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Complete List of Authors:
Al-Nuaimi, Yusur; Salford Royal , Salfor Royal Foundation Trust ; University of Manchester, The Dermatology Centre, Salford Royal NHS Foundation Trust and the Institute of Inflammation and Repair
Hardman, Jonathan; University of Manchester, Inflammation and Repair
Bíró, Tamás; Univeristy of Debrecen, DE-MTA "Lendulet" Cell Physiology group, Department of Physiology
Haslam, Iain; University of Manchester, The Dermatology Centre, Salford Royal NHS Foundation Trust and the Institute of Inflammation and Repair
Philpott, Michael; Queen Mary College, Centre for Cutaneous Research and the London School of Medicine and Dentistry
Tóth, Balázs; Univeristy of Debrecen, DE-MTA "Lendulet" Cell Physiology group, Department of Physiology
Farjo, Nilofer; Farjo Medical Centre, Medical Centre
Farjo, Bessam; Farjo Medical Centre, Medical Centre
Baier, Gerold; University of Manchester, Manchester Institute of Bioscience
Watson, Rachel; University of Manchester, The Dermatology Centre, Salford Royal NHS Foundation Trust and the Institute of Inflammation and Repair
Grimaldi, Benedetto; Italian Institute of Technology, Drug Discovery and Development
Kloepper, Jennifer; University of Luebeck, Department of Dermatology
Paus, Ralf; University of Manchester, The Dermatology Centre, Salford Royal NHS Foundation Trust and the Institute of Inflammation and Repair; University of Luebeck, Department of Dermatology

A meeting of two chronobiological systems: Period1 and BMAL1 modulate the human hair cycle

Yusur Al-Nuaimi[1,3], Jonathan A. Hardman[2,3], Tamás Bíró[4], Iain S. Haslam[3], Michael Philpott[5], Balázs I. Tóth[4], Nilofer Farjo[6], Bessam Farjo[6], Gerold Baier[7], Rachel E.B. Watson[3], Benedetto Grimaldi[8], Jennifer E. Kloepper[9]*, Ralf Paus[3,9] *§.

[1]Salford Royal Foundation Trust, Salford Royal Hospital, Salford, UK

[2]Doctoral Training Centre in Integrative Systems Biology, Manchester Interdisciplinary Biocentre, University of Manchester, UK

[3] The Dermatology Centre, Salford Royal NHS Foundation Trust and the Institute of Inflammation and Repair, University of Manchester, Manchester, UK

[4]DE-MTA ''Lendulet'' Cell Physiology Group, Department of Physiology, University of Debrecen, Debrecen, Hungary

[5]Centre for Cutaneous Research. Barts and the London School of Medicine and Dentistry, Queen Mary College London, UK

[6] Farjo Medical Centre, Manchester, U.K

[7]Manchester Interdisciplinary Biocentre, University of Manchester, UK

[8] Italian Institute of Technology, Geneva, Italy

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

*contributed equally

§corresponding author: ralf.paus@uksh.de, ralf.paus@manchester.ac.uk

Department of Dermatology,

University of Luebeck,

D-23538 Luebeck, Germany

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ABSTRACT

The hair follicle (HF) is a continuously remodelled miniorgan that cyclically switches between growth (anagen), regression (catagen) and relative quiescence (telogen). Since the anagen-catagen transformation of micro-dissected human scalp HFs can be observed in organ culture, this permits one to study the as yet unknown controls of autonomous, rhythmic tissue remodelling of the HF which intersects developmental, chronobiological and growth-regulatory mechanisms. Here, we have tested the hypothesis that the peripheral clock system is involved in the clinically important anagen-to-catagen transition. We show that in the absence of central clock influences isolated, organ-cultured human HFs show circadian changes in the gene and protein expression of core clock genes (*CLOCK, BMAL1, Period1*) and clock-controlled genes (*c-Myc*, *NR1D1, CDKN1A*), Period1 expression being hair cycle-dependent. Knock-down of *BMAL1* or *Period1* in human anagen HFs both significantly prolonged anagen and stimulated hair matrix keratinocyte proliferation. Moreover, individual silencing of these two core clock genes also stimulated HF melanogenesis in a hair cycle-independent manner. This provides the first evidence that peripheral core clock genes modulate human HF cycling and pigmentation, and are an integral component hair cycle. Specifically, our study identifies BMAL1 and Period1 as novel therapeutic targets for modulating human hair growth and pigmentation.

INTRODUCTION

The hair follicle (HF) is a highly dynamic mini-organ that undergoes a cyclical remodelling process, called the hair cycle (Kligman, 1959; Paus and Cotsarelis, 1999; Schneider *et al.*, 2009; Stenn and Paus, 2001). The chronobiological mechanisms that regulate the rhythmical transformation of the hair follicle have remained elusive. The HF cyclically undergoes massive cell death and subsequently regenerates owing to its rich endowment with various stem cell populations (Cotsarelis, 2006; Fuchs, 2009; Lavker *et al.*, 2003; Plikus, 2012; Plikus *et al.*, 2011; Plikus *et al.*, 2012). The hair cycle comprises three phases; the growth stage (anagen) is characterised by long-lasting epithelial proliferation and production of a pigmented hair shaft. Anagen is followed by rapid, apoptosis-driven organ involution (catagen) where the lower two thirds of the HF regress, and then by a phase of relative quiescence (telogen) (Fig. S1). Due to its autonomous oscillatory behaviour, the hair cycle represents an ideal model for studying complex mesodermal-neuroectodermal tissue interactions at the intersection of chronobiology, developmental biology, regenerative medicine and systems biology (Al-Nuaimi *et al.*, 2010; Al-Nuaimi *et al.*, 2012; Halloy *et al.*, 2002; Plikus, 2012; Stenn and Paus, 2001).

While numerous molecular players are known to impact on HF cycling, the basic controls of this oscillatory mechanism (“hair cycle clock”) remain unknown (Lin *et al.*, 2009; Paus and Foitzik, 2004; Plikus *et al.*, 2008; Schneider *et al.*, 2009). Investigating these controls promises new insights into general principles that impact on mammalian organ remodelling, and is of major clinical relevance as the vast majority of hair growth disorders can be attributed to altered HF cycling; most notably to deviations in the anagen-catagen transition (Cotsarelis and Millar, 2001; Paus, 2006; Paus and Cotsarelis, 1999; Paus and Foitzik, 2004; Peters *et al.*, 2006; Schneider *et al.*, 2009). Yet, none of the currently available major anti-hair loss drugs (e.g. finasteride, minoxidil) counteract undesired anagen shortening with satisfactory efficacy and reliability. Therefore, more effective “hair drugs” that target the anagen-catagen transformation **(Fig. S1)** are needed urgently (Cotsarelis and Millar, 2001; Paus and Foitzik, 2004).

There is growing consensus that the regulatory mechanisms governing the human hair cycle are based on an intra-follicular oscillatory system (Al-Nuaimi *et al.*, 2010; Kwon *et al.*, 2006; Lin *et al.*, 2009; Paus and Foitzik, 2004; Robinson *et al.*, 1997). One candidate is the circadian clock, a molecular oscillatory system with a 24-hour periodicity (Bass, 2012; Brown *et al.*, 2012; Dardente and Cermakian, 2007; Dunlap *et al.*, 2004; Feng and Lazar, 2012; Lowrey and Takahashi, 2004; Miller *et al.*, 2007; Ota *et al.*, 2012; Schibler and Sassone-Corsi, 2002) **(Fig. S2)**. The circadian clock is synchronised by the 'master regulator' the suprachiasmatic nucleus (SCN) which receives external cues, e.g. light and temperature, which synchronises the molecular clock found in peripheral tissues via sympathetic, parasympathetic and glucocorticoid signals, even though the exact mechanisms of this synchronisation are not fully understood (Bass, 2012; Brown *et al.*, 2012; Dunlap *et al.*, 2004; Feng and Lazar, 2012; Lowrey and Takahashi, 2004; Miller *et al.*, 2007; Ota *et al.*, 2012; Schibler and Sassone-Corsi, 2002; Sporl *et al.*, 2011). With the recent focus on peripheral clock activity and its profound influence on tissue functions, separate from the SCN, chronobiology research has entered into the field of peripheral tissue physiology (Albrecht, 2012; Dardente and Cermakian, 2007; Ota *et al.*, 2012; Saini *et al.*, 2011; Sporl *et al.*, 2011; Tonsfeldt and Chappell, 2012). Since clock dysfunction may cause tissue pathology (Chen-Goodspeed and Lee, 2007; Geyfman and Andersen, 2010; Geyfman *et al.*, 2012b; Lee, 2005; Sahar and Sassone-Corsi, 2009; Takahashi *et al.*, 2008; Takita *et al.*, 2012), a greater understanding of the clock system and the ability to modulate it pharmacologically, may have therapeutic benefits.

Since cultured murine or human keratinocytes, fibroblasts and melanocytes express clock genes and show 24-hour circadian rhythmicity (Kawara *et al.*, 2002; Sporl *et al.*, 2011; Tanioka *et al.*, 2009), and, furthermore, since murine and human skin express clock genes (Zanello *et al.*, 2000), the molecular clock may be implicated in human hair growth control as well **(Fig S2)**. Indeed, murine HFs express clock and clock-controlled genes (CCGs) which may regulate the hair cycle (Geyfman and Andersen, 2010; Geyfman *et al.*, 2012a; Lin *et al.*, 2009), since the deletion of core clock genes delayed anagen onset (Lin

et al., 2009). In addition, clock genes also impact on the cell cycle, where they temporally segregate metabolic and cell cycle activity, and control apoptotic genes (Chen-Goodspeed and Lee, 2007; Fu *et al.*, 2002; Geyfman *et al.*, 2012b; Lee, 2005; Matsuo *et al.*, 2003; Sahar and Sassone-Corsi, 2009; Takahashi *et al.*, 2008), key processes during HF cycling (Al-Nuaimi *et al.*, 2012; Paus and Foitzik, 2004; Schneider *et al.*, 2009; Stenn and Paus, 2001). Clock genes co-ordinate the activation of murine HF stem cells, which exist in a heterogeneous state, i.e. those that express high Period 1 (Per1) and those with low Per1 (Janich *et al.*, 2011). Finally, plucked hair shafts permit one to study the human peripheral circadian clock (50).

