Chronobiology of the hair follicle:

Dissecting the role of BMAL1 and PER1 in the control of human hair growth and pigmentation

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List of Abbreviations

Abbreviation	Full name
BDNF	Brain derived neutrophic factor
BMAL1	Brain and muscle aryl hydrocarbon receptor nuclear translocator-like
BMP	Bone morphogenic protein
cAMP	Cyclic adenosine monophosphate
CCG	Clock controlled genes
CDKN1A	Cyclin-dependent kinase inhibitor
cDNA	Complimentary DNA
c-kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (SCF receptor)
CLOCK	Circadian-locomotor output cycle kaput / clock circadian regulator homolog (Murine)
c-MYC	V-myc avian myelocytomatosis viral oncogene homolog
CRY	Cryptochrome circadian clock
CTS	Connective tissue sheet
DAPI	4',6-diamidino-2-phenylindole
Dbp	D site albumin promoter binding protein
dH2O	Deionised H2O
DNA	Deoxyribonucleic acid
DP	Dermal papilla
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERK	Elk-related tyrosine kinase
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gp100	Melanocyte protein PMEL also known as premelanosome protein (PMEL) or silver locus protein homolog (SILV)
HF	Hair Follicle
HFPU	Hair follicle pigmentary unit
Hr	Hairless
HRLM	High-resolution light microscopy
HTA	Human tissue act
IFNγ	Interferon-gamma
IHC	Immunohistochemistry
IRS	Inner root sheet
Ki-67	Proliferation marker antigen ki-67
MAPK	Mitogen-activated protein kinases
MAPK	Mitogen-activated protein kinases
MITF	Microphthalmia-associated transcription factor
MK	Matrix keratinocytes
mRNA	Messenger RNA
MSX2	MSH homeobox 2
NF-κB	Nuclear factor Kappa B
NIH	National institute of health
NR1D1	nuclear receptor subfamily 1, group D, member 1 / REV-Erbα alpha
OCT	Optimal cutting temperature compound
ORS	Outer root sheet
P21	Cyclin-dependent kinase inhibitor 1

P53	Tumour protein 53
P75	Low-affinity nerve growth factor receptor
PBS	Phosphate buffered saline
PER	Period circadian protein homolog
PlGF	Placental growth factor
PPIA	Peptidylprolyl isomerase A
qRT-PCR	Quantitative real-time polymerase chain reaction
REV-ERB	Nuclear receptor subfamily 1, group D, member 2 (NR1D1)
RNA	Ribonucleic acid
RORα	RAR-related orphan receptor-alpha
ROS	Reactive oxygen species
SC	Stem cell
SCF	Stem cell factor
SCN	Suprachiasmatic nucleus
SEM	Standard error of mean
siRNA	Short interfering RNA
T3	Triiodothyronine
T4	Thyroxine
TBS	Tris buffered saline
TGF	Transforming growth factor
TSH	Thyroid-stimulating hormone
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TYR	Tyrosinase
TYRP1,2	Tyrosinase-related protein
UNG	Uracil-DNA glycosylase
UV	Ultraviolet light
VEGF	Vascular endothelial growth factor
Wnt	Wingless

Abstract

"Chronobiology of the hair follicle: Dissecting the role of clock genes BMAL1 and PER1 in the control of human hair growth and pigmentation"

A Thesis submitted for the degree of Doctor of Philosophy to the University of Manchester
By Jonathan Alan Hardman (September 2014)

The hair follicle (HF) is a human mini-organ that autonomously cycles between phases of growth (anagen), regression (catagen) and relative quiescence (telogen). Whilst many molecular controls are now appreciated to influence hair cycle, what ultimately choreographs the switch between each cycle stage is yet to be elucidated. With the increasing link between molecular clock activities in controlling local tissue physiology, we began by studying the hypothesis that the human HF has a functional molecular clock. Utilising human HF organ culture, qRT-PCR and immunofluorescence we found that the HF does indeed have oscillating clock gene expression over 24 and 48 hours *in situ*, separate from the suprachiasmatic-nucleus. Moreover, core clock proteins BMAL1 and PER1 are expressed in the human HF with PER1 increasing as HFs enter catagen. Next utilising siRNA mediated gene knock-down of either *BMAL1* or *PER1 in situ,* we were able to show that silencing either clock gene leads to anagen prolongation in cultured HFs, demonstrating that the molecular clock modulates the human hair cycle, namely the anagen-catagen switch *in situ.*

As human pigmentation is tightly coupled to the hair cycle and both human HFs and epidermal melanocytes express clock genes/proteins, this led us to investigate the hypothesis that the molecular clock modulates human pigmentation. By silencing *BMAL1* or *PER1* in HFs an increase in melanin content (Masson-Fontana) was observed in a hair-cycle independent manner. Furthermore, tyrosinase expression/activity as well as *TYRP1* and *2* expression, gp100 protein expression, melanocyte dendricity and the number of HF melanocytes were all significantly increased in *BMAL1* and/or *PER1*-silenced HFs. Mechanistically, *BMAL1* knockdown reduced *PER1* transcription, and *PER1* silencing was found to induce phosphorylation of the master regulator of melanogenesis, MITF, thus stimulating human melanogenesis and melanocyte activity. This provides the first evidence that the peripheral molecular clock influences human pigmentation.

Finally, the thyroid hormone (T4) has strong links with peripheral clock activity and has been shown to prolong anagen and increase human HF pigmentation. Moreover, T4 is a commonly prescribed treatment for thyroid disorder. As such, we investigated the hypothesis that T4 influences HF clock gene activity. It was observed that transient T4 treatment reduces the amplitude of clock gene oscillations whilst circadian rhythmicity is maintained. Conversely with longer term treatment clock gene activity was significantly increased compared to a scrambled oligo-control.

Here we have demonstrated that the human HF has peripheral molecular clock activity which influences the human hair cycle and pigmentation. Finally we were able to uncover a potential novel target, T4, whose pulsatile administration may potentially be used to treat not only hair growth and pigmentation disorders but may be able to modulate circadian activity in peripheral tissues and treat clock-related disease.

Declaration

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Chapter 1: Structure and scope of thesis

This thesis was written in the alternative format and so the chapters are presented in the format of research articles. Before each chapter there is a brief description of the work undertaken by each author, and the contribution of the PhD candidate is clearly defined. All research chapters in this thesis have either been published or are currently under consideration in a peer-reviewed journal. As there has been a strong focus on publication throughout this PhD project, the alternative format was felt to be most appropriate. Although each chapter can be understood independently with their own specific introduction, methodology and discussion, they also come together to form a coherent investigation into the role of chronobiology on hair follicle biology.

Chapter 2 provides an overview of hair follicle biology, pigmentation, endocrine interactions with the hair follicle and chronobiology. Throughout this chapter it is evident that there is a clear link between chronobiology and the hair follicle. Despite this a functional role for the circadian clock in hair follicle biology is yet to be elucidated. This chapter has been written with the intention of condensing it to form a comprehensive review article discussing current research on chronobiology in dermatology. Following this, **Chapter 3** describes all methodologies used throughout this project. Additional details on specific methodologies are presented here elaborating on the more condensed methods sections included in each research chapter.

Chapter 4 presented the first research article which uncovers that not only does the human HF have functional, oscillatory clock gene expression it is functionally relevant in modulating the human hair cycle. This conclusively demonstrates that silencing core clock genes (*BMAL1 or PER1*) leads to anagen prolongation. Throughout this thesis the clock genes/proteins BMAL1 and PER1 have been the main focus of study. Both of these are core clock genes, one from the positive loop of circadian systems (*BMAL1*) and one from the negative (*PER1*). *BMAL1* was specifically chosen as its knock-out leads complete disruption of molecular clock activity (Bunger et al. 2000).

Leading on from chapter 4, as the molecular clock was shown to be important in human hair cycle control, **Chapter 5** next asked if the molecular clock also has a functional role in modulating the (hair cycle-coupled) process of human hair pigmentation. Not only was hair pigmentation increased in clock knock-down HFs, this occurred in hair cycle- independent manner. This led us to ask; how does the molecular clock mediate human pigmentation? By studying melanocyte biology; including

melanocyte number and dendricity, and the process of melanogenesis we were able to demonstrate that the molecular clock influences hair pigmentation on multiple levels. Indeed silencing core clock genes on the one hand increases melanocyte number, dendricity melanosome number and melanosome transfer, and on the other increases the enzymatic activity of those proteins involved in melanin production. To elucidate whether this was restricted to human hair pigmentation and to uncover whether this was a melanocyte intrinsic molecular clock or an emergent property of cross-talk between melanocytes and other sub-populations in the hair follicle pigmentary unit (HFPU), the effects of clock knock-down was assessed in skin biopsies and cultured epidermal melanocytes. This demonstrated that clock knock-down induced hyperpigmentation does appear to be innate to both follicular and epidermal melanocytes. However, while an increase in melanin production attributed to an increase in enzymatic activity appears to be intrinsic to melanocytes, also occurring in cultured primary melanocytes; changes in melanocyte number and dendricity seems to be influenced by communication in the melanocytes niche i.e. the HFPU.

Following this, as both the human hair cycle and human pigmentation are modulated by clock activity, **Chapter 6** asked whether the molecular clock could be modulated *in situ*, potentially leading to novel therapeutic interventions. The thyroid hormone thyroxine (T4) was chosen as a test compound, given that it is both commonly prescribed as a therapeutic agent for thyroid dysfunction and has also been shown to influence the human hair cycle and human HF pigmentation *in situ*. In these experiments we asked whether these observations are actually caused by thyroxine mediated modulation of clock activity. Intriguingly, short term T4 treatment did reduce the amplitude of clock expression yet maintained clock rhythmicity. Conversely longer treatment with T4 increased clock activity. This lead us to postulate that perhaps diurnal T4 administration may reset clock activity and act as a novel therapy for both clock disruption-related pathologies, but also a potential treatment for hair loss and hair greying.

Finally, in **Chapter 7** the overall conclusions drawn from the experimental chapters of this thesis are summarised and potential avenues for future research are discussed.

Chapter 2: Introduction and literature review

This section has been condensed and used to form a draft review article (Appendix 4) with an aim for submission to Bio Reviews once input from the co-authors has been received.

Introduction

Chronobiology is the study of biological timing systems that control living processes including metabolism, DNA replication and hormone production (Gérard and Goldbeter 2012; Baron and Reid 2014; Dardente et al. 2014; Orozco-Solis and Sassone-Corsi 2014). The purpose of chronobiology is to dissect how environmental time cues produce daily rhythms and long term seasonal rhythms that mediate and adapt biological processes (Giebultowicz 2004). It is now understood that most cells have an intrinsic timing system that is essential for living processes (Karatsoreos et al. 2011; Engelberth et al. 2014; Orozco-Solis and Sassone-Corsi 2014). When these normal biological timing systems are altered, e.g. during nightshift work, psycho-emotional stress and diet, normal tissue functions are disrupted which can trigger or aggravating diseases, including metabolic syndrome, Alzheimer's disease, hypertension, diabetes, and cancer (Kondratov and Antoch 2007; Baron and Reid 2014; Kalsbeek et al. 2014; Mazzoccoli et al. 2014; Robinson and Reddy 2014; Sheikh-Ali and Maharaj 2014). It is clear that both a better understanding of biological time-keeping and a more easily accessible model that has intrinsic clock activity is required.

The human hair follicle (HF) is a cyclically regenerating clinically relevant mini-organ which traverses three stages of growth, apoptosis-driven regression and relative quiescence (Schneider et al. 2009). The HF is structurally, molecularly and temporally complex undergoing life-long cyclic transformations. While there are many molecular components appreciated to be involved in hair cycle control, the intrinsic timing-mechanism or "hair cycle clock" that drives this long-term oscillatory system is not understood making the HF a key target and model for chronobiology research (Paus and Foitzik 2004; Kloepper et al. 2010). That in many animals the HF will undergo seasonal changes leading to shedding or colour changes further supports this (Hazlerigg and Loudon 2008; Yoshimura 2013).

Experimentally the human HF can be cultured, during which, it will encapsulate both hair growth and the anagen-catagen stages of the HF cycle at rates close to that of *in vivo* HFs (Philpott et al. 1990). By studying the complex system that is the HF we can begin to understand how biological timing systems affect cellular and physiological processes and how different cellular populations

interact. Moreover, a better understanding of how the HF functions and how dysfunction can lead to pathologies including androgenic alopecia can be gained (Inui and Itami 2011; Higgins et al. 2013).

Literature Review

2.1 The human hair follicle

2.1.1 Hair follicle anatomy

The HF is a complex three dimensional appendage composed of multiple different cell populations with a variety of functions and can so be termed a 'mini-organ' (Knorr et al. 2009; Al-Nuaimi et al. 2010). The HF can be split into morphologically distinct sections from the proximal infundibula, which associates with the epidermis region, to the more distal bulb region (**Figure 2.1**) (Schneider et al. 2009; Schembri et al. 2013). The two most proximal sections, the infundibulum and the isthmus are part of the non-cycling portion of the HF whereas the suprabulbar and the bulb region, that undergo cyclic remodelling through the hair cycle, are considered the cycling region (Knorr et al. 2009).

The infundibulum or top most region opens to the skin surface extending down to the point of insertion of the sebaceous gland duct and acts as both a reservoir for moisture and topically applied compounds and mediates substance entry into the HF (Vogt et al. 2007; Schneider et al. 2009; Mangelsdorf et al. 2014). The infundibulum is of ectodermal origin and can itself be further sub-divided into two sections, the acroinfundibulum and the infrainfundibulum (Blume-Peytavi and Vogt 2011). The acroinfundibulum is continuous with the epidermis and is covered by the *stratum corneum*, an impermeable keratinized layer that mediates the penetration of substances into the follicle (Pinkus et al. 1981; Blume-Peytavi and Vogt 2011; Horita et al. 2014; Mangelsdorf et al. 2014). The infrainfundibulum however is more permeable being composed of trichilemmal tissue as opposed to the epidermal cells found in the acroinfundibulum (Blume-Peytavi and Vogt 2011).

Descending down the HF, the isthmus is defined as the region descending from the sebaceous gland duct to the arrector pili muscle insertion point, a smooth muscle responsible for erecting the hair shaft (Schneider et al. 2009). Within the isthmus is the stem cell-containing bulge region

(Cotsarelis et al. 1990; Purba et al. 2014). Originally the bulge region was believed to be a cluster of homogenous cells however, it has recently come to light that the bulge stem cells show a large amount of heterogeneity in activation state and cell marker expression (Janich et al. 2011; Wang et al. 2012; Purba et al. 2014). During the hair cycle the bulge stem cells undergo rounds of activation followed by extended periods of quiescence. The main two activation periods of the bulge stem cells are during the onset of anagen and mid-anagen to replenish those cells lost in earlier anagen (Greco et al. 2009; Janich et al. 2011; Plikus 2012). Also found in the bulge region are the melanocyte stem cells which reconstitute the melanocyte population as anagen resumes (Plonka et al. 2009; Nishimura 2011).

Entering the cycling portion of the HF, in the suprabulbar region, the outer root sheath (ORS) and inner root sheath (IRS) are morphologically thicker. Within this region the ORS becomes fully keratinised (Whiting 2004). Descending to the hair bulb, this region contains various different cell types including the hair matrix keratinocytes (MKs), melanocytes and the dermal papilla (DP). The bulb itself is often referred to as the 'factory' of the HF containing actively proliferating and differentiation transient amplifying cells that are derived from the bulge stem cell during anagen onset which rapidly proliferate into MKs (Al-Nuaimi 2011; Plikus 2012). As these cells exit the cell cycle they terminally differentiate into either hair shaft trichocytes or one of the cell types that constitute the IRS (**fig. 2.1**) (Schneider et al. 2009).

The HS, the IRS and the ORS are concentric ring structures made up of various epithelial cell types (Legué and Nicolas 2005). Figure 2.1 shows the various structural components that make up the complete HF. The HS itself is split into an inner medulla and an outer cortex which is surrounded by a cuticle layer totalling 50-100μm in diameter (Kreplak 2001; Schneider et al. 2009; Joshi 2011). The cuticle is roughly 5μm thick and is composed of overlapping sheet-like cells (Vogt et al. 2007). These cells can be referred to as scales and interlock with the IRS (Kreplak 2001; Schneider et al. 2009; Joshi 2011).

