins (Chen *et al.*, 2012; Chun *et al.*, 2014; Kojetin and Burris, 2014)—can also be utilized therapeutically to manage human pigmentary disorders, ranging from HF graying to epidermal depigmentation.

MATERIALS AND METHODS

Human HF and skin samples

Discarded human scalp HFs and abdominal skin were obtained with informed, written consent following the 'Declaration of Helsinki Principles'. All tissues were received and stored with ethical and institutional approval from the University of Manchester and stored and tracked in a human tissue biobank (see Supplementary Table S4 for patient information).

In vitro silencing of BMAL1 and PER1 in human HFs and skin

Micro-dissected human HFs (Kloepper *et al.*, 2010) or 4 mm full-thickness human skin punch fragments (Lu *et al.*, 2007; Knuever *et al.*, 2012) were transfected with siRNA specific for *BMAL1* or *PER1* with a scrambled-oligo control, as previously described in detail (Al-Nuaimi *et al.*, 2014; see Supplementary Table ST1).

Masson-Fontana stain for melanin

HF cryosections were stained for melanin using a 10% silver nitrate solution with ammonia following the protocol as described by Kloepper *et al.* (2010).

HF fixation for high-resolution light microscopy

After gene knockdown, samples were fixed in half strength Karnovsky's fixative with a secondary fixation in osmium tetroxide (Tobin *et al.*, 1998; Bodó *et al.*, 2007) and 0.5 μm semi-thin sections were counterstained with toluidine blue. Images were taken using a $\times 600$ magnification ($\times 60$ digital zoom) on a Keyence Biozero 8000 microscope (Osaka, Japan).

gp100 immunofluorescence

OCT-embedded cryosections (5 μm) were fixed in acetone and stained for gp100 (premelanosome protein, silver-locus protein; Singh *et al.*, 2008, 2010) by immunofluorescence microscopy using an Alexafluor 488 (Life Technologies, Paisley, UK) goat anti-rabbit secondary antibody following previously described protocol (Gáspár *et al.*, 2011; Samuelov *et al.*, 2013; see Supplementary Table S2).

MITF immunofluorescence

Human HF or skin cryosections were fixed in a 1:1 mix of methanol and acetone and washed with TBS. After blocking with 10% normal goat serum in TBS, HF/skin sections were incubated at room temperature overnight with primary antibody directed against MITF or phosphorylated (that is, transcriptionally active MITF [MITF-P]) (Sigma-Aldrich, Dorset, UK; see Supplementary Table S2). Subsequently, HFs were incubated with a goat anti-rabbit Alexafluor 594 secondary antibody for 45 minutes and counterstained with 4′,6-diamidino-2-phenylindole before mounting.

Tyrosinase activity *in situ* assay

Tyrosinase was assessed using the protocol as described by Han *et al.* (2002).

Immunohistomorphometry

Immunoreactivity was quantified on images from multiple cryosections (6 μm) and measured with Image J (NIH, Bethesda, MD) software using defined set reference areas, as described before (Kloepper *et al.*, 2010; Gáspár *et al.*, 2011; Samuelov *et al.*, 2013). For individual cell measurements (Figure 2a), measurements were corrected for cell size and background (Burgess and Vigneron, 2010).

Total RNA extraction and qRT–PCR

RNA was extracted from five human HFs or one human skin biopsy per group, using an RNeasy micro kit (Qiagen, Crawley, UK). RNA was used to synthesize cDNA using a Tetro cDNA Synthesis kit (Bioline, London, UK). Quantitative PCR was performed using a StepOne real-time PCR system (Applied Biosystems, Foster City, CA) and the probes listed in Supplementary Table S3 (see Supplementary Text ST1). Steady-state transcript levels were normalized to the least regulated housekeeping gene, *PPIA*, and verified using *GAPDH* (Figure 3a).

Human epidermal melanocyte culture

Isolated human scalp epidermal melanocytes were seeded at a density of $1.5 \times 10^6\,\mathrm{ml}^{-1}$ and cultured in melanocyte growth media (Promocell, Heidelberg, Germany). For knockdown experiments, melanocytes were seeded on chamber slides for immunofluorescent staining and a 6-well plate for RNA extraction following the same protocol as for HF/skin knockdown.

Statistical analysis

A Student's *t*-test was used for all statistical analysis using the Holm-Sidak correction for multiple comparisons unless stated otherwise. Where the data were nonparametric, the Mann–Whitney test was used.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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

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