On this basis, we hypothesised that clock genes, (Albrecht, 2012; Bass, 2012; Dardente and Cermakian, 2007; Dunlap *et al.*, 2004; Lee, 2005; Lowrey and Takahashi, 2004; Lowrey and Takahashi, 2011; Saini *et al.*, 2011; Sporl *et al.*, 2011; Tonsfeldt and Chappell, 2012) may function as molecular components of the human "hair cycle clock" (Paus and Foitzik, 2004). Since previous studies (Akashi *et al.*, 2010; Lin *et al.*, 2009) have not distinguished between the effects of the central and peripheral clock on the HF, we have asked:

1. Does the expression of clock genes/proteins in intact, isolated human scalp HFs, i.e. in the *absence* of central clock inputs, show circadian and/or hair cycle-dependent variations?

2. Does silencing core molecular clock components affect human HF cycling, hair growth and/or pigmentation *in vitro*?

RESULTS

Human anagen HFs transcribe core clock and CCGs with circadian rhythmicity

We first investigated whether the core clock genes, *CLOCK, BMAL1*, and *PER1* are transcribed in human anagen scalp HFs. As expected from previous data generated in murine and human skin and plucked human hair shafts (Akashi *et al.*, 2010; Brown *et al.*, 2008; Geyfman *et al.*, 2012b; Sandu *et al.*, 2012), human anagen scalp HFs expressed CLOCK, BMAL1 and PER1 mRNA and protein (**Fig. 1, 2, 3b, S3 S4**). In addition, human anagen scalp HFs transcribed the CCGs, *c-Myc, NRD1* and *CDKN1a* (**Fig. 1**).

Next, we determined by qRT-PCR whether human HFs also exhibit a circadian expression pattern for any of these genes. Following dexamethasone synchronisation of clock gene activity (Balsalobre *et al.*, 2000), HFs were sampled every 4 hours over a 24-hour period. All three core clock genes were expressed in the HFs of three separately tested patients, and showed a circadian variation of their transcription patterns. In two separately tested individuals, circadian rhythmicity was also seen for the intrafollicular transcription of CCGs (*NR1D1, c-Myc and CDKN1a* (*P21)*) (**Fig. 1**). Despite the expected inter-individual variation, their phase distribution and mean overall relative expression values were similar. Subsequently, all HFs were harvested within the same time window (7-10 p.m.; see Materials and Methods), if not stated otherwise.

CLOCK, PER1 and BMAL1 are also expressed on the protein level in human HFs

By immunohistochemistry/immunofluorescence microscopy, we then checked whether the core clock genes are also expressed at the protein level. As shown in **Figs. 2**, **S3 S4**, CLOCK, BMAL1 and PER1 protein immunoreactivity (IR) was restricted mainly to the HF epithelium, where it was most prominent in

the outer root sheath (ORS). However, BMAL1 IR was also detected in the dermal papilla and connective tissue sheath (CTS) (**Fig. 2d**, **Fig. S1**).

Intrafollicular PER1 gene and protein expression is hair cycle-dependent

To probe, whether clock gene expression in organ-cultured human scalp HFs is hair cycle-dependent, we compared gene and/or protein expression between anagen VI and catagen HFs. This showed that the mRNA steady-state levels (**Fig. 3b**, qRT-PCR) and the intrafollicular PER1 protein expression for *PER1* (**Fig. 2c**, quantitative immunohistomorphometry) were significantly higher in catagen HFs compared to anagen VI HFs. In order to check whether the observed increase in PER1 expression was indeed hair cycle-dependent and did not just result from circadian expression changes, intrafollicular PER1 IR was also compared at two different time points (9 a.m., 3 p.m.) between anagen and catagen HFs. This showed that, irrespective of the time of day analyzed HFs were analyzed, PER1 expression was always higher in catagen than in anagen HFs (**Fig. S3c**). Also, when clock gene activity in anagen VI HFs was synchronized by dexamethasone treatment, the amplitudes of the relative *PER1* mRNA levels differed significantly between anagen and catagen (**Fig. 3b**). While minor transcriptional amplitude differences between anagen and catagen HFs were also seen for *CLOCK* and *BMAL1*, these did not reach significance (**Fig. 2e and 3b**). Taken together this shows that expression of at least one core clock gene product, PER1, is hair cycle-dependent.

PER1 silencing in human HFs significantly prolongs anagen

Therefore, we next investigated the functional consequences of reducing *PER1* gene activity on human HF cycling by intrafollicular gene knock-down (Samuelov *et al.*, 2012) with *PER1* siRNA transfection. Successful PER1 knock-down in human anagen HF organ culture was demonstrated at the mRNA and protein level (**Fig. S4a**). Given that PER1 expression was low in anagen and sharply rose during catagen,

we hypothesized that PER1 silencing would prolong anagen duration. Indeed, 96 hours following *PER1* knock-down, a significantly greater proportion of human HFs transfected with *PER1* siRNA had remained in anagen (71.4%) than in the scrambled oligo-treated control group (4.3%) (**Fig. 4a**). This observation was confirmed in 4 separate experiments with HFs from 4 different individuals (**Fig. 4a** shows pooled data from all four experiments). The anagen-prolonging effect of PER1 silencing was confirmed by the demonstration that the test group also showed a higher number of Ki-67 positive (i.e. proliferating) hair matrix keratinocytes compared to the scrambled oligonucleotide-treated HFs (**Fig. 4b**). This identifies PER1 as a novel catagen-inducing signal in human cycle control, whose silencing prolongs the duration of anagen.

BMAL1 or CLOCK silencing in human HFs also prolongs anagen

To assess whether anagen prolongation by PER1 silencing is PER1-specific, or an effect of the peripheral core molecular clock, organ-cultured human HFs were transfected with a *BMAL*1-specific siRNA probe, and knock-down was achieved on the mRNA and protein level (**Figure S4**). This experiment was important as BMAL1 is essential for the core clock oscillations and induces the transcription of PER1 (see **Fig.S2**), and since silencing of BMAL1 leads to asynchrony of the peripheral clock (Balsalobre *et al.*, 2000; Bunger *et al.*, 2000).

96-hours after BMAL1 knock-down a significantly greater proportion of silenced HFs (42%) remained in anagen VI than in the control group (10%) (**Fig. 5a**). Although BMAL1-silencing slightly modulated hair matrix keratinocyte proliferation/apoptosis, this did not reach significance. Pilot data from an additional CLOCK knock-down experiment (1 patient) also demonstrated anagen prolongation (**Fig. S5a**). Taken together, this suggests that the molecular clock as a system, rather than just individual clock components, controls the human “hair cycle clock”.

PER1 and BMAL1 both inhibit human HF melanogenesis

As HF melanogenesis is tightly coupled to anagen (Slominski *et al.*, 2005; Tobin *et al.*, 1998), HF pigmentation was assessed as another marker for anagen prolongation. This confirmed that, 96 hours after PER1 or BMAL1 knockdown, as expected, the melanin content of both control and silenced HFs was significantly decreased ($p < 0.001$) as HFs in both groups entered catagen (**Fig. 4c & 5c**). However, when only anagen VI HFs were compared between silenced and control groups, the melanin content of PER1- or BMAL1-silenced anagen HFs was significantly increased compared to control anagen VI HFs (PER1: $p=0.034$, BMAL1: $p=0.014$). These quantitative histomorphometry experiments show that PER1 and BMAL1 also operate as hair cycle-*independent* inhibitors of human HF pigmentation. Again, the pilot CLOCK knock-down mirrored the results from the PER1 and BMAL1 knock-down, showing an increased melanin content of CLOCK-silenced anagen VI HFs (**Fig. S5c**).

DISCUSSION

Following up on prior *in vivo* work in mice (Lin *et al.*, 2009) and human scalp hair shafts (Akashi *et al.*, 2010), our study provides the first evidence that intact human scalp HFs show both circadian and hair cycle-dependent clock gene activity *in the absence of central clock influences*. Moreover, we demonstrate that both *peripheral clock* PER1 and BMAL1 can regulate human HF cycling without input from the central clock. Specifically, we show that both PER1 and BMAL1 produce anagen-terminating signals and, independent of this hair cycle-regulatory effect, also inhibit human HF pigmentation *in situ*. Importantly, the organ-cultured human HFs in the current study lack the bulge region (Ito *et al.*, 2004; Philpott *et al.*, 1994). Therefore, the observed inhibition of both catagen HF pigmentation by PER1 and BMAL1 silencing occurred independently of epithelial and melanocyte stem cells in the bulge (Nishimura, 2011; Plikus, 2012; Plikus *et al.*, 2012) and of potential changes in clock gene activity in these HF stem cells (Janich *et al.*, 2011; Plikus, 2012; Plikus *et al.*, 2011).

That the silencing of both PER1 and BMAL1, who's deletion disrupts the clock (Bunger *et al.*, 2000), had the same hair growth- and pigmentation-stimulatory effects strongly suggests that these are not isolated events, but reflect core peripheral clock activity (**cf. Fig. S2**). This concept is corroborated by our CLOCK knock-down pilot data (**Fig. S5a**).