The IRS interlocks with and surrounds the HS and is responsible for guiding the hair as it grows (Paus and Foitzik 2004). It is composed of three distinct layers: Huxley's, Henle's and the

companion layer which are keratinised, stiffening the follicle and allowing it to successfully guide the HS to the skin surface (Alonso and Fuchs 2006; Joshi 2011). The outermost layer of the IRS, the companion layer, acts as a slippage layer allowing the hair shaft to slide past the ORS and grow upwards (Paus and Foitzik 2004; Sequeira and Nicolas 2012). Both the HS and the IRS are the last of the cell layers created from the differentiation of the MKs (Legué and Nicolas 2005; Sequeira and Nicolas 2012). Alternatively, the ORS, although from epithelial origin is created from local progenitor cells and acts as a local paracrine signal source within the HF (Yu et al. 2008).

The MKs of epithelial origin surrounds the DP. The DP is a collection of densely packed fibroblast cells which, unlike most of the HF cells, are of mesodermal origin (Plikus et al. 2008). This region is believed to have an important role in signalling throughout the hair cycle (Blanpain et al. 2004; Morgan 2014). For example, at the onset of anagen there is believed to be extensive signalling from the DP to the epithelial cells of the bulge leading to resumed growth (Blanpain et al. 2004). Interestingly there is considerable communication between the two mesenchymal components of the HF, the DP and the connective tissue sheath (CTS), and Oliver *et al.* have shown that the CTS is able to reconstruct the DP when ablated (Oliver 1966). Furthermore, the DP has been shown to be indispensable for HF cycling. Indeed recent studies have demonstrated the possibility of regenerating human HFs from isolated DP cells (Higgins et al. 2009; Inoue et al. 2009; Ohyama and Veraitch 2013). However, many of the studies have focused on mouse models and whilst it is assumed to be similar in humans, this remains unexplored.

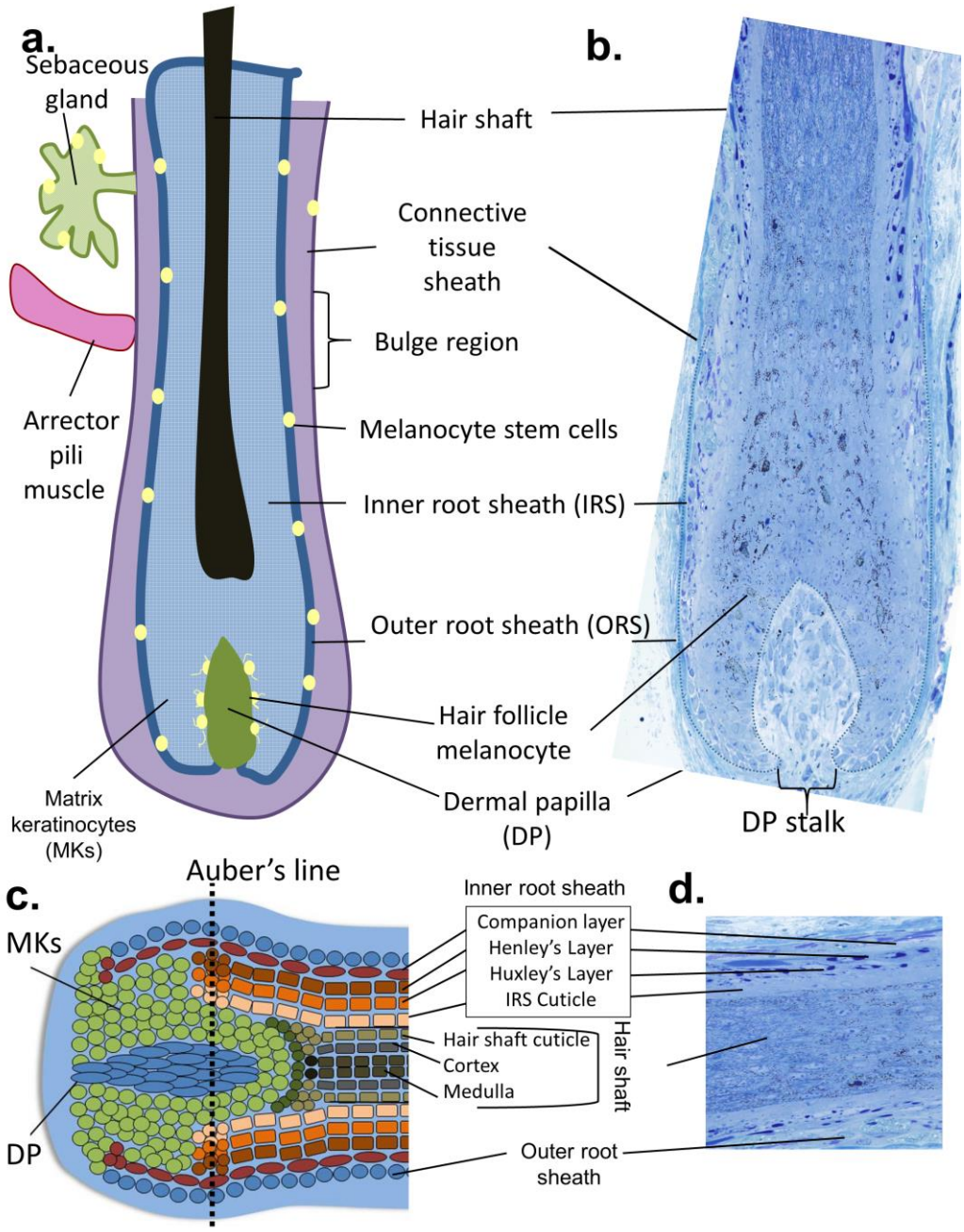

Fig. 2.1: Basic hair follicle anatomy.
(a.) A schematic of the basic structures of the hair follicle and associated structures including the arrector pili muscle and sebaceous gland. This schematic highlights the bulge region (at the site of the arrector pili muscle insertion point) containing keratin15 positive hair follicle stem cells. (b.) A 0.5µm thick high-resolution section of a hair follicle counterstained with toluidine blue showing some of the equivalent structures on a HF. The different cellular layers of the inner root sheath and outer root sheath are also highlighted. (c.) The different cell types of the HF with a HF section showing the equivalent layers on a sectioned HF (d.) [Panel c. modified after Schneider et al. 2009].

2.1.2 Hair follicle morphogenesis

Development of the HF begins *in utero* during the eighth week of gestation. HF morphogenesis begins as dermal cells cluster and initiate bi-directional communication between themselves and the undifferentiated epidermis and subsequently cause clustering and proliferation of epidermal keratinocytes (Whiting 2004; Alonso and Fuchs 2006; Rishikaysh et al. 2014). As the placode forms, cells in the immediate vicinity are prevented from forming a HF. Continued epidermal signalling encourages the dermal cells to form the DP which becomes surrounded by keratinocytes. Finally, cell differentiation occurs forming the IRS and hair shaft (Alonso and Fuchs 2006; Rishikaysh et al. 2014). During this time melanocytes migrate to the HF and hair pigmentation begins. This process occurs in a synchronized wave-like pattern travelling from the frontal to occipital scalp creating a coat of fine lanugo hairs which are shed by week 36 of gestation (Millar 2002; Whiting 2004; Schmidt-Ullrich and Paus 2005). Normal hair cycling begins in catagen after shedding a second coat of lanugo hairs around 3-4 months post-natal (Whiting 2004). Unlike morphogenesis in mice, normal human hair cycling is desynchronised (Schneider et al. 2009). Molecular controls of this process are detailed in **Figure 2.2**.

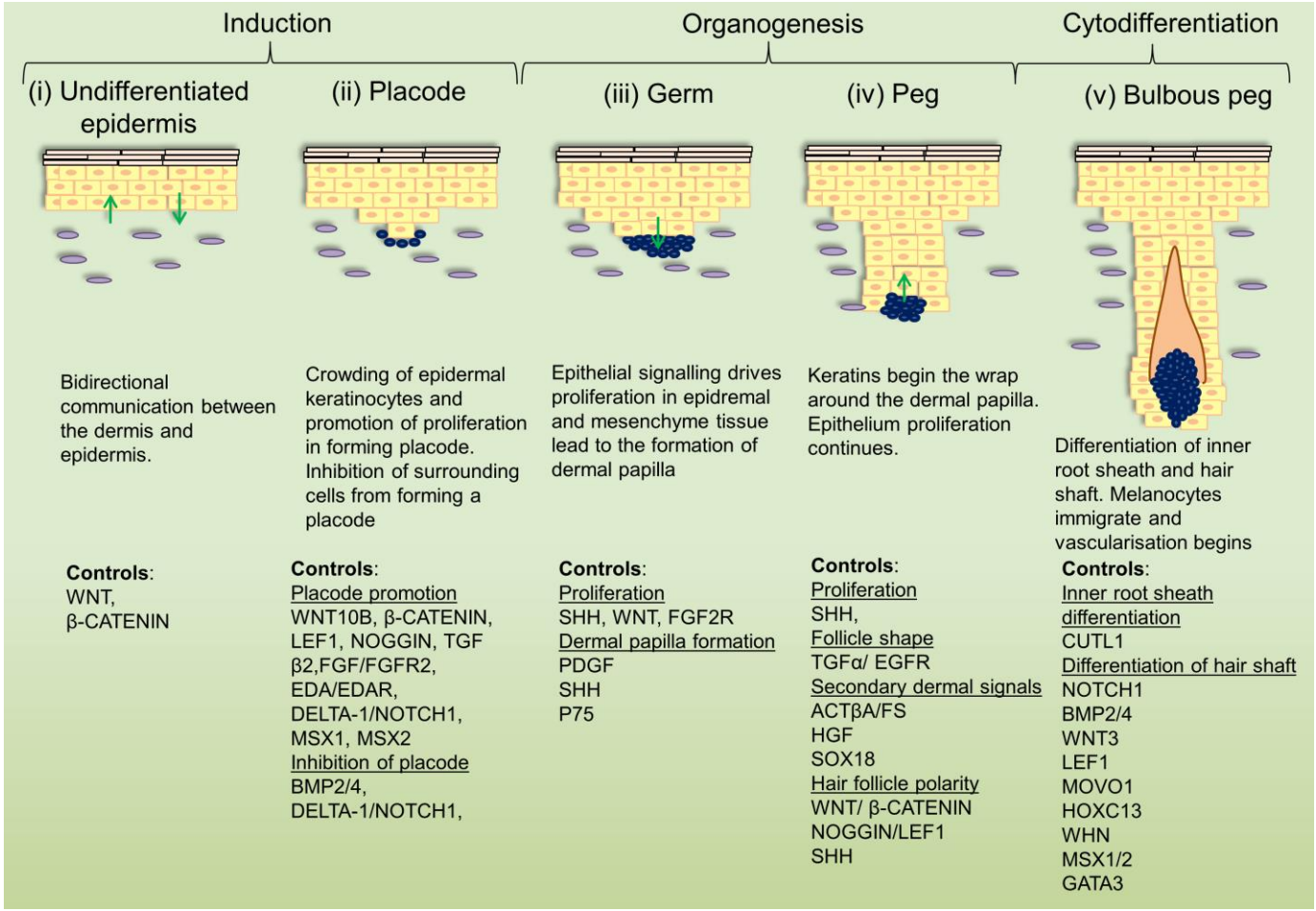

Fig. 2.2: Molecular controls of hair follicle morphogenesis.
(i) Bidirectional communication between the undifferentiated epidermis and dermis inducing the condensation of fibroblasts. (ii) The formation of this hair placode inhibits the formation of additional placodes in surrounding cells. (iii) Cross talk between the mesenchyme and the epithelial placodes initiates the formation of the dermal papilla which is surrounded by the developing HF (iv). (iv) Cell differentiation follows forming the inner root sheath and hair shaft as melanocytes migrate to the HF and the vasculature begins to develop forming a mature HF. [Modified after Millar 2002 and Schmidt-Ullrich and Paus 2005)].

2.1.3 The Hair cycle

Once formed, the HF will cyclically and continuously oscillate through three distinct stages throughout life; maintaining their cycling capacity when isolated from a neurovascular supply at rates comparable to that of *in vivo* growth (Paus et al. 1999b; Rogers and Hynd 2001). This emergent oscillatory behaviour coupled with the complex mesodermal, neuro-ectodermal interactions make the HF an ideal, clinically relevant model for many areas of biology from molecular biology, stem cell biology to systems biology and chronobiology (Al-Nuaimi et al. 2010, 2014; Purba et al. 2014)

HF cycling is a highly conserved yet the mechanisms as to why it cycles still remain a mystery (Paus et al. 1999b). Proposed theories include seasonal change, limitation of hair shaft length, cleansing the skin surface of toxins, disposing of chemicals from the body in trichocytes and to prevent the accumulation of mutations by shedding cells (Paus and Foitzik 2004; Cotsarelis 2006a). HF cycling has been extensively studied in murine models but there is minimal data available on this cyclic behaviour in the human HFs which, unlike the murine follicle, undergoes asynchronous cycling.

By gaining a better understanding this cyclic transformation process we can not only understand how to treat hair growth pathologies such as androgenic alopecia but also utilise the HF as a model system to understand how cyclic on/off switching of local tissue physiologies can be timed.

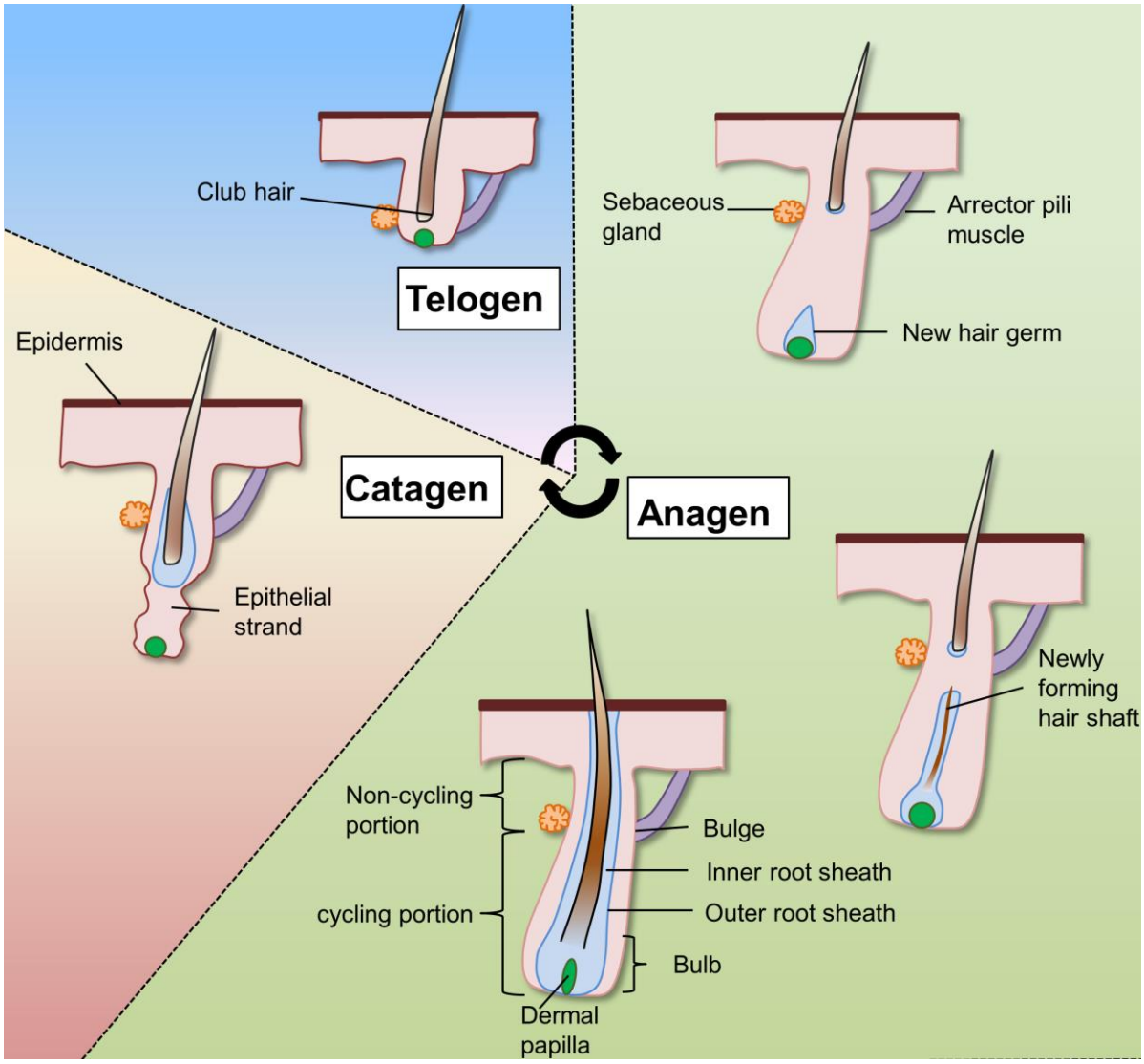

Fig. 2.3: The human hair cycle.
The hair follicle (HF) undergoes continuous cyclical transformations after morphogenesis. This process, named the hair cycle, consists of three stages, firstly catagen in which the cycling portion of the HF undergoes apoptosis driven regression. Following this the HF remains in telogen a phase of relative quiescence. Subsequently, after stem cell activation, the hair follicle enters anagen, the growth phase, which involves the production of a new hair and lasts 2-7 years on the human scalp.