Our findings correspond to a growing body of evidence that clock genes regulate physiological processes including infradian rhythms, such as the cell cycle (Khapre *et al.*, 2010; Matsuo *et al.*, 2003), metabolism (Bass, 2012; Geyfman *et al.*, 2012b), tumour growth (Chen-Goodspeed and Lee, 2007; Fu *et al.*, 2002; Yang *et al.*, 2009), seasonal rhythms (Hazlerigg and Loudon, 2008), breeding patterns (Miller *et al.*, 2004), reproductive cycle (Ware *et al.*, 2012) and complex disease states such as diabetes mellitus and depression (de Bodinat *et al.*, 2010; Etain *et al.*, 2011). Thus, the autonomous oscillations of PER1 and

BMAL1 observed in human scalp HFs are in line with an evolving paradigm shift in the importance of "circadian" clock functions in peripheral tissue physiology (Geyfman and Andersen, 2010; Geyfman *et al.*, 2012b; Janich *et al.*, 2011; Plikus *et al.*, 2011). Moreover, they suggest that the core peripheral clock is an integral component of the elusive "hair cycle clock" (Al-Nuaimi *et al.*, 2012; Paus and Foitzik, 2004), which may operate under the control of both transcriptional and posttranscriptional circadian clock mechanisms (Koike *et al.*, 2012) (**Fig. S1**). While murine *in vivo* work had already implicated clock gene activity in the control of murine HF cycling (Lin *et al.*, 2009), this pioneering study had not been able to exclude a role of the central clock. The current study settles this question by showing that the central clock is dispensable for clock-controlled hair cycle modulation.

The differences in expression of PER1 protein and mRNA between anagen and catagen reported here in human scalp HFs are mirrored in the murine hair cycle: *Per1* mRNA expression in mouse skin increases during the anagen-catagen transformation *in vivo* (Lin *et al.*, 2009), though less dramatically than during the human anagen-catagen transformation *in vitro*. In contrast to previous work, the current organ culture study clearly excludes the central clock, suggesting that peripheral *Period* family activity is not only directly important for human, but also for murine HF cycling (Geyfman *et al.*, 2012a; Lin *et al.*, 2009). While the catagen-telogen and telogen-anagen transformations cannot be studied in human HF organ culture, recent microarray analyses of synchronized murine HF cycling suggest that peripheral clock gene activity also impacts on other transformation stages of the cycling HF (Geyfman and Andersen, 2010; Geyfman *et al.*, 2012a; Lin *et al.*, 2009).

In human HFs, BMAL1 shows consistent protein expression throughout the anagen-catagen transformation, which differs from the murine system, where *BMAL1* mRNA and protein expression peaked in late anagen (Lin *et al.*, 2009). Therefore, potential species-specific differences in how and when the peripheral core clock impacts on HF cycling may exist. The significant variations of clock and

CCG activity observed confirms earlier work (Akashi *et al.*, 2010), however, has shown that the clock genes will continue to oscillate in absence of the SCN.

While the (likely complex) mechanisms through which PER1 and BMAL1 exert their hair growth- and proliferation-modulatory effects remain to be dissected in follow-up studies, these effects are well in line with the established concept that clock genes and CCGs control cell cycling (Geyfman *et al.*, 2012b; Lowrey and Takahashi, 2004; Miller *et al.*, 2007). Interestingly, two recognised hair cycle-regulatory genes, c-Myc (Bull *et al.*, 2001; Bull *et al.*, 2005) and p21 (Mitsui *et al.*, 2001; Ohtani *et al.*, 2007), are not only key cell cycle regulators, but both are also reduced by PER1 knock-down. Also, the reduction of P21 by PER1 silencing corresponds well to the reduced p21 expression in BMAL1 knock-out mice which show delayed anagen onset (Lin *et al.*, 2009). PER2 has further been shown to control cyclin D (Fu *et al.*, 2002), a recognized modulator of human HF cycling (Xu *et al.*, 2003). Thus it is reasonable to hypothesize that both PER1 and BMAL1 regulate proliferation and apoptosis, and thus the anagen-catagen transformation (13) by impacting on the cell cycle and apoptotic machinery of hair matrix keratinocytes , similar to role of CCGs Nr1d1 and Dbp in the murine hair matrix (Lin *et al.*, 2009).

To the best of our knowledge, this study also provides the first evidence in any system and species that peripheral core clock genes regulate melanogenesis, and identifies PER1 and BMAL1 as novel, endogenous inhibitors of human pigmentation *in situ*. Our demonstration that the peripheral core clock also regulates human HF pigmentation invites the question whether excessive clock activity may in any way be related to human HF greying (Inomata *et al.*, 2009; Paus, 2011; Tobin and Paus, 2001), and whether antagonizing PER1 and BMAL1 activity could be exploited as an anti-greying strategy.

Evidently, another important translational aspect of the current study is that our results designate the peripheral core clock, specifically PER1 and BMAL1 activity, as promising novel targets for therapeutic

hair growth modulation, e.g. with topically applied, HF-targeting (Chourasia and Jain, 2009; Liu *et al.*, 2011; Patzelt *et al.*, 2011) small molecule clock modifiers (Chen *et al.*, 2012), thus circumventing undesired effects on the central clock. Antagonising the activity of PER1, BMAL1, CLOCK and/or selected down-stream CCGs may serve to counteract hair loss (alopecia, effluvium), while promoting activity of these targets may suppress unwanted hair growth (hirsutism, hypertrichosis) (Cotsarelis and Millar, 2001; Paus, 2006).

In summary, we show that the peripheral core clock modulates human HF cycling and pigmentation under clinically relevant *in vitro*-conditions, that BMAL1, PER1, and likely also CLOCK form an integral component of the elusive human "hair cycle clock" and that these clock genes are novel targets for the therapeutic modulation of human hair growth and pigmentation. Moreover, we demonstrate that HF organ culture (59, 83) offers a highly instructive, clinically relevant model for preclinical peripheral clock research in a complex, oscillating human mini-organ where two chronobiological systems meet.

MATERIALS AND METHODS

Human skin and HF collection

Redundant human scalp skin was obtained from the temporal or occipital regions from females undergoing routine facelift surgery (total n=3, 31-69 years) and scalp occipital hair follicular units from males undergoing hair transplantation surgery (total n=10, 28-48 years). Tissue was obtained following ethical approval (University of Luebeck; University of Manchester) and informed subject consent. Skin or HFs were fixed in 10% phosphate buffered formalin or snap-frozen in liquid nitrogen or first embedded in Shandon Cryomatrix (Fisher Scientific) prior to snap freezing.

Human HF organ culture

Human scalp HFs in anagen stage VI of the hair cycle (**Fig. S1**) were micro-dissected and organ-cultured under serum-free conditions in the presence of insulin and hydrocortisone as described (Philpott *et al.*, 1990; Philpott *et al.*, 1994) **(supplementary text S1a).** Under these conditions human anagen HFs continue to produce a pigmented hair shaft and eventually spontaneously enter a catagen-like state (Kloepper *et al.*, 2010; Sanders *et al.*, 1994). The telogen hair cycle phase cannot be captured n human HF organ culture.

24-hour time series experiment

Circadian rhythmicity of the expression of core clock genes and selected CCGs was investigated in human scalp occipital anagen VI HFs from three male subjects (**Supplementary table S2** a, b, c). Surgery was performed during the time window of 09:30-13:00 GMT. Micro-dissection and organ culture of the HFs was set-up within 2 h after surgery, and incubated without further manipulation for 24 hours (equilibration period) for 2 subjects and after 7 days for the third. Follicular clock activity was then synchronized (100nM dexamethasone, 30 minutes) (Balsalobre *et al.*, 2000), after which HFs were

harvested every 4 hours for a 24 hour period, stored in RNAlater (Sigma) and then processed for qRT-PCR analysis.

In a second time series experiment HFs were cultured and staged according to macroscopic staging criteria (Kloepper *et al.*, 2010). For subject C (**Supplementary table S2**); once half the HFs were in anagen and the others had entered catagen, the samples were synchronised as above and both anagen and catagen HFs were collected every 5 hours. For subjects D and E this was repeated however, all HFs entered catagen (**Supplementary table S2**). This took between 7 and 14 days for the HFs to enter the correct stage. The HFs were maintained in RNAlater until processed for qRT-PCR analysis to check the expression of *CLOCK*, *BMAL1* and *PER1*.

Quantitative immunohistomorphometry

Immunohistochemistry or immunofluorescence microscopy (IHC/IF) staining for localisation and quantification of clock proteins (CLOCK, BMAL1, PER1) *in situ* was established and performed on whole human scalp skin cryosections (8 µm) or isolated human scalp HFs (6 µm). [See **Supplementary Table S1** for details.(Ackermann *et al.*, 2007)] Primary antibodies were incubated overnight at 4°C. Sections were washed in either phosphate-buffered saline or TRIS-buffered saline between steps. IHC staining for Masson-Fontana and Ki-67/TUNEL double-immunofluorescence microscopy were carried out as previously described (Ito *et al.*, 2005; Kloepper *et al.*, 2010; van Beek *et al.*, 2008). Quantitative immunohistomorphometry in defined reference area, using standardized light exposure, was performed with Image J (NIH) software as described (Ito *et al.*, 2005; Kloepper *et al.*, 2010).

PER1 and BMAL1 knock-down in organ cultured human HFs

Micro-dissected human anagen VI HFs were transfected with either *PER1* siRNA (PER1 FsiRNA (h): sc-38171) or *BMAL1* siRNA (FsiRNA (h): sc-38165) in organ culture, following the previously described lipofectamine-based knock-down method (Chen and Roop, 2012), using scrambled oligo as a parallel control. (See **Supplementary Table S3** for details). Results were analysed by combining the data from

three subjects for the siRNA *BMAL1* data. A pilot knock-down of a third member CLOCK further supported this data showing an increased number of HFs in anagen when compared to a control **(Fig. S5a)**.

Quantitative RT-PCR

All qRT-PCR for *CLOCK*, *BMAL1, PER1, NR1D1*, *c-Myc* and *CDKN1A* was performed as described in the supplement, normalized to a housekeeping gene (PPIA) (**supplementary text S1b**, **supplementary table S4**).

***In situ* hybridisation: *CLOCK* mRNA**

Intrafollicular clock gene transcription was assessed by *in situ* hybridization, using digoxigenin-labelled *CLOCK* sense and antisense probes as previously described (Langmesser *et al.*, 2008) (see **supplementary text S1c** for details).

Conflict of Interests

The authors state no conflict of interest

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FIGURE LEGENDS

Figure 1: Circadian expression profiles of clock transcripts CLOCK, BMAL1, PER1 and clock controlled genes NR1D1, C-MYC and CDKN1A in isolated human anagen hair follicles.

Transcript levels of the above candidates was quantified using qRT-PCR in whole hair follicles synchronised with dexamethasone and sampled at time points 4, 8, 12, 16, 20 and 24 hours post-synchronisation. Data shown are the mean relative expression levels for each gene compared to housekeeping gene *PPIA* in individual male subjects (a), (b) and (c) (black dots).

Figure 2: CLOCK, BMAL1 and PER1 expression in human hair follicles (HF).

(a) CLOCK protein/mRNA expression was found in the HF by IHC and RT-PCR in the outer root sheath (ORS). **(b)** PERIOD1 (PER1) protein expression in isolated HF cryosections was found in the nucleus and cytoplasm and localising to the matrix keratinocytes (MKs) and ORS. In cultured anagen/catagen HFs PER1 fluorescence was found in the ORS and MKs of anagen HFs increasing through catagen **(c)**. **(d)** BMAL1 expression was found in anagen HFs in cell nuclei localising to the dermal papilla, connective tissue sheath, the MKs and the inner root sheath and ORS showing little differences between anagen and catagen **(e)**. (Mann-Whitney test (Holm-Bonferroni correction) *p <0.05, ***p<0.001). Male patients were used.

Figure 3: Time series expression of clock mRNA in anagen and catagen human hair follicles

(a) The average relative expression levels by qRT-PCR (from five male patients) against housekeeping gene PPIA over 24-hours showed the mean *CLOCK* expression was significantly higher in anagen than catagen (*p=0.046*). However, there was no significant difference in between anagen and catagen

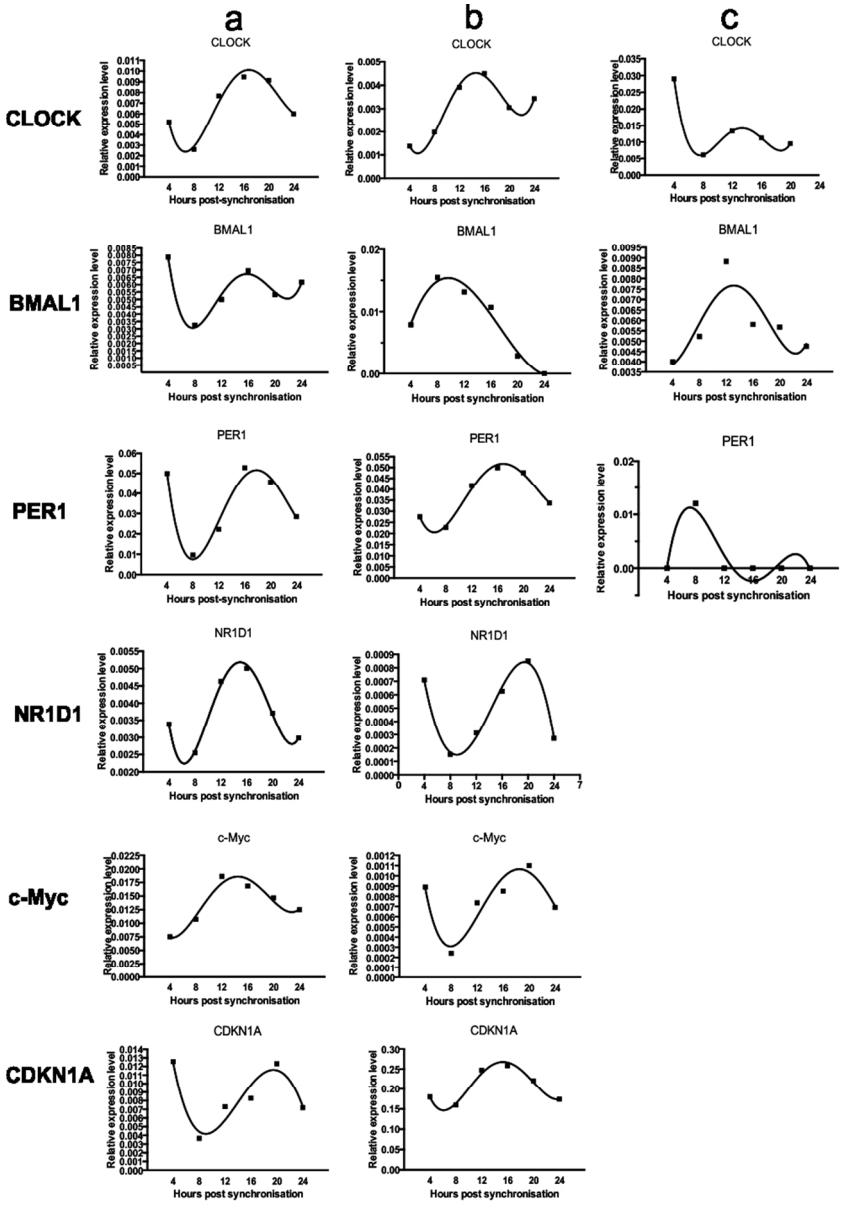
samples for *BMAL1* and *PER1.* On qualitative assessment the wave form appeared to be different between the two cycle stages in all three genes **(b).** Therefore difference in amplitudes of anagen and catagen was calculated by averaging the maxima and minima expression for each gene. Results show PER1 amplitude was significantly greater in catagen $p<0.05$. Student's t-test was used for significance, error bars are ±*SEM*.

Figure 4: Effects of PER1 knock-down in human hair follicles (HF)

(a) 96-hours post-PER1 knock-down, cycle stages were determined by morphology. A significantly higher number of HFs remained in anagen in silenced HFs ($p<0.05$, Fisher's exact test). **(b)** PER1 knock-down in HFs also increased proliferation (46.7%) 24-hours following transfection (assessed by Ki-67/TUNEL) compared to a control (36.0%). However, this was not significant (Mann-Whitney, $p=0.2$). **(c)** To confirm hair cycle stages melanin content was assessed by histomorphometry which showed a significant decrease in melanin between anagen and catagen in both the control and knock-down (Mann-Whitney, $p<0.001$). **(d)** Analysis of melanin content in only anagen HFs showed an increase in melanin of silenced HFs (Mann-Whitney, $p=0.016$). Error bars ± SEM. Results from four patients (3 male/1 female).

Figure 5: Effects of BMAL1 knock-down in human hair follicles (HF)

(a) 96-hours post-BMAL1 knock-down, cycle stages were determined by morphology. A significantly higher number of HFs remained in anagen in silenced HFs ($p=0.028$, Fisher's exact test). **(b)** BMAL1 knock-down in HFs also increased proliferation (41.6%) 24-hours following transfection (assessed by Ki-67/TUNEL) compared to a control (33.9%). However, this was not significant (Mann-Whitney, $p=0.29$). **(c)** To confirm hair cycle stages melanin content was assessed by histomorphometry which showed a significant decrease in melanin between anagen and catagen in both the control and knock-down (Mann-Whitney, $p<0.001$). **(d)** Analysis of melanin content in only anagen HFs showed an increase in melanin of silenced HFs (Mann-Whitney, $p=0.014$). Error bars ± SEM. Results from four patients (2 male/1 female).



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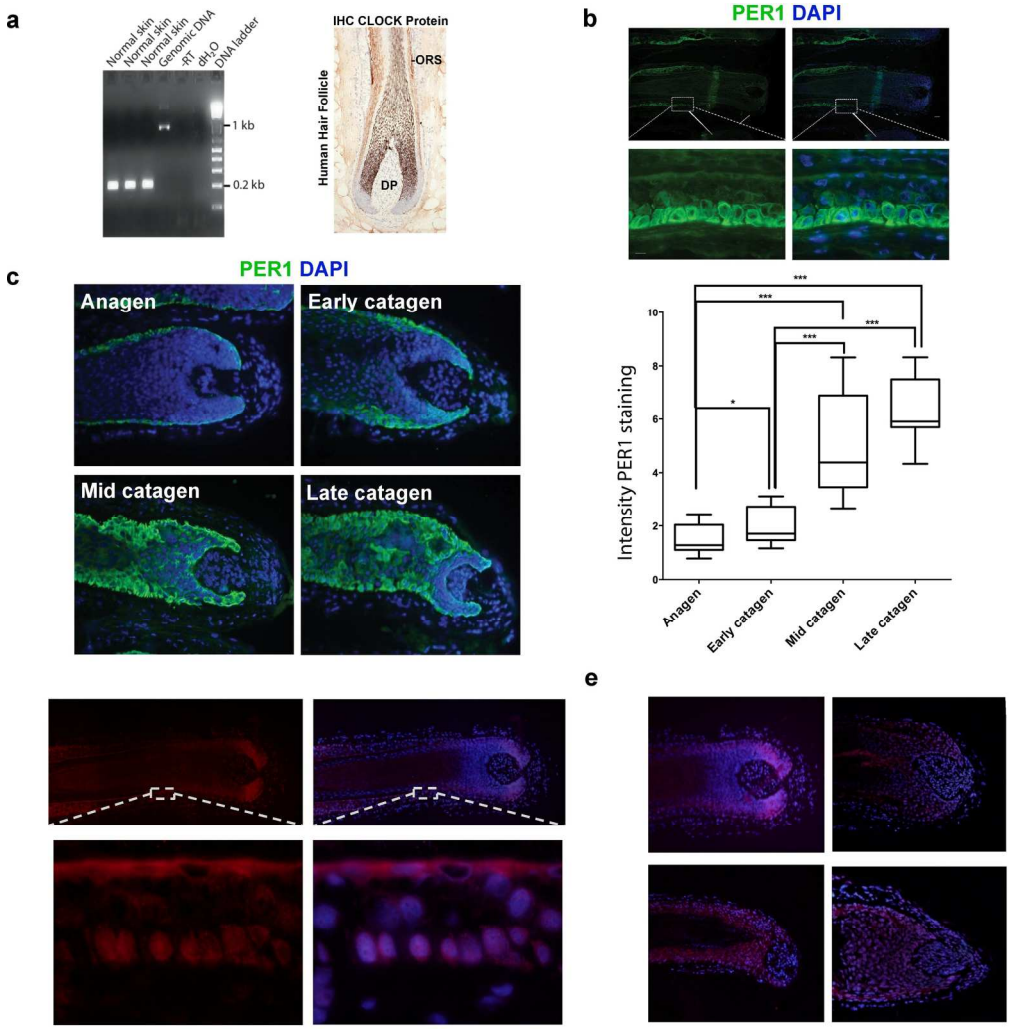