Catagen

The hair cycle begins in catagen, the destructive phase, once morphogenesis is complete (Geyfman and Andersen 2010). As catagen starts, the HS and the IRS of the lower two thirds of the HF, the cycling portion, regresses leaving behind a sack-like epithelial strand from the remnants of the ORS (Paus and Foitzik 2004; Whiting 2004; Schneider et al. 2009). The previous HS becomes a club hair as those cells which have committed to differentiate complete this process (Paus and Foitzik 2004; Schneider et al. 2009). The bottom of the HS seals, becomes surrounded by a pocket of trichilemmal keratin and ascends up the HF becoming anchored in the non-cycling portion (Whiting 2004; Alonso and Fuchs 2006). As the cycling portion regresses the DP comes into contact with the epithelial stem cell region, the bulge (Schneider et al. 2009). The bulge activation theory states that it is this contact that activates the bulge stem cells causing them to proliferate and triggering the hair cycle to repeat (Sun et al. 1991). The induction of catagen is believed to be related to a substantial increase in transforming growth factor-β1 (TGF-β1) which increases at the end of anagen and is found in high levels in early catagen HFs, where it co-localises with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), a marker for cell apoptosis (Foitzik et al. 2000). Experimental deletion of tgf-β1 in mice prolongs anagen whereas administration of it induces catagen, demonstrating its essential role in inducing catagen (Foitzik et al. 2000). While the actual mechanisms that lead to catagen induction in HFs are not fully understood, factors such as epidermal growth factor (EGF), fibroblast growth factor-5 (FGF-5) and P53 are thought to be involved (Hébert et al. 1994; Hansen et al. 1997) (**Table 2.1**).

Catagen is identifiable morphologically by the loss of MKs and epithelial structures. During early catagen the diameter of MKs in the bulb region decreases and the DP becomes more rounded in shape. Coupled to this the DP stalk opens and fibroblast cells can be seen migrating, a key characteristic of this stage (Kloepper et al. 2010) (**Fig. 3.2**). To identify this stage more quantitatively, markers for proliferation (ki-67) and apoptosis (TUNEL) can be utilised. During early catagen, which can appear morphologically similar to anagen, there is an increase in TUNEL positive cells observed below Auber's line (the MKs found below the widest part of the HF) whilst, the number of DAPI stained nuclei and ki-67 positive cells will decrease (Kloepper et al. 2010).

Furthermore, the number of migrating fibroblasts in the DP stalk will also increase in early catagen (Kloepper et al. 2010).

Telogen

Following catagen is telogen, a phase of 'relative quiescence' (Paus and Foitzik 2004). Although telogen was originally thought to be a 'resting phase', it is now understood that telogen is a crucial stage in the hair cycle and is of regulatory importance (Paus and Foitzik 2004). Telogen itself can be subdivided into two stages, refractory telogen and competent telogen (Plikus et al. 2008). During refractory telogen a high level of bone morphogenic proteins (BMPs) from the DP, HF and the surrounding macroenvironment (Blanpain et al. 2004; Greco et al. 2009), coupled with high levels of Wnt antagonists from the macroenvironment (surrounding dermal tissue and adipocytes) prevent anagen initiation (Plikus 2012). Fibroblast growth factor 18 (fgf18) expressed by the bulge stem cells and the DP during this stage is also necessary for the maintenance of refractory telogen (Kimura-Ueki et al. 2012; Plikus 2012; Purba et al. 2014). Ablation of the fgf18 producing bulge stem cells leads to anagen initiation in mice, which can be rescued by fgf18 administration demonstrating the important role of fgf18 in telogen maintenance (Kimura-Ueki et al. 2012). After approximately one month, there is a drop in Bmp ligands, namely Bmp2 & 4 and a reduction in Wnt antagonists and fgf18 (Plikus et al. 2008). With a reduction in Wnt antagonist, the HF becomes primed for anagen initiation and enters competent telogen (Plikus 2012).

It is interesting to note that whilst telogen was originally thought to be a resting phase, mRNA profiling has identified genes involved with proteolysis, rhythmic processing and cholesterol metabolism increase 1.5-fold during this stage (Geyfman et al. 2011). Moreover, microarray analysis has shown 425 genes that show differential expressions levels in telogen when compared to other phases of the hair cycle, which highly supports the dynamic role of telogen in the hair cycle (Geyfman et al. 2011). By the end of telogen, the HF-stem cells provide the necessary cells for the next round of anagen (Geyfman and Andersen 2010). Interestingly circadian genes, which have a potential role in the hair cycle (Lin et al. 2009), primarily act at the telogen-anagen switch (Tiede et al. 2007). Other important processes also take place during telogen including shedding of the club

hair. Arguably hair shedding can be described as a separate hair cycle stage termed exogen (Stenn et al. 1998). Although originally thought to be a passive phase it is now believed that exogen is an active process initiated by specific signals that may act by decreasing the cellular adhesions between the club fibre and the HF (Stenn 2005; Higgins et al. 2009). As telogen progresses and enters competent telogen the bulge stem cells are temporarily activated Wnt/β-catenin signalling giving rise the transient amplifying cells of the bulb (Lo Celso et al. 2004). There are many processes that occur during telogen supporting its role as more than just a resting phase in the hair cycle.

Morphologically telogen is identifiable in murine HFs by the significant reduction in size and localization of the entire follicle in the dermis (Müller-Röver et al. 2001). Other notable characteristics include the lack of an IRS and the DP being circular in shape. In human HF culture however, the telogen hair cycle stage cannot be captured.

Anagen

Once Bmps and noggin, which usually maintain the bulge SCs in their quiescent state by the antagonising tgf β2 produced by the DP (Yu et al. 2008; Fuchs 2009; Schneider et al. 2009), are reduced, the HF is responsive to activation signals for anagen initiation. The transcription factor Tmeff which transcribes tgfβ2 is essential for this step, with its deletion delaying anagen onset (Plikus 2012) . Anagen initiation begins with a transient increase in Wnt from the DP followed by expression of Wnt and an increase in phospho-smad 2/3 in the epithelial progenitor cells of the newly formed hair germ (Plikus 2012). This coincides with an activation in the MAPK pathway leading β-catenin stabilisation (Greco et al. 2009). Fgf7 is another key transcription factor for anagen initiation. Once produced by the DP, Ffg7 induces hair germ proliferation (Greco et al. 2009). Interestingly, whilst the bulge stem cells show moderate levels of activation by fgf7, it is the hair germ that responds first (Greco et al. 2009). In response proliferation and differentiation of the transient amplifying cells to the MKs occurs (Alonso and Fuchs 2006). These MKs will subsequently differentiate into the trichocyte cells that compose the hair shaft and the cell types of the IRS (figure 2.1) (Stenn and Paus 2001; Legué and Nicolas 2005; Schneider et al. 2009). This differentiation is

thought to be reliant on BMP signalling from surrounding tissues of the HF including the ORS (Kulessa et al. 2000).

The anagen stage of the HF is identifiable by the high levels of proliferating cells (Ki-67 positive) found below Auber's line (cells below the widest part of the HF). Morphologically the diameter of MKs in the hair bulb is significantly higher than those observed in any other stage. Coupled to this the dermal papilla is 'onion' shaped and relatively enclosed by surrounding MKs (Müller-Röver et al. 2001; Kloepper et al. 2010). During anagen HF pigmentation (melanogenesis) becomes active and a noticeable increase in pigment/melanin content of HFs can be observed (Slominski et al. 1991, 2004).

Controls of the hair cycle

While there has been substantial work in dissecting the molecular controls of HF cycling, much of this has tended to focus on the murine hair cycle. Whilst mice provide an accessible model in which all cycle stages can be observed there are key differences between this and the human system. One key difference being that HF cycling in synchronised in mice. Table 2.1 lists some of the know controllers of the murine hair cycle and highlights those that have also been demonstrated in the human system.

Modulator	Role in the hair cycle	Reference
BDNF *(Brain-derived neutrophic factor)*	Induces catagen.	(Botchkarev et al. 1999; Peters et al. 2005)
BMP2/4 *(Bone morphogenic protein)*	Induce catagen. Maintain stem cells in quiescent state. Reduction of BMP2/4 levels allows the HF to pass from refractory to competent telogen.	(Kulessa et al. 2000; Kobielak et al. 2003; Guha et al. 2004; Plikus et al. 2008)
CGRP *(calcitonin gene-related peptide)*	CGRP has been shown to be a strong inducer of catagen in culture human HFs and also inhibits MK proliferation.	(Samuelov et al. 2012a)
Decorin	Decorin is a member of the proteoglycan family and has been is expressed in the epithelial cells of the HF and in the DP. The levels of decorin were high in anagen decreasing in catagen and telogen. Furthermore, administration of decorin can prolong anagen.	(Inui and Itami 2014)
Dopamine	Dopamine can act as a catagen promoter and inhibitor of MK proliferation in cultured human HFs. Furthermore dopamine receptor transcripts are present in the human HF.	(Langan et al. 2013)
EGF *(Epidermal growth factor)*	EGF promotes the anagen to catagen transition.	(Alonso and Fuchs 2006)
Endocannabinoids	Endocannabinoids inhibit hair shaft elongation, MK proliferation and induces catagen.	(Telek et al. 2007)
Estradiol	Oestrogen arrests HFs in telogen and induces catagen.	(Conrad et al. 2005; Ohnemus et al. 2005, 2006; Hu et al. 2012)
FGF-18	Inhibits growth of HF keratinocytes with its loss promoting anagen. Furthermore FGF-18 is expressed in human HF cells such as dermal papilla cells.	(Kawano et al. 2005; Subramanya et al. 2010; Plikus 2012; Leishman et al. 2013)
FGF-5 *(Fibroblast-growth Factor)*	FGF-5 is expressed in the upper ORS where it induces catagen by binding to FGF-R1 in the DP. Overexpression of FGF5 induces catagen in human HFs.	(Suzuki et al. 1998; Schneider et al. 2009; Higgins et al. 2014)
Follistatin/ Activin	Mice with either follistatin or activin deletion demonstrate a retardation in HF morphogenesis that can be rescued by the addition of follistatin or activin respectively.	(Nakamura et al. 2003)
Galanin	Galanin immunoreactivity, mRNA and its receptors (GalR2, 3) were detected in human HFs. Furthermore, culturing with galanin decreases hair shaft elongation and anagen duration.	(Holub et al. 2012)
HGF *(Hepatocyte growth factor)*	HGF is important for anagen maintenance. It is involved in communication with the dermal papilla and can promote hair shaft growth.	(Lindner et al. 2000; Paus and Foitzik 2004; Kloepper et al. 2010)
Hr *(Hairless)*	Loss leads to premature catagen entry and rapid HF degeneration.	(Panteleyev et al. 1999, 2000; Krause and Foitzik

		2006)
IFN-γ *(Interferon gamma)*	Induces catagen, primarily via up-regulating TGF-β. It is expressed in high levels during catagen.	(Ito et al. 2005; Fessing et al. 2010)
IGF-1 *(Insulin-like growth factor)*	Maintains Anagen and can promote hair growth.	(Rudman et al. 1997; Paus and Foitzik 2004; Schneider et al. 2009)
MSX-2 *(MSH homeobox 2)*	MSX-2 promotes anagen. MSX-2 deficient mice have a short anagen phase coupled with prolonged catagen and telogen. Furthermore these mice have increased hair-loss and the hair shaft structure is abnormal.	(Ma 2003; Krause and Foitzik 2006)
NF-κB *(Nuclear factor kappa-light-chain-enhancer of activated B)*	Is required for anagen maintenance. Inhibiting NF-κB *in situ* promotes catagen in human HFs.	(Kloepper et al. 2014)
NGF *(Nerve growth factor)*	NGF is significantly up-regulated at the beginning of anagen where it induces MK proliferation. NGF levels drop as HFs enter catagen.	(Paus et al. 1994; Peters et al. 2006)
Noggin	Noggin is an antagonist to BMP. As its levels rise, it reduces BMP levels allowing stem cell differentiation. Furthermore, noggin is up-regulated during anagen.	(Kobielak et al. 2003; Schneider et al. 2009; Al-Nuaimi et al. 2010)
NT3 /4 *(Neutrophin)*	Levels of NT3 & 4 increase towards the end of anagen where they promote catagen and inhibit hair shaft elongation. NT3&4 are significantly up-regulated in catagen	(Botchkarev et al. 1998, 1999; Alonso and Fuchs 2006)
p75NTR *(Nerve growth factor receptor)*	Induces catagen.	(Botchkarev et al. 2000; Krause and Foitzik 2006)
P-cadherin	Silencing of P-Cadherin inhibits hair growth, induced catagen and inhibits MK proliferation. P-cadherin acts via Wnt signalling and suppresses the catagen inducing effect of TGFβ2	(Samuelov et al. 2012b)
PlGF *(Placental growth factor)*	Placental growth factor s expressed in the DP, MKs and ORS. Organ culture with PlGF increases hair shaft elongation and promotes anagen.	(Yoon et al. 2014)
Shh *(Sonic hedgehog)*	Shh is up-regulated in early anagen. Shh up-regulation allows bulge stem cells to become active. Up-regulation in anagen may be due to inhibition of BMP4 (its antagonist).	(St-Jacques et al. 1998; Rogers and Hynd 2001; Botchkarev and Paus 2003; Lin et al. 2009)
T3 and T4 *(triiodothyronine / Thyroxine)*	Culturing HFs with T4 promotes MK proliferation and co-culture with T3 and T4 inhibits apoptosis. T4 can furthermore prolong anagen by down-regulation of TGF-β2. Both T3 and T4 can also stimulate hair follicle pigmentation.	(van Beek et al. 2008)
TGF-β *(Transforming*	TGF-β is up-regulated at the end of anagen where it inhibits growth of keratinocytes and induces	(Mori et al. 1996; Foitzik et al. 2005; Lin et al. 2009; Gáspár et al. 2010; Plikus

growth factor β	catagen.	2012)
TNF-α *(Tumour necrosis factor)*	Induces catagen. Polymorphisms in the TNF-α promoter leading to its overproduction may induce alopecia areata in humans.	(Philpott et al. 1996; Schneider et al. 2009; Mauricio et al. 2012)
TRH *(Thyrotropin-releasing hormone)*	The human HF expresses both TRH and TRH-receptors. Culturing HFs with TRH promotes HF elongation, anagen prolongation and hair pigmentation. The anagen prolongation effects act via antagonizing the catagen induction effect of TGF-β2. Furthermore, TRH can induce MK proliferation and inhibit apoptosis by inducing P53 activation. Furthermore TRH has been shown to promote intrafollicular mitochondrial energy metabolism.	(Gáspár et al. 2010, 2011; Vidali et al. 2014)
VEGF *(Vascular endothelial growth factor)*	Prolongs anagen.	(Kozlowska et al. 1998; Lachgar et al. 1998; Yano et al. 2001)
Wnt/ β-Catenin *(wingless)*	Wnt/ β-Catenin signalling can induce stem cell differentiation. They are expressed in late telogen to anagen and are necessary for anagen. Loss of Wnt from these cells leads to a reduction in SC proliferation.	(Kulessa et al. 2000; Myung et al. 2012; Fang et al. 2014; Kandyba and Kobielak 2014)

Table 2.1: Molecular controls of the hair cycle (examples).