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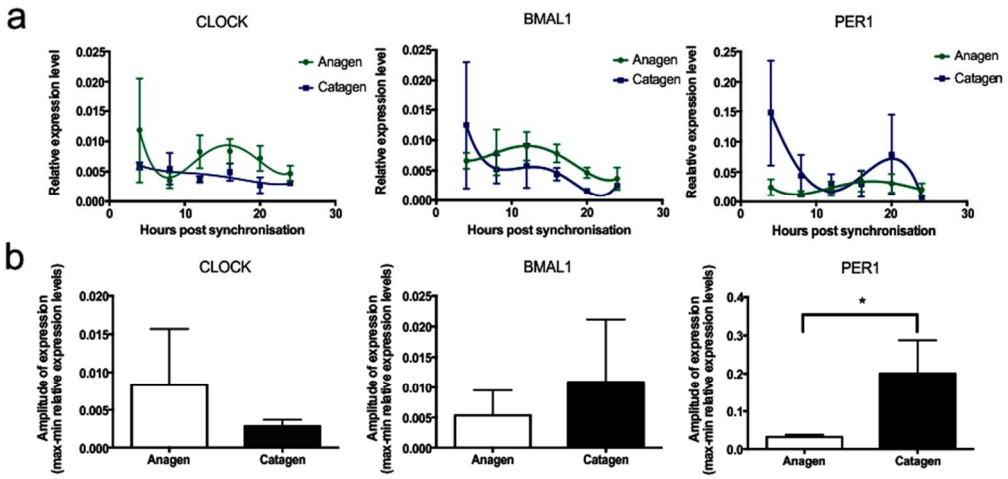

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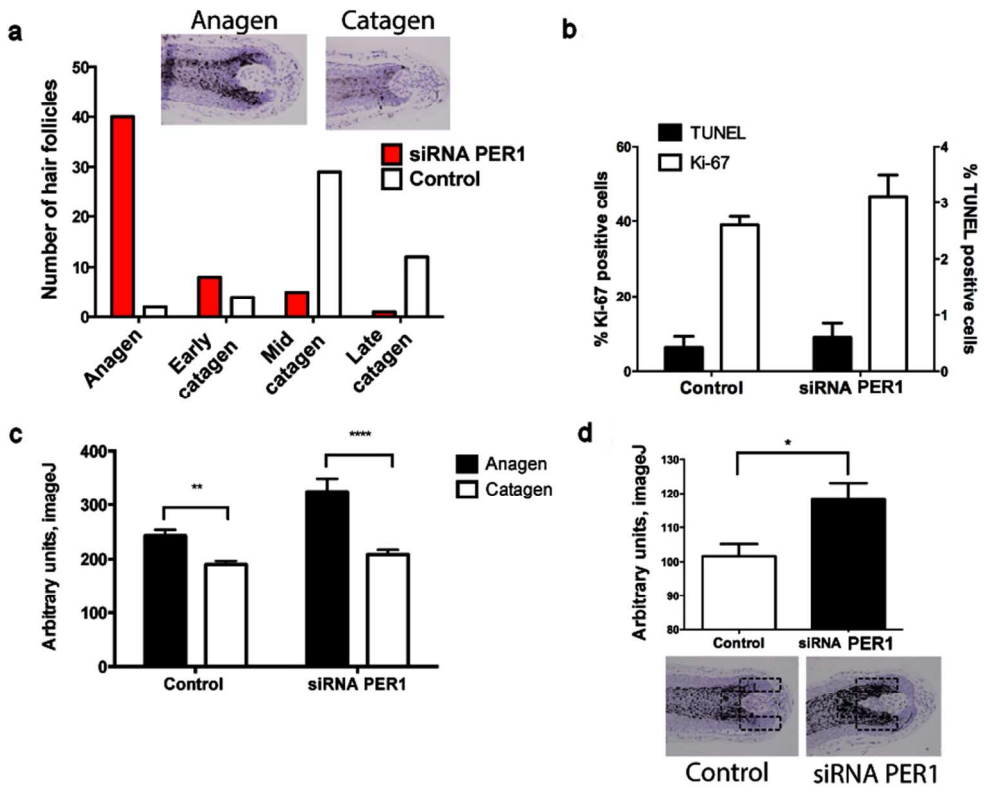

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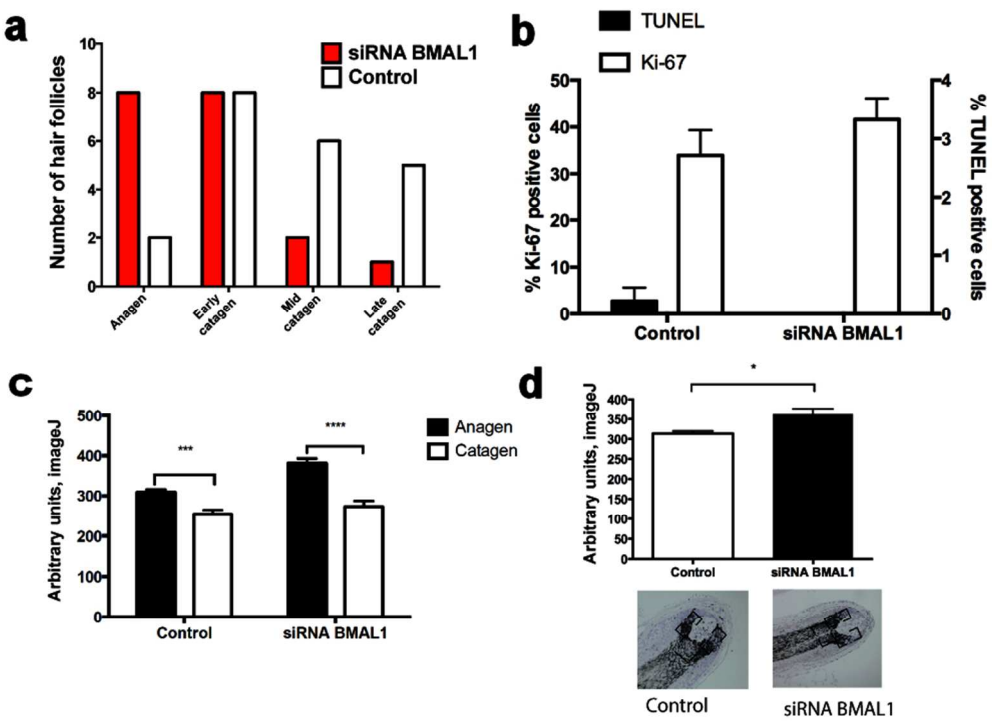

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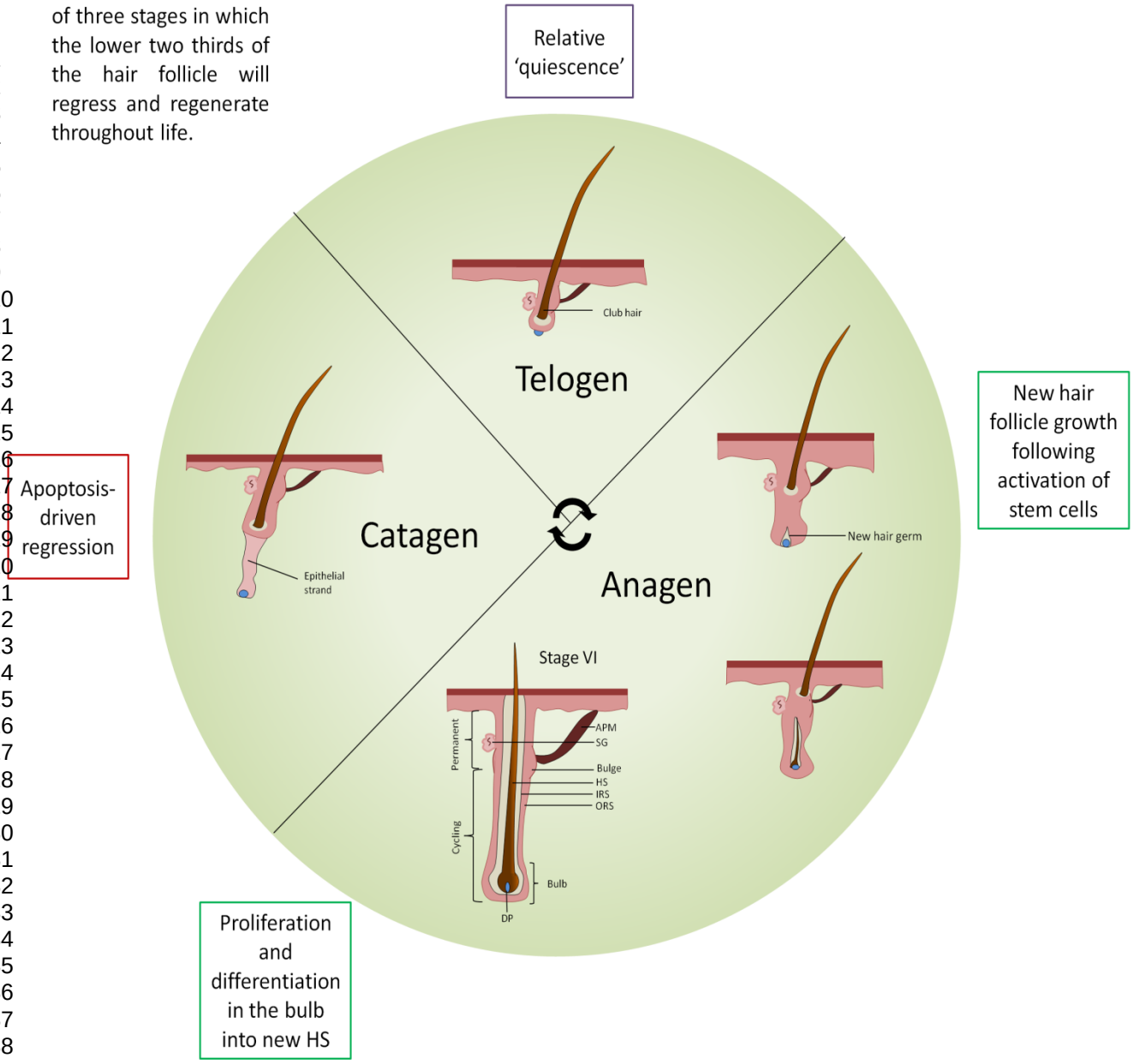
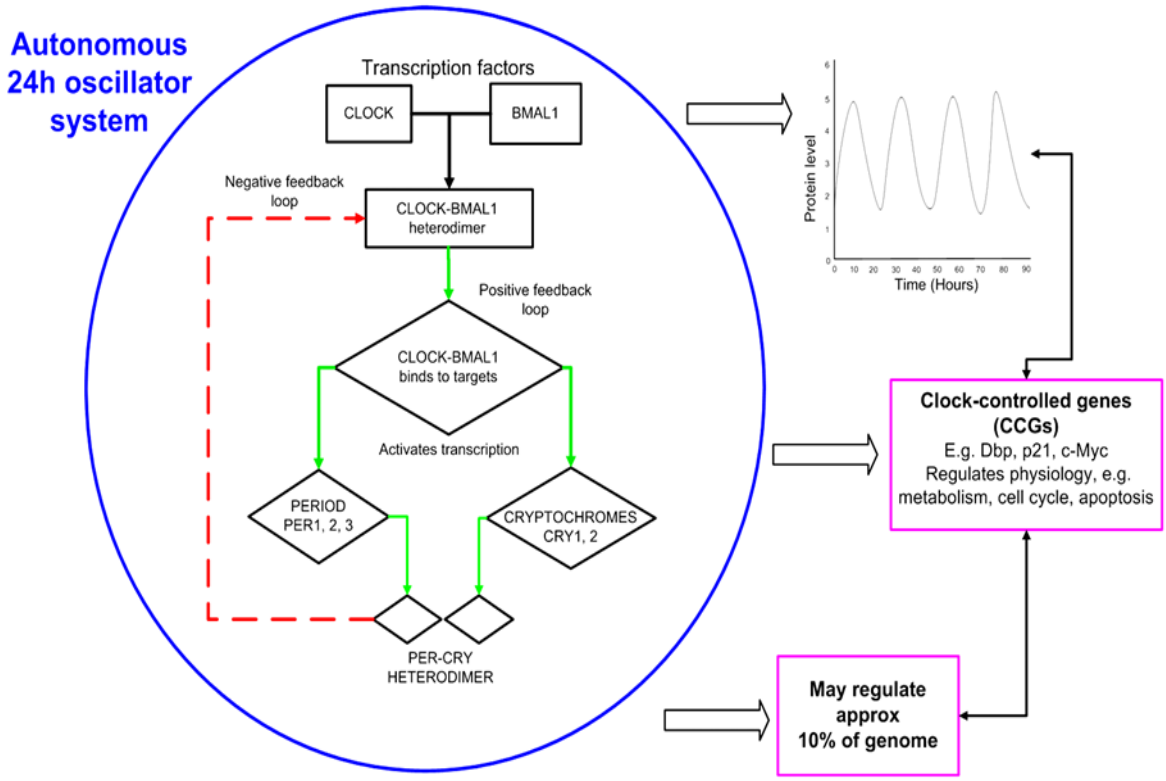
The hair cycle consists of three stages in which the lower two thirds of the hair follicle will regress and regenerate throughout life.



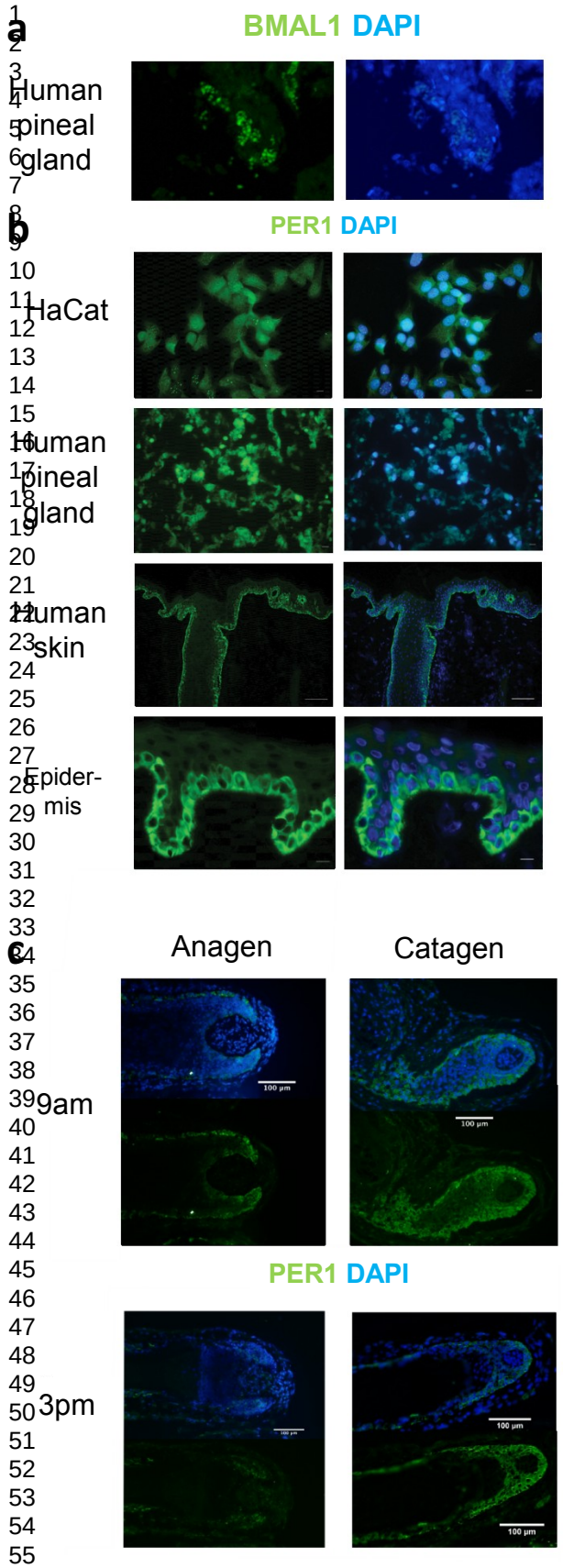
Supplementary Figure S1: The hair cycle

The hair follicle undergoes a continuous cyclical process after morphogenesis involving regression and regeneration of the lower two-thirds of its structure. This process, named the hair cycle, consists of three stages, firstly catagen is initiated (red) involving apoptosis of the epithelial compartment of the hair follicle causing the cessation of hair shaft production and leaving behind an epithelial strand. This stage lasts approximately two weeks in humans. The dermal papilla rests next to the bulge region (epithelial stem cell niche) in the telogen phase (yellow) of the hair cycle and a club hair formed. Shedding of the hair is described as exogen, but is a side path off the cycle stages. After approximately 2-4 months in humans, stem cells are activated and the anagen phase commences (green). This involves the total regeneration of the lower two-thirds of the hair follicle as rapid proliferation and differentiation occurs. Anagen involves the production of a new hair and lasts 2-7 years on the human scalp. DP = dermal (follicular) papilla, HS = hair shaft, ORS = outer root sheath, SG = sebaceous gland, APM = arrector pili muscle.