Table 2.1 shows a list of some of the molecular controls of the murine HF that affect HF cycling. Those controls highlighted in blue have been documented in the human HF or in both human and mouse HF cycling.

2.2 Chronobiology

Although there are many appreciated controls involved in modulating the rhythmic process that is the human hair cycle (**Table 2.1**), the timing mechanism controlling the switch between each cycle stage is yet to be fully described (Paus et al. 1999b). Chronobiology, the study of biological timing systems is becoming increasingly appreciated in tissue physiology (Giebultowicz 2004). With the molecular clock now established as a novel candidate in controlling many biological processes including metabolism, the cell-cycle, the immune system and the murine hair cycle amongst other things (Lin et al. 2009; Bellet and Sassone-Corsi 2010; Bailey et al. 2014; Ben-Shlomo 2014; Kalsbeek et al. 2014), it may be that this oscillatory molecular system is one of the main regulators of the elusive 'hair cycle clock'.

Chronobiology has long been associated with the study of daily rhythms and the impact on physiology and health when normal rhythms are perturbed. Key examples of this are jetlag and night-shift work which are frequently associated with an increasing prevalence of diseases including obesity, diabetes and cancer (Davis and Mirick 2006; Buxton et al. 2012; Kalsbeek et al. 2014). Since the discovery of the genes and proteins that produce the biological clock, the 'molecular biological clock', this system is now understood to be intrinsic to all cells (Partch et al. 2014). Furthermore, the molecular clock is now appreciated to be integral in choreographing complex processes involved in tissue maintenance including balancing reactive oxygen species (Lai et al. 2012).

This section will begin by outlining how clock disruption can promote the onset of diseases. Following this, the molecular clock and how it is synchronised will be described. Finally the section will end by discussing current literature linking the hair follicle and chronobiology, highlighting major open questions specifically in the human HF.

2.2.1 Chronobiology in health and disease

It is increasingly appreciated that disruption to the molecular clock and loss of synchronisation from external cues can lead to tissue malfunction and clinical pathologies. Such pathologies include hypertension, with *bmal1* knock-out mice having low blood pressure. It is currently thought that the molecular clock balances the level of vascular reactive oxygen species (ROS) via nicotinamide adenine dinucleotide phosphate-oxidases (NADPH-oxidases) such as Nox1,2,4,5 leading to vascular tissue maintenance including vasodilation and vasoconstriction and endothelial remodelling (Kunieda et al. 2008; Takac et al. 2012). Further examples were demonstrated in clock knock-out mice which show an increased number of age-related co-morbidities such as ROS accumulation, decreased life span and loss of bone mass (Kondratov et al. 2006; Geyfman and Andersen 2010; Kondratova and Kondratov 2012).

In humans, a similar increase in the prevalence of hypertension has been observed in epidemiological studies of night-shift workers (Ohlander et al. 2014). Other pathologies include metabolic disease, which is also more prevalent in shift workers (Sheikh-Ali and Maharaj 2014), and an increased prevalence of age-related pathologies. Furthermore, there is a growing link between Alzheimer's disease and clock dysfunction and patients with this disease often show decreased amplitude of clock gene expression. Potentially this may lead to increased amyloid-beta deposit in the brain, exacerbating the condition (Bedrosian and Nelson 2012; Buratti et al. 2014). Moreover, there is a strong link between hypothalamic-pituitary-adrenal axis (HPA) induced stress and circadian dysregulation (Nicolaides et al. 2014).

With an increasing understanding of how clock, specifically the molecular clock, dysfunction can exacerbate diseases (Karatsoreos 2014) and by gaining a better understanding of how the molecular clock controls normal tissue functions may help to uncover novel therapeutic targets in future. Indeed more recently chronobiology based therapies have been successful in treating illness. Studies having shown that administering circadian-based chemotherapy to cancer sufferers reduced clinical fatigue, prevented a loss in body weight and showed other general improvements when compared to patients with fixed-time therapy (Innominato et al. 2014; Ortiz-Tudela et al. 2014).

Moreover, prenatal babies treated in incubators with a light/ dark cycle showed a significant gain in body mass compared to those in traditional incubators (Vásquez-Ruiz et al. 2014).

It is clear external influences are important for healthy tissue function. However, many of these studies are association based and focus on the role of external cues such as light and dark and sleep/wake cycles and therefore fail to take into account the molecular clock which produces these daily rhythms in peripheral tissues (Stokkan et al. 2001; Roelfsema and Pijl 2012; Frank and Cantera 2014; Vásquez-Ruiz et al. 2014).

2.2.2 The molecular clock

While the term chronobiology refers to biological timing systems, it often refers specifically to the molecular clock, which modulates local tissue physiology. It is this molecular system that may be the elusive candidate that choreographs rhythmic processes in peripheral tissues i.e. the human hair cycle. It is now appreciated that most if not all cell types have a molecular clock, with organs including the liver, heart and kidney having peripheral molecular clock activity that with oscillate irrespective of the central pacemaker and external cues (**Fig. 2.4**) (Schmutz et al. 2011; Bass 2012; Sato et al. 2013).

The molecular clock is a series of molecular feedback loops that are intrinsically involved in controlling the cell cycle, with all check point proteins being under circadian control (**Fig. 2.6**). Moreover the circadian system temporally segregates DNA replication, occurring during the night to reduce potential damage from UV, whilst metabolic genes are up-regulated during light hours. Furthermore, that apoptotic genes are also under clock control (Gaddameedhi et al. 2012), including p53 (Hamada et al. 2014), further suggests the potential role for the molecular clock in mediating the switch between the HF growth stage anagen, marked by high-levels of proliferation and the apoptotic driven catagen.

The core molecular clock is a series of cellular feedback loops that are synchronised by the master clock, the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN receives external cues or zeitgebers such as light and dark cycles and synchronises the circadian activity of the molecular clock found in peripheral tissues (**Fig. 2.4**) (Amir and Robinson 2006; Schroeder and Colwell 2013; Robinson and Reddy 2014).

The core molecular clock begins when CLOCK and BMAL1 proteins come together, forming a heterodimer complex (Kondratov et al. 2003). This heterodimer translocates to the cell nucleus where it binds to those genes with an EBOX motif in their promoter region (Verastegui et al. 2000; Yoo et al. 2005). Genes with such a motif are collectively termed clock controlled genes (CCGs) and it's thought that up to 10% of the genome may be controlled in this manner (Panda et al. 2002). The binding of the CLOCK:BMAL1 heterodimer leads to the transcription of CCGs (Gekakis 1998; Bunger et al. 2000; Robinson and Reddy 2014). Two examples of CCGs are the period family (PER1,2 3) and the cryptochrome family (CRY1,2) (Darlington 1998; Sangoram et al. 1998; Robinson and Reddy 2014). As PER and CRY accumulate they form a second heterodimer which inhibits the formation of the initial CLOCK:BMAL1 heterodimer thereby inhibiting their own transcription. As these proteins degrade the CLOCK:BMAL1 heterodimer can reform, restarting the cycle (Kondratov et al. 2003; Robinson and Reddy 2014) (**Figure 2.4**). This process occurs over approximately 24 hours producing a circadian expression pattern of genes/proteins. Additional CCGs are the retinoic acid receptor related orphan receptors RORα and REV-ERBα/β, which form accessory loops to the 'core' clock. These additional loops help maintain the 24 hour rhythmicity along with input from the SCN.

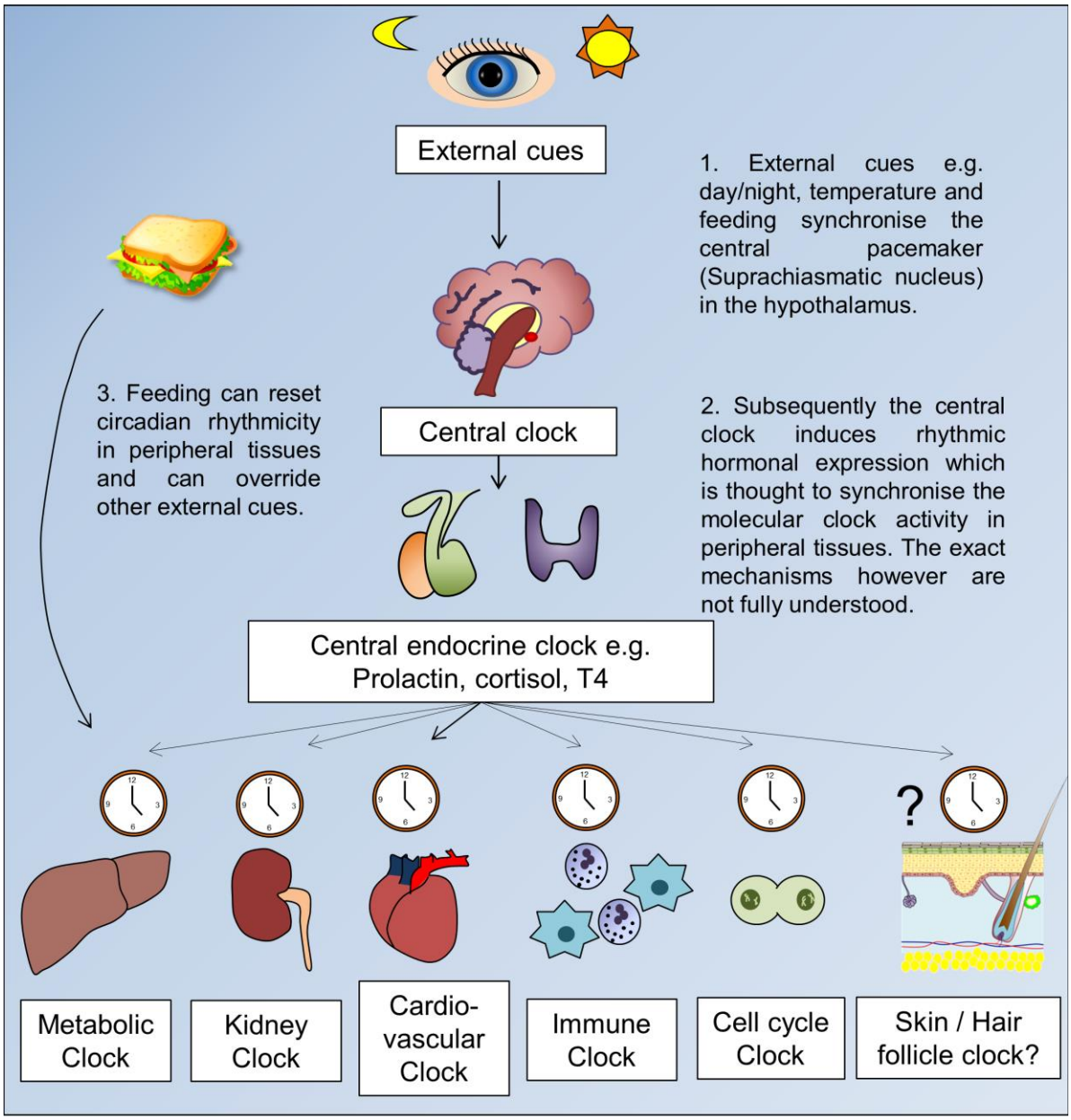

Fig 2.4: Organisation of the central and peripheral clocks.
(1.) The external cues such as the light and dark cycle are detected by the eye and conveyed to the suprachiasmatic nucleus (SCN) in the hypothalamus. (2.) The hypothalamus induces rhythmic expression of systemic hormones, which subsequently synchronise the autonomous clock activity in peripheral tissues. (3.) Feeding is also one key external cue that can synchronise peripheral clock activity.

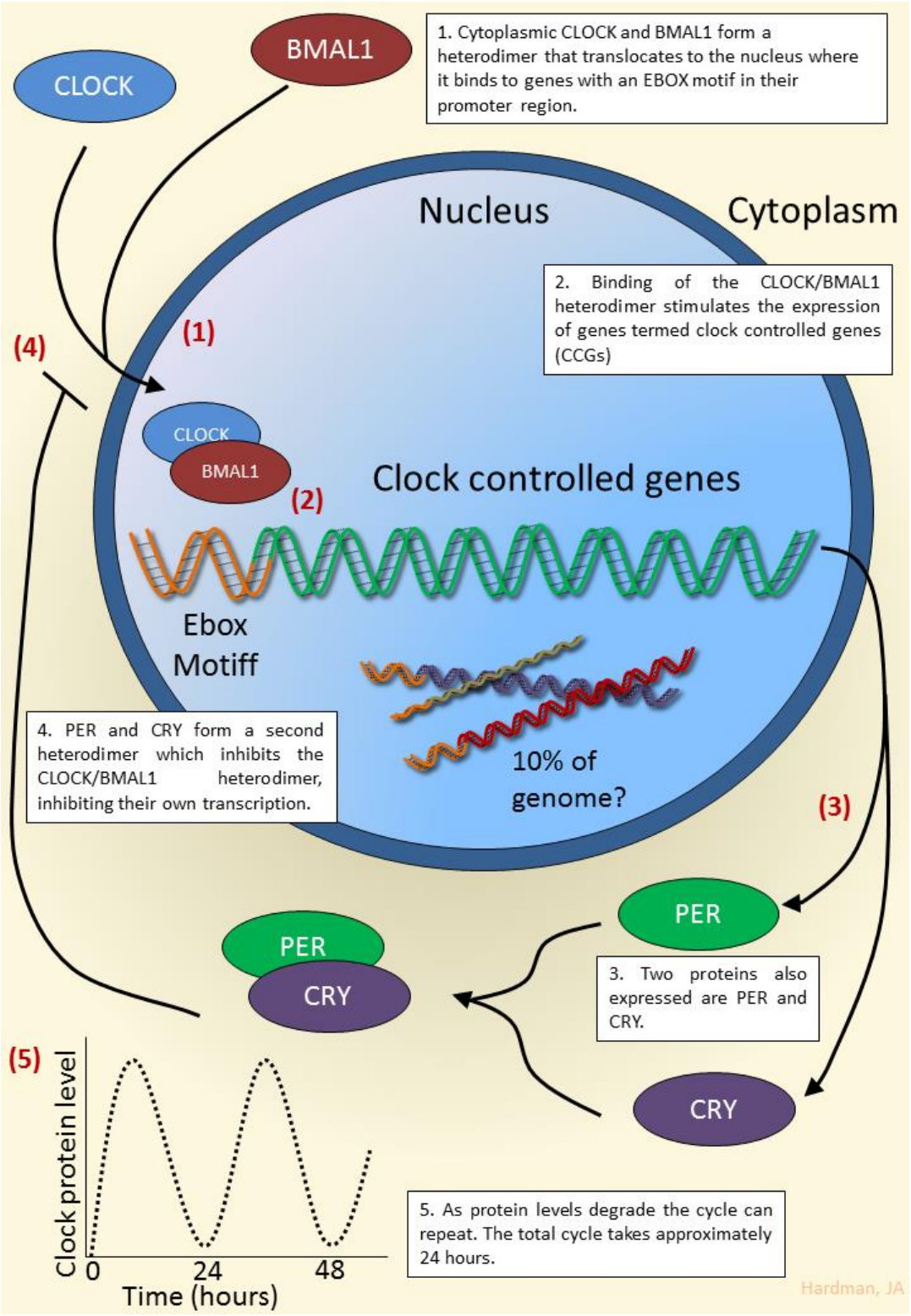

Fig. 2.5: The core molecular clock system.
The core molecular clock is an oscillating system lasting 24-hours. (1) The transcribed protein CLOCK and BMAL1 form a heterodimer which translocate to the cell nucleus where it binds to genes containing an EBOX motif in its promoter region or clock controlled genes (CCGs). (2) This binding leads to the transcription of CCGs and two families of proteins, the Period (PER) and Cryptochrome (CRY) family. (3) Proteins PER and CRY form a second heterodimer which prevents the formation of the first heterodimer and thereby inhibiting its own transcription. (4) This cycle leads to a 24-hour oscillatory rhythm.