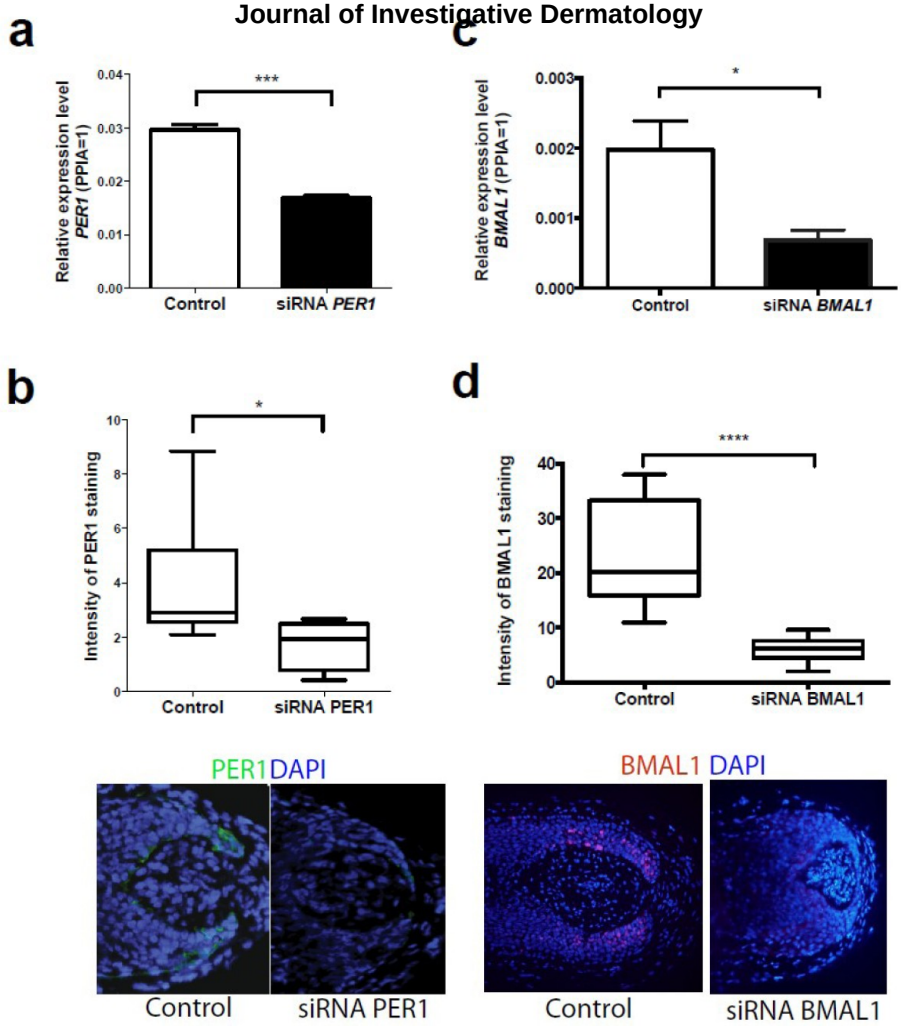
Supplementary Figure S2: Basic schema of the core clock mechanism with regulatory components

The mechanism governing the circadian system involves self-sustaining endogenous oscillators which consist of interlocking transcriptional feedback loops synchronized via a central pacemaker ("master clock") in the suprachiasmatic nucleus (SCN). The molecular components of this core clock mechanism (components within the blue circle represent this at a very simplified level) involve auto-regulated expression of a class of PAS (PER-ARNT-SIM) domain transcription factors including CLOCK, BMAL1, two Cryptochrome genes (CRY1 and CRY2) and three Period genes Period1, Period2 and Period3. CLOCK and BMAL1 proteins form a heterodimer which bind to the E-box response element for PER and CRY and induce their transcription. The translated PER and CRY proteins (negative loop, red dashed line) in turn inhibit CLOCK-BMAL1 heterodimerisation via interaction with the PAS domain of CLOCK-BMAL1. Other genes that are not involved in the clock mechanism, CCGs, such as D-box binding protein (Dbp), are regulated directly by the CLOCK-BMAL1 heterodimer. These CCGs have been found to be involved in physiological processes with 10% of genes within a tissue found to be CCGs. Importantly the expression of these genes have also shown tissue-specific expression. The resulting output of the clock mechanism is that mRNA and protein levels within this basic and extended system oscillate.



Supplementary figure S3: Immunofluorescent staining of BMAL1 and PER1 in positive control tissue and skin cryosections

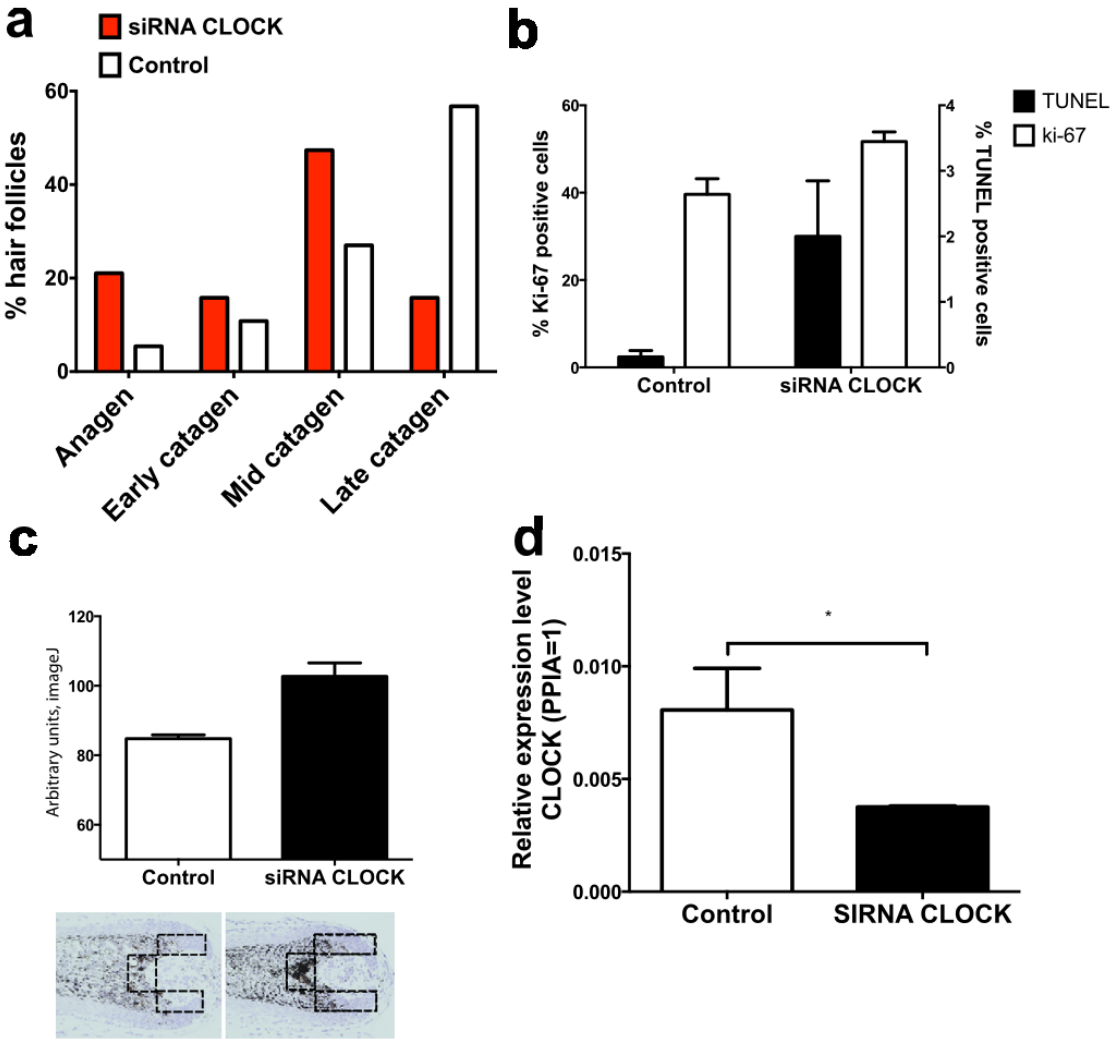
(a) BMAL1 immunofluorescent staining (green) in positive control tissue pineal gland. The right side picture shows the use of blocking peptide as a negative control. **(b) PER1 immunofluorescent staining in positive control tissues.** PER1 (lower picture set, green fluorescence) is localised to the nucleus and cytoplasm. Cell nuclei are counterstained with DAPI (blue). Basal cell expression of PER1 is found in the epidermis of whole human skin and in nuclei and cytoplasm of positive control tissues HaCaT cells and human pineal gland. **(c) PER1 expression in anagen and catagen at two time points.** To show the changes in PER1 expression levels between anagen and catagen were caused by the hair cycle stage as opposed to daily oscillations in expression anagen hair follicles were synchronised with dexamethasone. When half the follicles showed morphological criteria consistent with catagen, half of the total number of HFs were snap frozen. Six hours later the remaining HFs were snap frozen. Immunohistomorphometry shows no difference between HFs sample at 9am and 3pm.



Supplementary Figure S4:

PER1 and BMAL1 mRNA knock-down in human scalp hair follicles

(a) Relative expression of *PER1* mRNA in human hair follicles transfected with *PER1* siRNA (n=24, mean 0.018) and corresponding control (n=24, mean 0.03). Housekeeping gene *PPIA*. (b) Quantitative immunohistomorphometry of PER1 immunofluorescent staining 96 hours following PER1 knock-down compared to the control (random oligonucleotides). Hair follicles in the same hair cycle stage (mid catagen) were compared in the two groups to ensure that any differences in protein expression were not connected to the cycle stage differences in the two groups. (b) Shows a box plot of the intensity of PER1 fluorescent staining in the matrix keratinocytes (reference area below Auber's line). The hair follicles transfected with siRNA *PER1* showed a statistically significant reduction in the expression of PER1 protein. (c) Relative expression of *BMAL1* mRNA in human hair follicles transfected with *BMAL1* siRNA (n=5, mean 0.00068) and corresponding control (n=5, mean 0.0.0019). Housekeeping gene *PPIA*. (d) BMAL1 protein level knock-down confirmation. Protein level knock-down was assessed by immunohistomorphometry. BMAL1 protein levels were significantly reduced compared to control HFs (transfected with random oligonucleotides) (Mann-Whitney U-test, $p<0.0001$).



Supplementary Figure S5:

Figure 5: Hair cycle stages in CLOCK knock-down hair follicles and control.

(a) Number of hair follicles (HFs) in each hair cycle stage 96 hours post-CLOCK knock-down. Data shows combined staging results from CLOCK knock-down experiments performed for 96 hours on one patient. 21% of HFs in the CLOCK knock-down group remained in anagen compared to only 5% of HFs in the scrambled-oligo control group. **(b) CLOCK knock-down in human hair follicles also results in an increase in proliferation 24 hours post transfection**. Double immunostaining for ki-67 and TUNEL and the control and CLOCK knock-down HFs were compared. An increased percentage of ki-67 positive cells was found in the matrix keratinocytes in the CLOCK knock-down hair follicles (52%) when compared to the control group (40%). **(c) CLOCK knock-down increases melanin content in human anagen scalp hair follicles 24 hours following transfection.** Images show Masson Fontana staining on one patient. (d) CLOCK siRNA successfully reduced CLOCK transcript levels in the HF 24 hours post transfection. All average intensities were obtained over multiple cryosections and error bars are ±*SEM*.