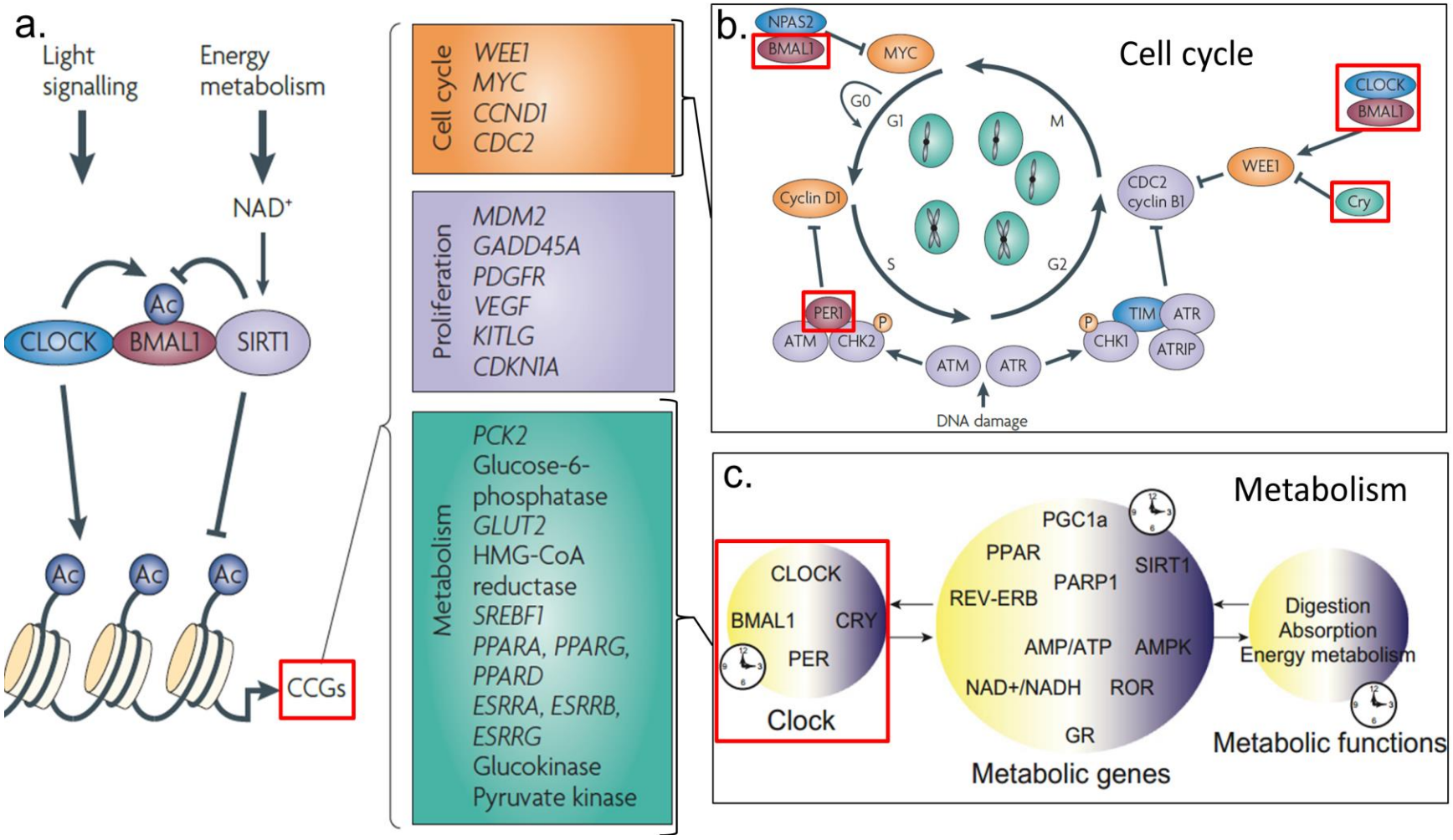
a.
Light signalling
Energy metabolism
NAD+
Ac
CLOCK
BMAL1
SIRT1
Ac
Ac
Ac
CCGs
Cell cycle
WEE1
MYC
CCND1
CDC2
Proliferation
MDM2
GADD45A
PDGFR
VEGF
KITLG
CDKN1A
Metabolism
PCK2
Glucose-6-phosphatase
GLUT2
HMG-CoA reductase
SREBF1
PPARA, PPARG, PPARD
ESRRA, ESRRB, ESRRG
Glucokinase
Pyruvate kinase
b.
Cell cycle
NPAS2
BMAL1
MYC
G0
G1
M
S
G2
Cyclin D1
CDC2 cyclin B1
WEE1
CLOCK
BMAL1
Cry
PER1
P
ATM
CHK2
ATM
ATR
P
CHK1
TIM
ATR
ATRIP
DNA damage
c.
Metabolism
CLOCK
BMAL1
CRY
PER
Clock
PGC1a
PPAR
SIRT1
PARP1
REV-ERB
AMP/ATP
AMPK
NAD+/NADH
ROR
GR
Metabolic genes
Digestion
Absorption
Energy metabolism
Metabolic functions

Fig. 2.6: Molecular clock and target systems.
(a) A schematic highlighting some of the genes and mechanism by which the molecular clock regulates tissue physiology. (b) Specifically the molecular clock is strongly implicated in cell-cycle control, regulating many of the checkpoint proteins. (c) The molecular clock has also been shown to regulate metabolism in a complex manner, particularly in the liver. Image modified from (Sahar and Sassone-Corsi 2009; Tahara and Shibata 2013).

2.2.2 Clock genes and the hair follicle.

Although circadian clock gene/protein expression was demonstrated in many of the cell types found in the skin (keratinocytes, melanocytes and fibroblasts) (Zanello et al. 2000; Sandu et al. 2012) it wasn't until Lin *et al.* in 2009 published their study that a link between circadian biology and the HF was established (Lin et al. 2009). In this murine study, distinct clock activity was observed throughout the hair cycle with Dbp, a CCG which acts as a robust marker for clock activity, showing the highest amplitude of expression in the secondary hair germ during telogen. Utilising a targeted knock-down of *bmal1* activity, thereby eliminating clock activity (Bunger et al. 2000), anagen onset was delayed when compared to littermate controls suggesting a role for the molecular clock in modulating hair cycle activity (Lin et al. 2009). It was further demonstrated that the bulb and bulge region of the murine HF had the highest level of clock activity, thereby identifying distinct, localised expression of clock genes/protein activity within the murine HF (Plikus et al. 2013). The clock activity within the bulge stem cells specifically coincides with the activation status of the stem cells. Utilising a *Per1* promoter attached to a fluorescent reporter has shown that high levels of *Per1* expression coincide with activated stem cells, which have high levels of Wnt and low tgfβ expression, whereas low *Per1* expression represented the dormant stem cells. This demonstrated that the HF bulge stem cells exist in a heterogeneous state, half being primed for activation whilst the other remain dormant (Janich et al. 2011; Lien and Fuchs 2014). Furthermore, the circadian clock has been implicated in controlling stem cell division, differentiation and is integral in maintaining stem cell heterogeneity (Brown 2014). These studies together first identified the robust circadian activity of the murine HF which has a distinct role in both the murine hair cycle and also in stem cell activity. However, work linking circadian biology and the HF has so far focused on murine studies that, while a useful model to highlight potential targets for further study, fail to appreciate the distinct differences between the murine and human systems.

In human HFs rhythmic clock gene expression was observed in plucked HFs identifying a novel tool by which to observe an individual's circadian profile however, this investigation failed to take into account the synchronising activity of the SCN, ignoring any potential intrinsically oscillating clock activity that may be present in the human HF (Akashi et al. 2010). Although there is a supported link

between the murine HF and clock activity, influencing both the hair cycle and bulge stem cells, whether the human HF has innate oscillating clock activity and therefore has peripheral clock activity is not known. Furthermore, it is not known whether this has a functional role in the human HF physiology including the human hair cycle, bulge stem cell maintenance and rhythmically time process of human HF pigmentation.

2.3 Human melanogenesis and melanocyte biology

2.3.1 Introduction

In addition to the hair cycle, the HF undergoes many other complex processes, one of which is the formation of hair pigmentation. Hair pigment is produced by melanocytes, which are specialised dendritic cells of the HF and epidermis. These are located adjacent to the DP and interact with the MKs of the HF bulb. Together this unit is collectively known as the HF pigmentary unit (HFPU) (Scott et al. 2002; Tobin 2011) (**Fig. 2.7**). Melanocytes produce hair pigment in the process of melanogenesis during which L-tyrosine is converted to the pigment molecule melanin (Chávez-Béjar et al. 2013). This process is tightly coupled to the hair cycle and occurs exclusively in anagen (Slominski et al. 1994).

Interestingly, like the hair cycle, melanocytes have been linked with the molecular clock having been demonstrated to express both clock genes and proteins when cultured (Zanello et al. 2000; Sandu et al. 2012). As the process of melanogenesis is tightly coupled to the hair cycle occurring exclusively in anagen, if the HF has peripheral molecular clock activity is likely it will control many aspects of melanocyte biology and melanogenesis also. Despite many candidates that control these processes being identified (**table 2.2**) what ultimately choreographs these systems remains elusive. This implicates the presence of an autonomously oscillating system, potentially the molecular clock, in controlling the complex on/off switch of melanogenesis.

2.3.2 Melanocyte biology

Melanocytes are derived from neural crest cells during development (Tobin 2011). These immature melanocytes, melanoblasts, are induced to differentiate, proliferate and migrate by WNT, endothelin

3 and stem cell factor (SCF) (Dupin and Le Douarin 2003; Tobin 2008), a process that is antagonised by BMP expression (Videira et al. 2013).

SCF expression is a key regulator of melanocyte biology and knock-out of its receptor C-kit results in an unpigmented hair shaft (Ito et al. 1999; Botchkareva et al. 2001; Videira et al. 2013). In the HF SCF is expressed by DP cells which cause melanocytes expressing its receptor (Videira et al. 2013) to migrate to the HF bulb where they will express adhesion molecules E-cadherin and P-cadherin and attach to the basement membrane between the DP and MKs of the bulb (Ito et al. 1999; Peters et al. 2002; Haass and Herlyn 2005; Samuelov et al. 2013). Alternatively, the amelanotic C-kit negative melanocytes will migrate to the ORS and bulge region (Ito et al. 1999; Peters et al. 2002). In a mature HF there are various sub-populations of HF melanocytes, separate from the bulb, in the ORS, infundibulum and the sebaceous gland (Slominski et al. 2005a) (**Fig. 2.7**).

During anagen these bulbar melanocytes will produce melanin and transfer it to those MKs of the bulb destined to differentiate into the HS (Seiberg 2001; Jian et al. 2014). In the HF bulb, one melanocyte will supply the pigment to approximately 5 MKs (Tobin and Paus 2001). As HFs enter catagen the melanocytes will undergo apoptosis (Tobin et al. 1998; Slominski et al. 2005a; Goding 2007) subsequently being replenished from the melanoblast progenitors found in the lower HF bulge during the next stage of anagen (Tobin et al. 1998; Goding 2007; Cichorek et al. 2013).

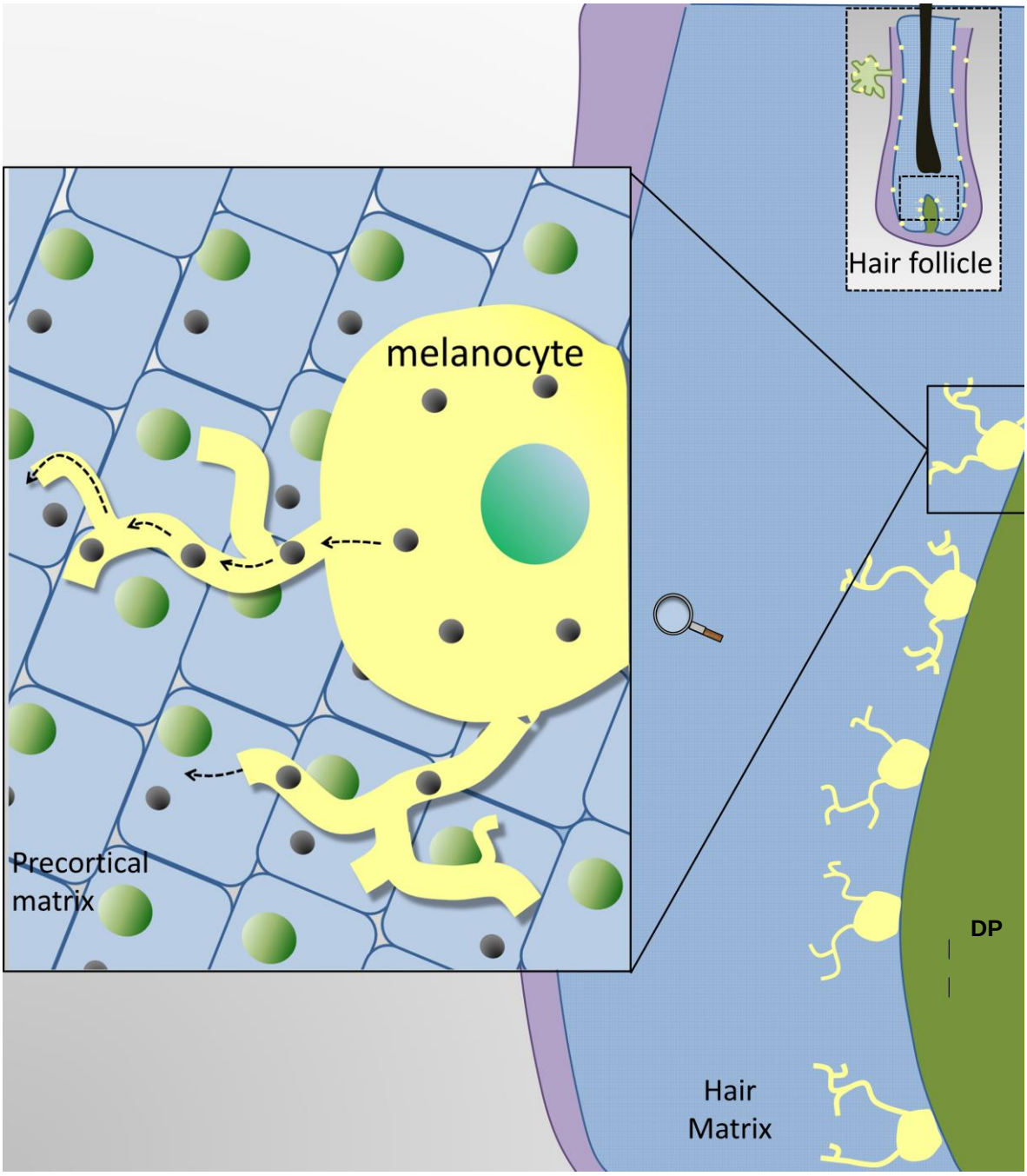

Fig. 2.7: Hair follicle pigmentation: Overview
Hair follicle pigmentation is a complex process collectively known as melanogenesis. The pigment molecule melanin is produced by specialised dendritic cells (melanocytes) that are located adjacent to the dermal papilla within the hair matrix. These melanocytes contain organelles known as melanosomes within which the reaction to create melanin occurs. Once the melanosome has matured, the organelle itself is transferred to those keratinocytes in the hair matrix destined to differentiate into the hair shaft via the melanocytes' dendrites.

Melanosome formation

The pigment molecules eumelanin and pheomelanin are produced in melanosomes, specialised organelles found specifically in melanocytes (Tobin 2008). As some of the products produced during melanin production are cytotoxic, that it occurs in these specialised membrane bound organelles protects the melanocyte from apoptosis (Sulaimon and Kitchell 2003; Plonka et al. 2009; Cichorek et al. 2013). Coupled with this MITF, the master-regulator of melanogenesis, up-regulates the anti-apoptotic gene BCL2 (Sulaimon and Kitchell 2003; Plonka et al. 2009; Cichorek et al. 2013). Interestingly while ROS are produced during melanin formation, melanin itself is a ROS scavenger ultimately absorbing any ROS produced during its creation (Bustamante et al. 1993).

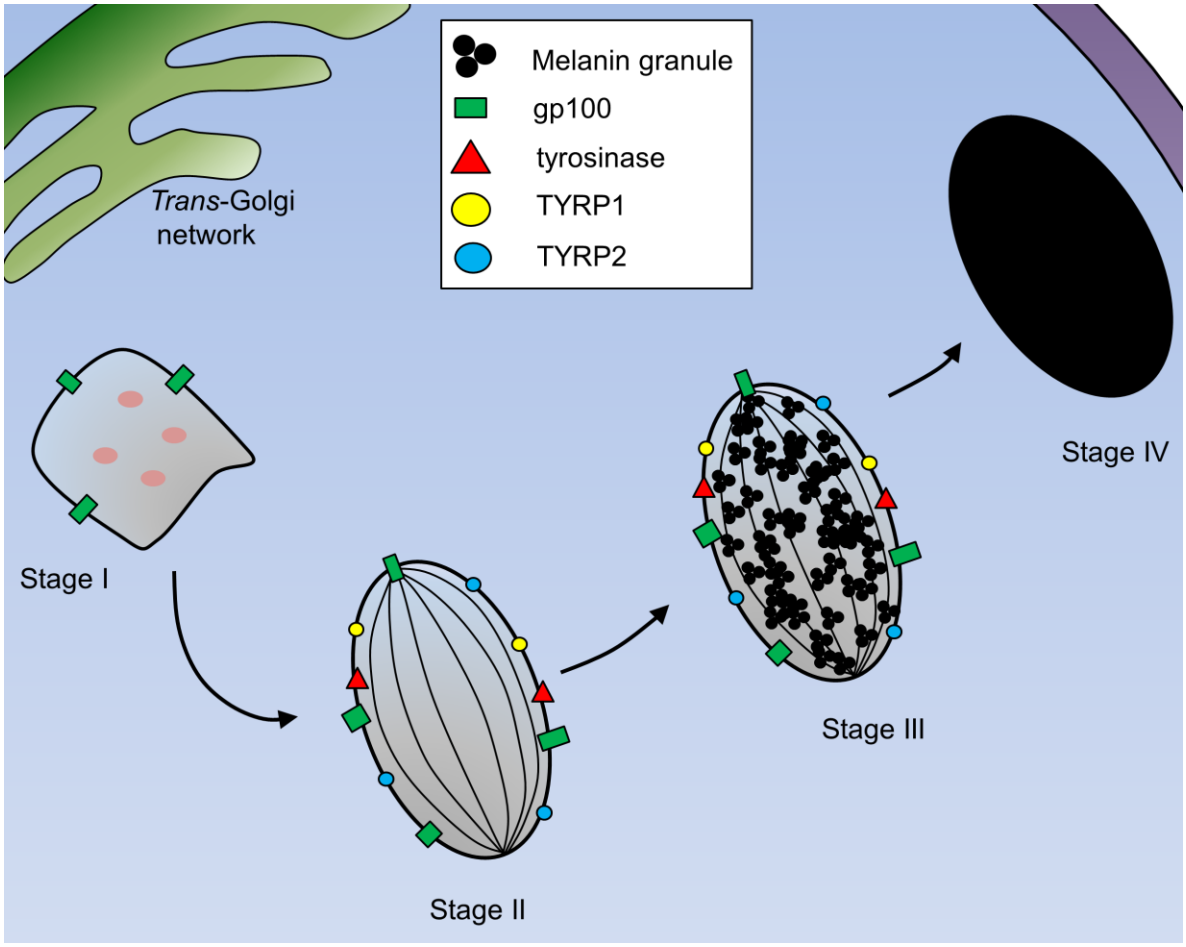

Fig. 2.8: Melanosome formation and maturation.
Stage I melanosomes from the trans-Golgi network resembling vesicular bodies are irregular in shape. During stage I the pre-melanosomal marker gp100 is delivered to the organelle. In stage ii melanosomes have a regular elongated shape and an intraluminal scaffold is laid down. Coupled to this, key enzymes tyrosinase, TYRP1 and TYPR2 are delivered to the melanosome by endocytosis. As melanin production begins in stage iii, it is deposited along the intraluminal scaffold. By stage iv melanin has fully accumulated in the melanosome obscuring the scaffold. Stage iv melanosomes are then transferred via filopodia to bulb MKs.

Melanosomes are believed to be formed as part of the vacuolar/endosomal pathway and melanosome maturation can be sub-divided into 4 stages (**fig. 2.8**) (Marks and Seabra 2001). Stage I melanosomes lack any pigment and can be identified by their irregular fibrous structure. During stage I gp100, a key protein in melanogenesis is delivered to the melanosome by endocytosis (Marks and Seabra 2001; Singh et al. 2008; Sitaram and Marks 2012). As melanosomes progress to stage II they develop a regular, ovular shape. Parallel intraluminal fibres are formed, connecting each end of the melanosome facilitated by gp100 which creates a scaffold for melanin deposition (Hearing 1999). In stage II other essential enzymes for melanin production are transferred to the melanosomes including the rate-limiting enzyme tyrosinase and tyrosinase-related protein 1 & 2 (TYRP1-2) (Marks and Seabra 2001; Slominski et al. 2005a). With all the enzymes present for melanin production melanosomes enter stage III (Sitaram and Marks 2012). Stage III is characterised by melanin granule deposition causing darkening of the melanosome (Marks and Seabra 2001). When melanin molecules fill the melanosome, obscuring the intraluminal scaffold, the melanosome is considered to be in stage IV and fully mature. Once mature melanosomes are transferred via filopodia to the surround MKs (Scott et al. 2002; Singh et al. 2010). This is mediated by PAR2 on keratinocytes (Slominski et al. 2005a; Cichorek et al. 2013).

It is melansomes that determine both skin colour and hair colour (Tobin 2008) (**Fig. 2.9**). Dark hair is characterised by large eumelanin rich melansomes, with brown hair having similar melanin rich melansomes, only smaller (Ozeki et al. 1996; Tobin 2008). In blonde hair, although the melanosomes do contain eumelanin they are poorly melanised and the fibrous intraluminal scaffold remains visible even when mature (Ozeki et al. 1996; Tobin 2008; Guenther et al. 2014). Alternatively, red hair is characterised by irregular deposition of pheomelanin and have misshapen and spherical melanosomes. Hair colour is mediated by the melanocortin-1 receptor (MC1R) which has as many as 7 variants leading to differences in pigmentation, whilst red hair results from mutations in this receptor (Frändberg et al. 1998; Cichorek et al. 2013; Abdel-Malek et al. 2014; Pasquali et al. 2014).

2.3.2 Melanogenesis

Melanin production is an essential protective mechanism against UV radiation (Costin and Hearing 2007) as it has the ability to absorb UV light, scavenge ROS and store ions (Costin and Hearing 2007). This provides the skin with a protective mechanism against UV damage and prevents DNA damage by reducing the likelihood of thymine dimers forming in the DNA helix, leading to cancer (Videira et al. 2013). Eumelanin is an excellent ROS scavenger and people with darker pigmentation have a significantly reduced risk of developing skin cancer (Hearing 2011).

The formation of melanin granules is a complex chemical pathway during which L-tyrosine is converted to either eumelanin or pheomelanin (**Fig.2.9**) (Slominski et al. 2004). The process first requires the delivery of L-tyrosine from the extracellular space or by hydroxylation of L-phenylalanine by the key melanogenesis enzyme tyrosinase (Schallreuter et al. 1998). Melanogenesis is a multi-step conversion of tyrosine to melanin. It is further catalysed by the rate-limiting enzyme tyrosinase and accessory enzymes tyrosinase-related protein 1 & 2 (TYRP1 and 2) which are 40% homologous with tyrosinase (Videira et al. 2013). All three of these enzymes are transcribed by phosphorylated MITF (Yokoyama 1997; Vachtenheim and Borovanský 2010) whose activation is controlled by the MAP-kinase pathway, specifically ERK1/2 phosphorylation (Buscà and Ballotti 2000; Yanase et al. 2001; Cichorek et al. 2013). These processes are reliant on cyclic-AMP (cAMP) to facilitate their activation (Otręba et al. 2012). Other key activators of melanogenesis include MSH and PKA (Costin and Hearing 2007; Cichorek et al. 2013).

While many of controls of human pigmentation and melanocyte biology are now appreciated (**table 2.2**) the timing mechanism that couples melanocyte cell function, melanogenesis and the human hair cycle is still not fully understood.

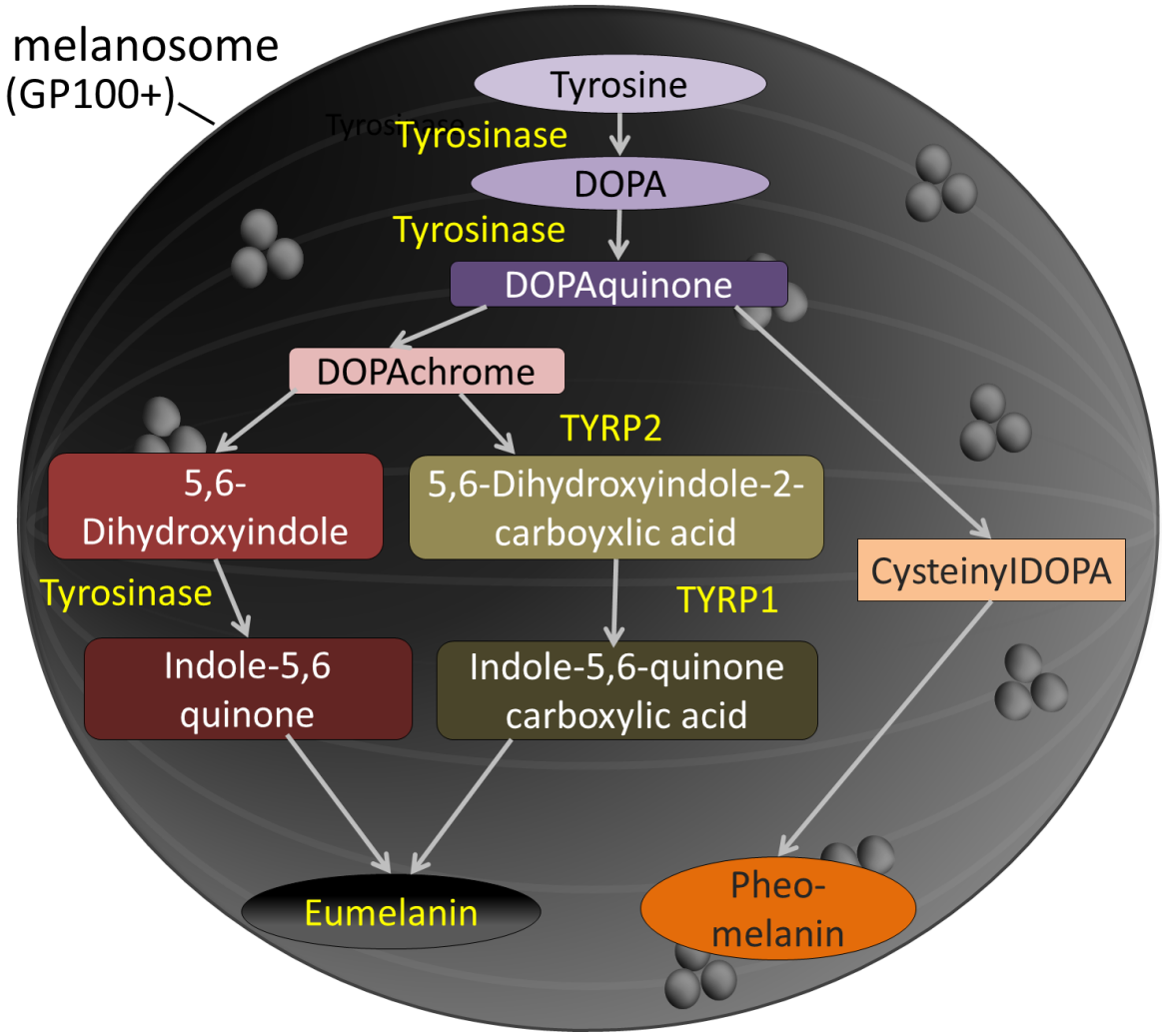

Fig 2.9: Melanin production pathway.
Melanin is produced in a multistep pathway through which tyrosine is converted to many intermediate molecular catalysed by the rate-limiting enzyme tyrosinase and TYRP1 and TYRP2. Through this process two types of melanin can be produced, the dark pigmented eumelanin and the red pigment pheomelanin.

Mediators of Melanogenesis	***Role in melanogenesis***	***References***
POMC *(proopiomelanocortin)*	POMC undergoes tissue-specific proteolytic processing to produce many small peptides two of which, (α-MSH and ACTH) have roles in melanogenesis.	(Paus et al. 1997; Takahashi and Mizusawa 2013)
α-MSH *(alpha- Melanocyte stimulating hormone)*	α-MSH is a neurohormone derived from POMC which regulates MITF expression by interacting with the melanocortin 1 receptor, which subsequently increases MITF levels via the cyclic-AMP pathway.	(Geschwind 1966; Böhm et al. 2006; Kim et al. 2013)
ACTH *(adrenocorticotrophic hormone)*	ACTH is derived from POMC and regulates melanogenesis by binding to melanocortin 1 receptor.	(Buscà and Ballotti 2000; Videira et al. 2013)
MITF *(Microphthalmia-associated transcription factor)*	MITF is a master regulator of melanogenesis. It is directly responsible for the transcription of key melanin synthesis related enzymes Tyrosinase, TYRP1 and TYRP2. MITF will bind to those genes with an EBOX or MBOX motif in their promoter region.	(Tachibana 2000; Du et al. 2003; Vachtenheim and Borovanský 2010)
C-kit / SCF	C-kit is the receptor for stem cell factor (SCF). It is required for melanocyte migration, differentiation and proliferation. SCF/c-Kit signalling is integral for the cyclic regeneration of the pigmentary unit of the hair follicle throughout the hair cycle.	(Botchkareva et al. 2001)
P-Cadherin	P-Cadherin is linked with melanoblast formation and melanoma progression. It is expressed mainly in the hair follicle pigmentary unit. Knock-down of this protein has shown to decrease GP100, MITF and tyrosinase expression.	(Samuelov et al. 2013)
BMP *(Bone morphogenetic protein)*	BMPs have various roles in melanogenesis. BMP4 and 6 influence filopodia related proteins. BMP 6 also stimulates melanin synthesis by increasing tyrosinase expression and can stimulate melanin transfer.	(Kulessa et al. 2000; Singh et al. 2012)

SOX10 *(SRY-related HMG-box)*	SOX10 is important for differentiation and survival of melanocytes during embryogenesis. SOX10 can activate MITF and co-operatively stimulate TYRP-2. SOX10 dysfunction in humans is associated with the hypopigmentation disorder Waardenburg-Shah syndrome.	(Buscà and Ballotti 2000; Lee et al. 2000; Harris et al. 2013)
Pax3 *(Paired box family 3)*	Pax3 is involved in mediating melanocytic stem cell activation and leads to the expression of MITF.	(Lang et al. 2005)
Endothelin	Endothelin (particularly endothelin 1) is an important mediator of melanogenesis. It has been linked with influencing melanocyte proliferation, dendricity and melanin synthesis.	(Imokawa et al. 1995; Nakajima et al. 2011)
BCL2 *(B-Cell lymphoma 2)*	BCL2 is a downstream target of MITF. Its anti-apoptotic ability is essential during melanin production as many cytotoxic products are formed.	(McGill et al. 2002; Nishimura 2011)
NGF *(Nerve growth factor)*	Keratinocyte derived NGF stimulates melanocyte dendricity, migration and survival in response to UV. Furthermore NGF can increase melanin production.	(Gilchrest et al. 1996; Marconi et al. 2006)
Histamine	Histamine induces melanin production by increasing tyrosinase activity. Histamine further increases cAMP levels in human melanocytes and increased their dendricity.	(Yoshida et al. 2000; Lassalle et al. 2003)
PGE2 *(prostaglandin E2)*	PGE2 is released from keratinocytes in response to UV-radiation. This stimulates cAMP production, tyrosinase activity and melanocyte proliferation.	(Gordon et al. 1989; Starner et al. 2010)
HGF *(Hepatocyte growth factor)*	HGF is necessary for melanocyte proliferation and differentiation with overexpression preventing hair greying in mice.	(Halaban et al. 1992; Lindner et al. 2000; Endou et al. 2014)

Table 2.2: Key molecular modulators of melanogenesis.
This table lists the key molecular modulators involved in modulating melanogenesis. Molecular modulators highlighted in blue have also been demonstrated to influence human melanogenesis.

2.3.3 Melanogenesis and the molecular clock

Although many of the molecular controls that control melanocyte biology and melanogenesis (**table 2.2**) are known, the molecular system that choreographs these processes and couples them to the hair cycle are still unknown. There is a developing link between human pigmentation and the molecular clock, however, whilst previous reports demonstrate 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 functionally impacts on melanogenesis or other melanocyte functions.