Supplemental Table S1: Protocol summaries for clock protein immunofluorescence and immunohistochemistry experiments

Protein	Primary Antibody	Secondary antibody, detection system	Negative control (besides omission of primary antibody)	Positive control tissue	Refs
CLOCK	Rabbit anti-human Clock (Clock AB5418P, Chemicon)	Horse anti-rabbit biotinylated (Vector Laboratories). ABC detection system		HaCaT cell line	(Zanello, Jackson et al. 2000)
BMAL1	Rabbit anti-human MOP3, 1:50 (MOP31 [N1N3] Genetex)	Goat anti-rabbit fluorochrome 1:200 (Jackson Immunoresearch)		Human pineal gland	(Ackermann, Dehghani et al. 2007)
PER1	Rabbit anti-human PER1, 1:100 (PER12-A, Alpha Diagnostics)	Goat anti-rabbit fluorochrome 1:200 (Jackson Immunoresearch)	Primary antibody pre-incubated with blocking peptide (PER12-P, Alpha Diagnostics)	Human pineal gland & HaCaT cell line	(Ackermann, Dehghani et al. 2007) (Zanello, Jackson et al. 2000)

Supplementary Table S2: Subject samples for 24 hour synchronised time series experiment

Subject ID	Age	Gender	Location	Hair cycle stage when samples taken
A	35	M	Occipital	Anagen
B	28	M	Occipital	Anagen
C	40	M	Occipital	Anagen
				Catagen
D	40	M	Occipital	Catagen
E	40	M	Occipital	Catagen

Supplementary Table S3: siRNA transfection experiments

All reagents were from Santa Cruz Biotechnology, Inc. and the transfections were performed according to manufacturer's gene silencing protocol. Control experiments were performed in parallel, using the same subject HFs, with random scrambled oligos. Whole HFs were collected from the siRNA transfected and control groups for qPCR or immunohistochemical analyses. HFs were stained for either PER1 or BMAL1 respectively, Masson Fontana and double staining for Ki-67/TUNEL were performed to decipher any effect on proliferation and apoptosis, melanin content and protein level after siRNA treatment at either 24 hours or 96 hours. HFs were staged and analyzed by immunohistomorphometry. The results from all patients were combined and tested for significance using Fisher's exact test between the siRNA and control groups. Significant differences were taken as $p<0.05$.

Gene	SiRNA probes used	Subject Age	No. of HFs	Location	Duration of knock-down
Period1	Period1 siRNA (h): sc-38171 and control siRNA	69 yr, female	90	Scalp, occipital	24 hrs
		53 yr, female	70	Scalp, temporal	96 hrs
		47 yr, male	75	Scalp, occipital	96 hrs
		48 yr, male	75	Scalp	96 hrs
		45 yr, male	83	Scalp	96 hrs
BMAL1	BMAL1 siRNA (h): sc-38165 and control siRNA			Scalp, occipital	24 hrs
				Scalp, temporal	96 hrs
				Scalp, occipital	96 hrs

Supplementary Table S4: Taqman® qPCR primers

Gene	GeneBank Accession No.	TaqMan® assay ID	Full gene name
Clock	NM_004898.2	Hs00231857_m1	clock homolog (mouse)
Bmal1	NM_001178.4	Hs00154147_m1	aryl hydrocarbon receptor nuclear translocator-like
Period1	NM_002616.2	Hs00242988_m1	period homolog 1 (Drosophila)
Cdkn1a	NM_000389.4	Hs00355782_m1	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
c-Myc	NM_002467.4	Hs00905030_m1	v-myc myelocytomatosis viral oncogene homolog (avian)
PPIA	NM_021130.3	Hs99999904_m1	peptidylprolyl isomerase A

(a) Human hair follicle organ culture.

In order to investigate changes in clock genes and proteins in accordance with the hair cycle, organ cultured anagen VI human HFs were isolated and maintained in organ culture conditions as described. HFs were assessed daily using a Nikon Diaphot inverted binocular microscope to determine whether they appeared, macroscopically, to be in anagen or early, mid- and late catagen phases. Once identified to be in the correct stage (Müller-Röver, Handjiski et al. 2001; Kloepper, Sugawara et al. 2009), HFs were immediately embedded in Shandon Cryomatrix and snap frozen in liquid nitrogen and 6µm thick cryosections cut by cryostat (Model CM 3050S, Leica) and stored at -80° C until required for immunohistochemistry analyses. HF stages (anagen versus catagen) were assessed by applying carefully defined morphological and immunohistological staging criteria (Kloepper, Sugawara et al. 2009). For qPCR analyses anagen and catagen HFs were stored in RNAlater and then processed as described in the qPCR section. This method was used for collecting HFs that were either maintained in anagen or had spontaneously entered catagen stages (early, mid and late catagen) and frozen for IF PERIOD1 and BMAL1. In addition, anagen and catagen HFs were obtained from one subject to check for any changes in expression between (non-synchronised) anagen and catagen HFs.

(b) Quantitative RT-PCR

Total RNA was extracted from whole HF samples using PureLink RNA Mini Kit (Invitrogen) following the manufacturer's instructions. Samples were all treated with Purelink DNase treatment Kit (Invitrogen) as directed by the instructions. RNA purity and concentrations were established by analysing the UV absorbance using the Nanodrop ND-1000 (Fisher Scientific, Loughborough, UK). Reverse transcription was carried out using the cloned AMV First Strand cDNA Synthesis Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. The volume of RNA added to the reaction mix was adjusted for each sample in each experiment to ensure the same concentration of RNA was loaded in the reverse transcription reactions for each sample set. This was to ensure that samples whose results were going to be compared by qPCR were subjected to the same conditions and to minimise any variation being due to different RNA amounts included in the reverse transcription reaction rather than true differences. Controls were included for each sample whereby the reverse transcription reaction was carried out without any reverse transcriptase. This was to test for any genomic DNA contamination present in the RNA samples during qPCR. Real time quantitative polymerase chain reaction (qPCR) was performed using human TaqMan® gene expression assays (Applied Biosystems, Warrington, UK) (Supplementary Table 4). The TaqMan® gene expression assays are detailed in (Supplementary Table 4). The StepOne Plus™ Real-Time PCR system was used (Applied Biosystems). Real-time quantification plots were collected and stored by the StepOne software. Relative expression was determined using the delta Ct and delta delta Ct methods against the housekeeper gene PPIA. Results were plotted and analysed using Graphpad prism and EXCEL (Microsoft®). Statistical analysis of paired or unpaired Student's t-tests were performed or one way ANOVA when appropriate. Results were considered significant if $p<0.05$. The HF samples; 6 day TRH experiment, non-synchronised anagen and catagen samples, non-synchronised three time point samples and 24 hour knockdown with Period1 and clock confirmation were all performed in the Biro lab, Hungary. The following method was used: Total RNA was isolated using TRIzol (Invitrogen) and 1 µg of total RNA was reverse-transcribed into cDNA by using 15 U of AMV reverse transcriptase (Promega) and 0.025 µg/µl random primers (Promega). Q-PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems) using the 5' nuclease assay as detailed previously (Bodo, Biro et al. 2005, Tóth, Géczy et al. 2009). PCR amplification was performed by using the TaqMan® primers and probes (Supplementary Table 4) and the TaqMan® universal PCR master mix protocol (Applied Biosystems). The internal control gene used to normalise the data for all cases apart from TRH-treated HFs was *peptidylprolyl isomerase A* (*PPIA*). *β-actin* (*ACTB*) was used for the 6 day TRH treated-HFs. Normalisation was carried out based on the delta Ct method. Experiments were performed in triplicates; the average relative expression levels were calculated and plotted using EXCEL (Microsoft®) or Graphpad prism. Statistical analyses were carried out with SPSS 9.0 statistical software (SPSS Inc.) using independent sample *t*-tests and one-way ANOVA. Pair-wise comparison was performed using both Dunnett and Bonferroni tests in order to compare the means of more than 2 samples.

(c) *CLOCK in-situ* Hybridisation.

6µm tissue sections were pre-hybridized in pre-hyb-solution (4x saline sodium citrate (SSC), 1x Denhardt's, 50% formamide, 500 mg/ml tRNA and 500 mg/ml salmon testes DNA, denatured at 100 ºC for 10 min and placed on ice before adding to the mix) and incubated at 42 ºC for 3-4 hours. Hybridisation was carried out using fresh pre-hyb-solution containing 80-100ng labelled probe (denatured at 65 ºC for 5 min) at 42 ºC overnight. Sections were washed in 2x SSC for 5 min (two times) and in 2x SSC, 1x SSC, 0.5x SSC, each containing 50% formamide, at 45 ºC – 55 ºC and in 0.1x SSC 50% formamide at 50 ºC – 60 ºC for 20 min. A final wash was in 2x SSC and rinsed in DIG buffer 1 (100mM Tris-HCl, 150mM NaCl pH 7.5). Sections were blocked with 10% normal sheep serum (NSS) in DIG buffer 1 and incubated with an anti-DIG alkaline phosphatase-conjugated antibody (Roche) diluted 1:400 in 1% NSS DIG buffer 1 for 2 hours, followed by washing in DIG buffer 1 (two times) and DIG buffer 2 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 min. The hybrids were visualised by incubating the section with BCIP/NBT (Sigma-Aldrich) liquid substrate in dark at 4 ºC overnight. The colour reaction was stopped by immersing the sections in 10 mM Tris-HCl pH8 1 mM EDTA for 30 min. The developed slides were mounted and examined under a light microscope.