The molecular clock is appreciated to be involved in the modulation of cellular metabolism and replication in response to UV light (Geyfman et al. 2012b) and can also relay light cues (Magalhães Moraes et al. 2014). Moreover, both *BMAL1* (Elshazley et al. 2012; Geyfman et al. 2012b; 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 (**Fig. 2.6**). In addition to this, the strength of cellular responses to DNA damage is under circadian control (Oklejewicz et al. 2008; Sancar et al. 2010). That melanocytes also respond to DNA-damaging UVR stimulation by increasing dendricity, hormone production and pigmentation, as well as acting as “sensory” cells within the epidermis implies that there may be a link between these two systems. Furthermore, 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 ROS accumulation and reduction of life-span, fat, muscle and bone mass (Kondratov et al. 2006; Geyfman and Andersen 2010; Kondratova and Kondratov 2012), that a loss in hair pigmentation, or hair greying, is one of the first signs of aging in the HF further supports this. Indeed ROS accumulation in HF melanocytes is believed to be involved in age-related greying (Arck et al. 2006; Wood et al. 2009).

Interestingly, clock genes and melanogenesis are linked with ROS homeostasis, which further supports a link between melanocyte biology and chronobiology (Geyfman et al. 2012b; Lai et al. 2012; Lee et al. 2013; Avitabile et al. 2014). Indeed, BMAL1 is activated by and is protective against

near-lethal doses of ROS (Tamaru et al. 2013), with BMAL1 deletion leading 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). Despite the strong associations between melanogenesis and the molecular clock a clear mechanistic link is not apparent.

The MAPK pathway, specifically ERK 1/2 activation, which subsequently activates MITF (Yanase et al. 2001; Jin et al. 2014), may suggests a mechanistic link between clock activity and potentially pigmentation control as ERK1/2 is regulated by the circadian clock (in *Neurospora*) (Bennett et al. 2013). Furthermore, the molecular clock has clearly defined links with cAMP levels; reduction of CRY1 and 2 increasing levels of cAMP. As cAMPs are integral for MITF activation (Zhang et al. 2010) highlights the potential role for the molecular clock in controlling the rhythmic on/off switch of HF pigmentation during the hair cycle.

With a potential link between melanogenesis and the molecular clock, and that both are linked with age related pathologies presents the idea that hair greying may occur due to uncoupling of these systems.

2.3.4 Hair greying

Hair greying is an age related pathology of the human HF. It is characterised by loss of pigment in the hair shaft that can be attributed to loss of melanocyte function by an increased accumulation of ROS which are cytotoxic (Wood et al. 2009). This is often caused by a loss of catalase activity, an antioxidant (Kauser et al. 2011; Shi et al. 2014) in the HF, coupled with down-regulation of MITF and Pax3 induced BCL2 in the melanocyte stem cells (Nishimura et al. 2005; Paus 2011). Unpigmented HFs have also been demonstrated to also have a significant reduction in tyrosinase, TYRP1, MITF and C-kit expression; Furthermore, a loss in melanoblasts in the HF bulge has also been reported (Shi et al. 2014).

It was initially thought that hair greying was attributed solely to depletion in the melanocyte stem cells found in the HF bulge (Nishimura et al. 2005; Tobin 2011). However, a loss of hair pigmentation is frequently seen in the anagen hair bulb much earlier than in the bulge stem cells in a manner reminiscent of catagen induction. As hair greying in the hair bulb is accompanied by a decrease in factors essential for melanocyte function such as BMP, SCF, HGF, MITF (Nishimura et al. 2005; Osawa et al. 2005; Paus 2011) and neuroendocrine hormones ACTH and α-MSH (**table 2.2**) (Paus et al. 1999a). Moreover, there is an increase TGFβ-2 which leads to for catagen induction and cessation of pigmentation (Tobin 2011). This implies that hair greying begins as a loss in maintenance in the HFPU homeostasis which eventually becomes permanent as the loss of function translates to the melanocyte stem cells in the hair bulge and amelanotic melanocytes of the ORS (Paus 2011). Greying may therefore be explained, at least in part by an a loss of HFPU promoting factors, increased oxidative damage leading to cytotoxicity and eventual damage to the HF melanocyte stem cells (Seiberg 2013; Shi et al. 2014).

There is increasing evidence that suggests that neuroendocrine hormones are essential for the maintenance of stem cell function (Paus 2011). ACTH and α-MSH produced from POMC are neuroendocrine hormones that have been shown to significantly increase HF pigmentation (Paus et al. 1999a; Takahashi and Mizusawa 2013). Moreover, both of these are produced in the HF along with their receptors (Kauser et al. 2005). Interestingly TRH, an additional neuroendocrine hormone produced in the hypothalamic-pituitary-thyroid axis (HPT), also influences HF pigmentation and is also produced locally (Gáspár et al. 2011). Together this highlights the potential role of locally produced neuroendocrine hormones in maintenance of hair pigmentation and loss of such hormones may exacerbate hair greying.

2.4 Hormonal regulation of the hair follicle

2.4.1 Hormones and the circadian clock

Hormones including neuroendocrine and thyroid hormones have been found to influence both hair follicle cycling and pigmentation in the human HF (Bodó et al. 2010; Gáspár et al. 2010). Furthermore, the SCN in the hypothalamus is not only the key centre of circadian activity in humans, synchronising molecular clock activity in peripheral tissues, but is also an important centre of hormone production. It is via the production of endocrine hormones including TRH, prolactin and melatonin that the SCN is able to synchronise the activity of clock genes and CCG in peripheral tissues (**Fig. 2.4**) (Charmandari et al. 2011). Indeed, many peripheral tissues have autonomous circadian behaviour and study of these systems may generate insights into mechanisms by which clock and CCGs might be modulated within peripheral tissues. This has the potential to uncover novel hormone treatments with which to alleviate clock related illnesses. Table 2.3 lists some hormones shown to have a diurnal expression pattern that are also known to be involved with HF biology.

Hormone	***Comments***	***References***
PRL (prolactin)	Prolactin demonstrates hair cycle-dependent expression and has been shown to induce catagen. Furthermore PRL is under direct circadian control with clock disruption preventing PRL surges in rats. PRL is also expressed in humans in a circadian manner.	(Foitzik et al. 2003; Geyfman and Andersen 2010; Poletini et al. 2010; Roelfsema and Pijl 2012)
BDNF (Brain Derived Neutrophic factor)	BDNF is a catagen induced in the hair follicle and shows strong sex-linked variations in its expression over 24 hours.	(Peters et al. 2005; Piccinni et al. 2008)
Leptin	Leptin is expressed in the murine DP and its induction initiates anagen. Furthermore, leptin shows distinct circadian expression profiles and is able to feedback to the central clock.	(Sumikawa et al. 2014; Tinoco et al. 2014; Watabe et al. 2014)
Adiponectin	Receptors for adiponectin produced by adipocytes can be found in the DP, ORS, CTS and sebaceous gland of human HF. Addition of Adiponectin to human HFs induces MK proliferation. Adiponectin is expressed in a circadian manner but is able to feedback to the central pacemaker.	(Won et al. 2012; Hashinaga et al. 2013)
Cortisol/ glucocorticoids	Glucocorticoids are expressed in a circadian manner and have a strong ability to feedback to the central clock and are able to reset the circadian phase. These are also linked with human DP cells where they reduce their viability in culture.	(Balsalobre 2000; Cordero et al. 2012; Choi et al. 2013; Kalsbeek et al. 2014)
TSH (Thyroid stimulating hormone)	TSH and its receptors are produced locally by the human hair follicle. Furthermore TSH has strong links with circadian biology showing circadian expression patterns and is reported to be under direct control of the accessory clock gene REV-ERB. Altered circadian variation of TSH in humans is linked with diseases including hypothyroidism.	(Aizawa et al. 2007; Bodó et al. 2010; Langan et al. 2010; Chen et al. 2013; Sviridonova et al. 2013; Aninye et al. 2014)

T3 and T4 (Triiodothyronine & thyroxine)	Culturing HFs with T4 promotes MK proliferation and co-culture with T3 and T4 inhibits apoptosis. T4 can furthermore prolong anagen by down-regulation of TGF-β2. Both T3 and T4 can also stimulate hair follicle pigmentation. Both of these hormones are expressed in a circadian pattern moreover, thyroidectomy can dampen the expression profile of core clock genes.	(McEachron et al. 1993; van Beek et al. 2008; Dardente 2012; Zhao et al. 2013)
αMSH	α-MSH regulates MITF expression, the master regulator of pigmentation and therefore pigmentation. Furthermore, it exhibits circadian rhythmicity in horses.	(Cordero et al. 2012; Kim et al. 2013)

Table 2.3: Hormones that influence the HF and demonstrate circadian expression patterns.
Table 2.3 highlights some of the hormones that are involved in modulating the hair follicle and demonstrate circadian rhythmicity in their expression profile. Those highlighted in blue are involved in both the human HF and show circadian expression in humans.

2.4.2 The HPT axis

From table 2.3 it is evident that TSH, T4 and T3, all members of the hypothalamic pituitary axis (HPT), demonstrate circadian rhythmicity and have been shown to be expressed in the HF. The HPT axis (**figure 2.10**), through which the thyroid hormones thyroxine (T4) and the more active triiodothyronine (T3) are produced, like circadian rhythms begin in the hypothalamus. In this pathway TRH produced by the hypothalamus travels to the anterior pituitary gland where it stimulates the production of thyroid stimulating hormone (TSH). The production of TSH inhibits further production of TRH and stimulates the production of thyrotropin in the thyroid gland. TRH, while directly stimulating the production of thyrotropin also regulates other downstream hormones including prolactin, growth hormone and insulin that also have roles in modulating the HF and hair cycle

It is now understood that human skin and the human HF express many of these hormones and the corresponding receptors and therefore there may be a peripheral HPT axis. TSH receptors for example have been found in cultured dermal and HF fibroblasts, HF and skin derived keratinocytes (Slominski et al. 2002). This was further confirmed in human skin which expressed TSH and TSH receptor protein and mRNA. Further supporting the presence of a peripheral HPT-axis is TRH and TRH–receptors which are expressed in human HFs, where they stimulate pigmentation, prolong anagen and lead to MK proliferation therefore increasing HF elongation *in-vitro* (Gáspár et al. 2010). Finally, key enzymes involved in converting the terminal product thyroxine (T4) to the more active triiodothyronine (T3) D2 and D3 can be found in the human hair follicle suggests that HFs are actively able to convert T4 to T3 separate from the thyroid gland (van Beek et al. 2008). Together, that the human HF and human skin expresses all components of the HPT-axis strongly suggests that there is indeed a local HPT-axis in human skin and its appendages.

Interestingly, many of the hormones produced systemically are under tight circadian control. Products of this process thyroxine (T4) and triiodothyronine (T3) (**Fig 2.8**) are of particular interest as they influence both peripheral clock activity and have a role in negative feedback, inhibiting upstream products of the axis. T3 and T4, like TRH, have been shown to have a considerable role

in the HF (van Beek et al. 2008) where they inhibit apoptosis, prolong anagen and have been shown to modulate keratin expression within the HF, namely K15 (van Beek et al. 2008). Moreover, there is a clear link with the circadian clock and thyroid hormones. Animal studies have found that systemic treatment with thyroid hormones can lengthen circadian periodicity in hamsters (Beasley and Nelson 1982). In contrast thyroidectomy can both reduced circadian periodicity but also dampen clock gene expression, specifically blunting PER2 oscillations (Beasley and Nelson 1982; McEachron et al. 1993; Amir and Robinson 2006). In addition, T4 is essential for seasonal rhythms and mating season timing in mammals and also has a diurnal expression patterns (Dardente 2012; Dardente et al. 2014). Furthermore, it has been implicated in regulating metabolism (Dardente et al. 2014), a process regulated by the molecular clock (Sahar and Sassone-Corsi 2009; Bass 2012; Buxton et al. 2012). However, direct evidence that T4 modulates the peripheral clock in human tissues *in situ* is still missing. While current studies highlight a potential link between thyroid hormones and the clock system, how thyroid hormones affect peripheral clock activity and whether it is through the molecular clock system that thyroid hormones influence the human HF requires investigation.

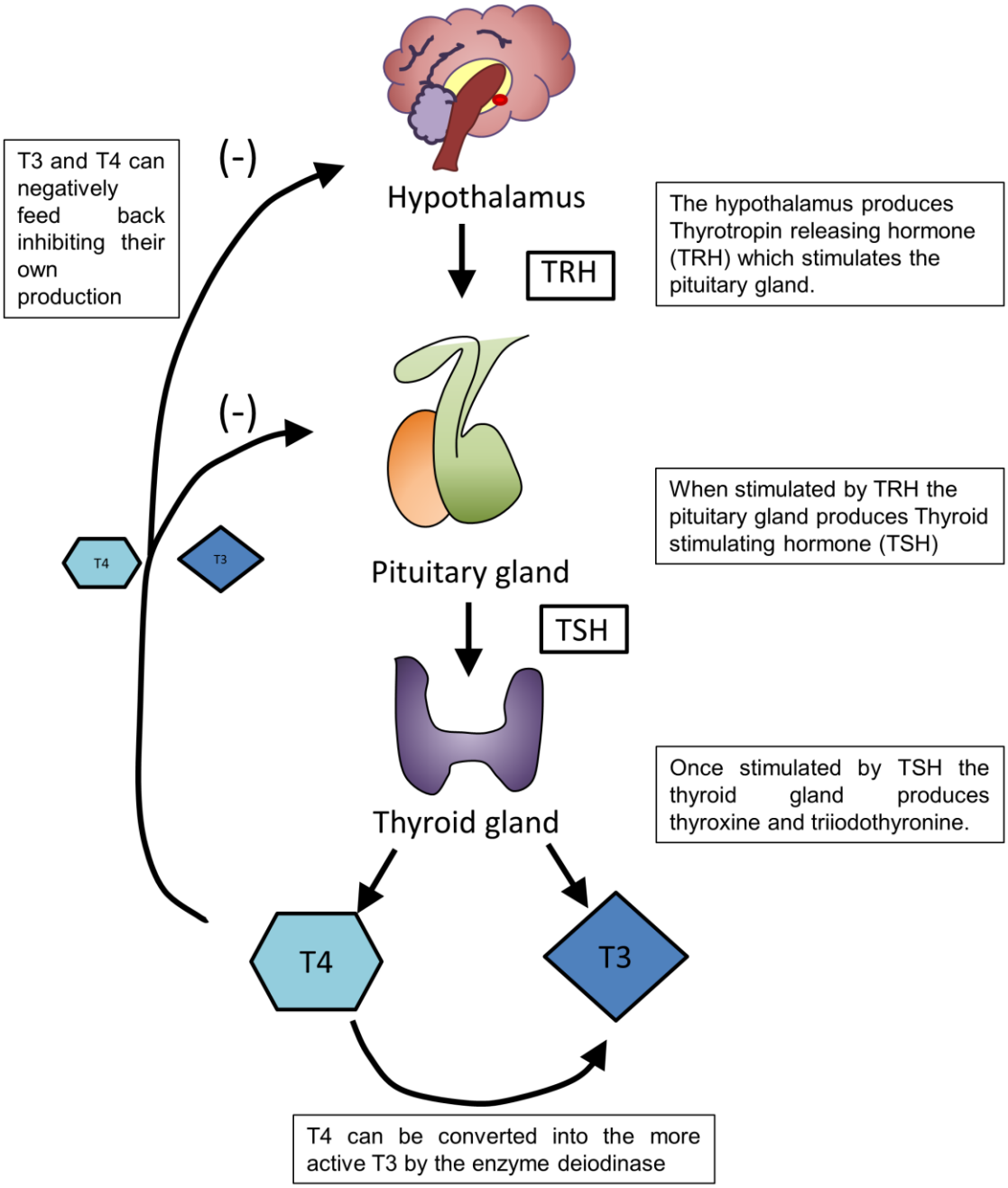

Fig. 2.10: The hypothalamic-pituitary-thyroid axis.
The neuroendocrine hormone thyrotropin-releasing hormone (TRH) produced in the hypothalamus induces the production of thyroid-stimulating hormone (TSH) from the anterior pituitary gland. TSH travels systemically to the thyroid gland where it induces the formation of thyroxine (T4) and triiodothyronine (T3). T4 can be converted by the enzyme deiodinase to its more active form T3. The formation of T4 and T3 negatively feeds back to the pituitary gland and the hypothalamus, thus inhibiting their own production.

2.5 Major open questions and aims of thesis

Does the human HF have rhythmic and functional clock gene/protein expression?

While there is a strong link between circadian biology and the HF, with it influencing the murine hair cycle and the distinct clock protein expression, there is limited data for the human HF. Although cell culture experiments have demonstrated that cell populations of the human HF do express clock genes, there is currently no data describing whether the human HF expresses clock genes *in situ* and whether this is functionally relevant, i.e. does it influence hair growth/hair cycle? Therefore the following aims and specific questions will be addressed:

1. To investigate whether the human HF has functional, oscillatory molecular clock activity.

- Does the human HF express clock genes and proteins in a hair-cycle dependent manner?
- Does the human HF have peripheral molecular clock activity?

2. To investigate whether the molecular clock controls the human hair cycle in situ.

Is human HF melanogenesis and melanocyte biology influenced by the molecular clock?

Although human melanocytes in culture have been shown to express clock genes and proteins and that the melanocytes and melanogenesis are tightly coupled to the human hair cycle (Slominski et al. 1994), a functional role for the molecular clock in human pigmentation, specifically HF pigmentation is not known. Therefore the influence of the molecular clock on the complex process of hair pigmentation will be investigated focussing on the following aims and experimental questions:

3. To investigate whether the molecular clock influences melanocyte biology and human pigmentation in situ.

- Is pigmentation controlled by the molecular clock?
- How does the molecular clock control pigmentation? What mechanisms are involved?
- Are these pigmentation effects specific to HF melanocytes or all melanocyte populations?
- Is the 'pigmentation clock' activity specific to melanocytes or an interaction with the niche environment?

Do thyroid hormones influence clock gene expression in the human HF?

Thyroid hormones have been demonstrated to influence both hair growth and pigmentation *in situ*. Furthermore, thyroid hormones, specifically thyroxine, are expressed in a circadian pattern with a thyroidectomy leading to clock dysfunction. Despite this, it is unknown whether thyroid hormones influence clock gene expression thereby prolonging anagen and increasing pigmentation. Therefore, if the molecular clock does have a distinct role in HF biology we will aim to uncover potential, clinically viable modulators of clock activity in the human HF. As many hormones are currently used in clinical therapies and as there is a strong link with hormones and circadian clock modulation, hormones may be ideal for therapeutic modulation of HF biology (Mitchell et al. 2009; Dardente et al. 2014).

4. To investigate whether hormonal modulators of the human hair cycle and pigmentation influence clock gene activity.

- Does short term T4 treatment influence clock gene and protein expression in human HFs *in situ*?
- How is circadian rhythmicity of clock genes affected by T4 treatment?
- How is clock gene and protein expression affected by longer cultures with T4?
- Do the stem cells found in the HF bulge express clock genes and is their expression influenced by T4?

2.6 Experimental design

To investigate '*whether the human HF has functional, oscillatory clock activity molecular clock activity',* cultured anagen and catagen HFs were assessed for clock proteins using immunofluorescence and the immunoreactivity was assessed between both cycle stages. Utilising immunofluorescence and quantitative immunohistomorphometry not only can the presence of clock proteins be documented; the localisation within the HF could be assessed. Following this, qRT-PCR was utilised on synchronised HFs to assess whether rhythmic clock gene expression was maintained over 24 and 48 hours. Finally siRNA mediated gene knock-down was used on cultured human HFs to assess whether the molecular clock had a functional role in modulating hair cycle stage *in situ.*

To investigate '*whether the molecular clock influences melanocyte biology and human pigmentation in situ',* siRNA mediated gene knock-down was again utilised to assess the functional role of the molecular clock in melanocyte biology and melanogenesis. Using a histochemical stain for melanin content (Masson-Fontana) allowed any change in melanin production to be observed. Following this high-resolution light microscopy (HRLM) was used to determine whether any difference in melanosome number and therefore the capacity for melanocytes to produce melanocytes was assessed. Following this, changes in melanocyte number and dendricity was assessed using gp100 as an immunohistochemical marker. Subsequently an enzymatic activity assay was utilised to investigate whether the rate-limiting step of melanogenesis was influenced by clock gene silencing. Quantitative-PCR was used to further support changes observed in gp100, key enzymes in melanogenesis (TYRP1, 2) and master regulator MITF. Following this, to gain a mechanistic understanding as to how the molecular clock influence pigmentation the activation status of MITF was assessed by quantitative immunohistomorphometry. Finally, to conclude these experiments skin and primary melanocyte culture was used to assess if the observed differences in pigmentation were specific to HF melanocytes or a feature of all melanocyte populations and whether these differences related to an intrinsic melanocyte clock, or are a feature of the surrounding niche, i.e. the hair follicle pigmentary unit (HFPU).

To investigate ‘whether hormonal modulators of the human hair cycle and pigmentation influence clock gene activity’, human HFs were cultured with thyroxine for 24 hours and 6 days and immunohistomorphometry and qRT-PCR was utilised to identify whether clock gene and protein levels were altered by thyroxine in both short-term (6/24 hours) culture are longer cultures (6 days). Following this, rhythmic changes in clock gene expression was assessed by qRT-PCR.

Chapter 3: Material and methods

3.1 Tissue culture methodologies.

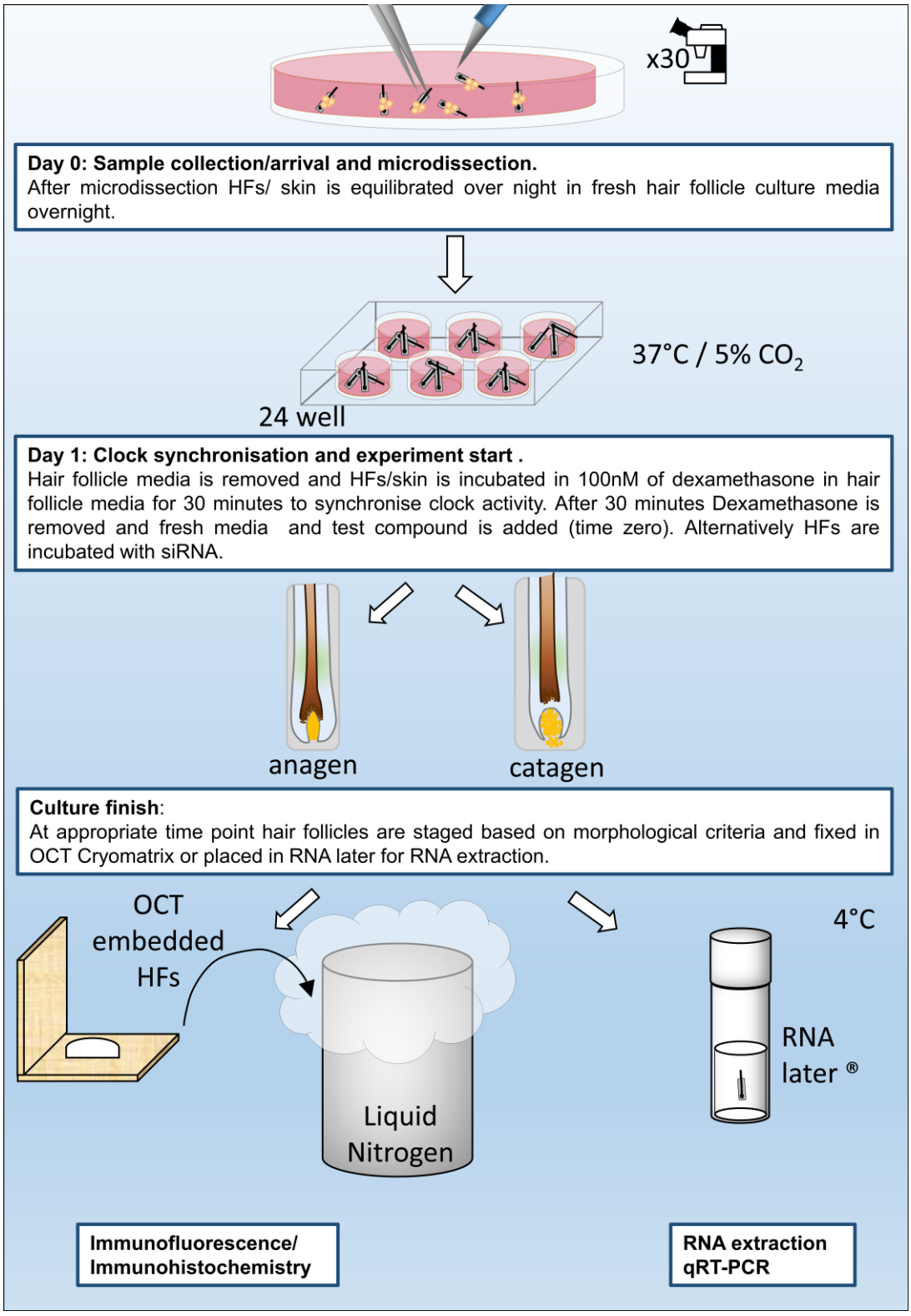

Fig 3.1: Basic experimental workflow.
A general overview of the research process used in this thesis. Additional details on each step are described below.

3.1.1 Tissue collection and ethics

Occipital scalp hair follicles (HFs) from hair transplant surgery were obtained from either the Farjo medical centre (Manchester, UK) or the Crown Cosma Clinic (Manchester, UK). All tissue was obtained with informed patient consent adhering to the 'Declaration of Helsinki Principles' with ethical and institutional approval from the University of Manchester. All samples, slides and biological material were tracked and stored according the 'Human Tissue Act' guidelines' in a HTA licensed freezer.

After collection HFs were stored at 4°C in William's E media (Gibco ®) supplemented with 2 x concentration of Penicillin and Streptomycin, prior to microdissection.

3.1.2 Hair follicle micro-dissection

Human HFs were observed at 30x magnification under a stereo light microscope and dissected from surrounding tissue using forceps and a scalpel. Full length HFs were used to ensure the stem cell containing bulge region was present. Only HFs showing morphological criteria consistent with that of anagen stage VI were using for further experimentation (Müller-Röver et al. 2001; Kloepper et al. 2010).

3.1.3 Hair follicle organ culture

For optimum growth HFs were equilibrated overnight in William's E media (Gibco ®) supplemented with 2 mM of L-Glutamine (Invitrogen, Paisley, UK), 10ng/ml hydrocortisone (Sigma-Aldrich®), 1 µg Penicillin/Streptomycin antibiotic mixture (Gibco®) (referred to as hair follicle media) in a 37° C incubator with 5% CO_2 level. After this equilibration period HFs were subsequently cultured for a period of 1-6 days depending on the experimental conditions required. This supplemented media (hair follicle culture media) was changed every two days as described by Philpott (1990) (Philpott et al. 1990, 1994). Three to five HFs were cultured in each well of a 24-well plate, with the addition of 0.5 ml of HF media per well.

3.1.4 Staging hair follicles

As there is a distinct difference in the genetic and protein profile of hair follicles between each cycle stage it is necessary during HF culture to assess the hair cycle stage of each HF. Hair cycle stage can be assessed qualitatively during culture based on morphological criteria using a Leica EZ4 stereo microscope at 30x magnification (**figure 3.2**). Cycle stage can be quantitatively confirmed by staining for the proliferation marker Ki-67 and the apoptosis marker TUNEL (Kloepper et al. 2010).

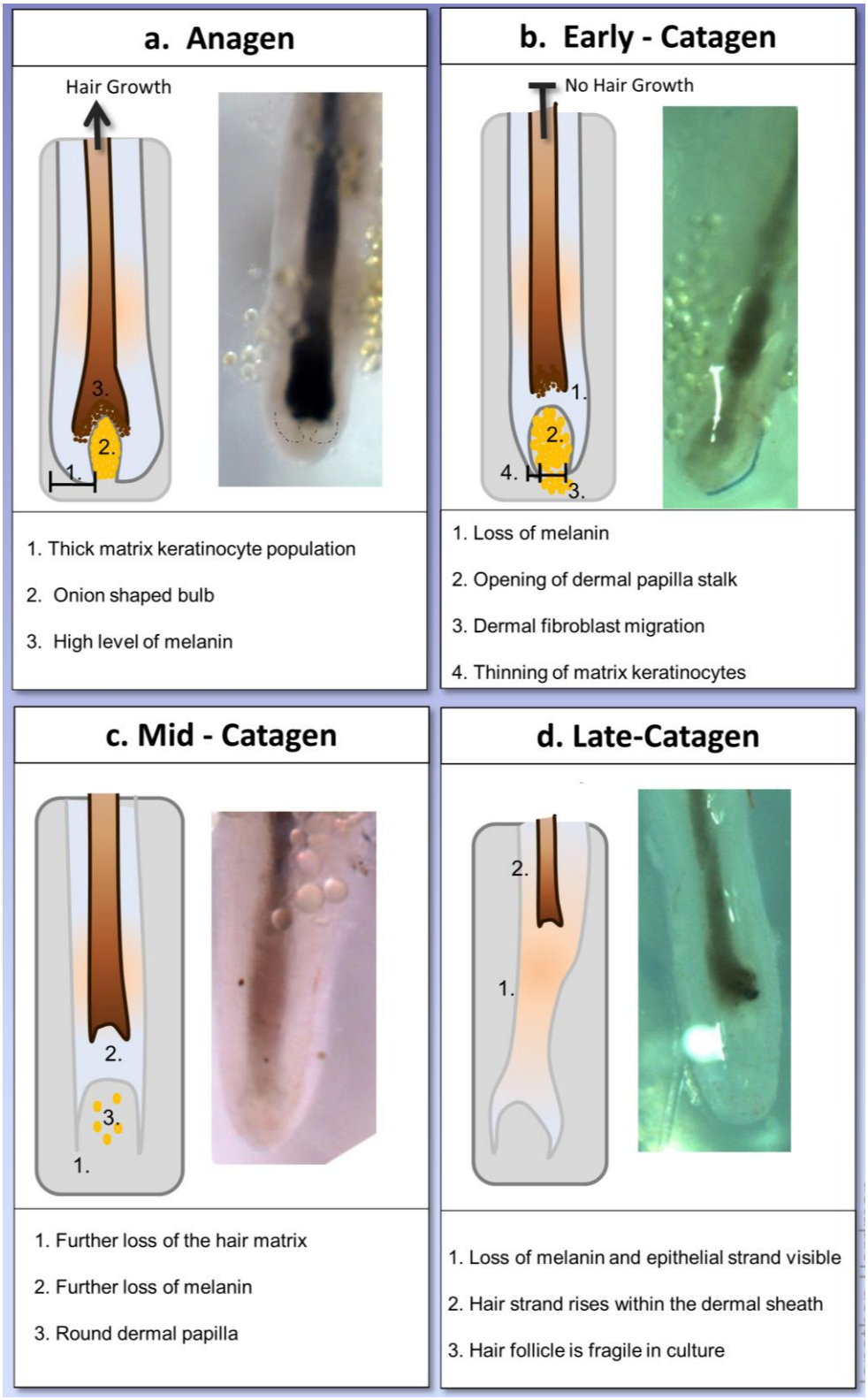

Figure 3.2: Staging the hair cycle of human hair follicles in vitro
Ex vivo human hair follicles will continue cycling *in vivo* with hair growth occurring at rates similar to in vitro. (a.) Anagen is identifiable by the onion shaped dermal papilla and thick hair matrix. (b.) Early catagen is morphologically similar to anagen. It can be identified by the thinning hair matrix and reduction of melanin. (c.) Mid-catagen is recognisable in culture by the round dermal papilla and very thin hair matrix. (d.) Late-catagen is identifiable by the rising hair shaft within the dermal sheath and the epithelial strand that begins to form. This image follows the guidelines described by Kloepper *et al.* 2010.

3.1.5 Hair follicle synchronisation

As clock gene/protein expression oscillates over a 24 hour time period it was necessary to synchronise molecular clock activity in HFs. By synchronising clock activity it can be concluded that any changes in readout parameters observed are due to the culture conditions and not an effect of the follicular molecular clock oscillating out of synchronisation. There are two demonstrated methods to synchronise peripheral molecular clock activity, serum shock (removal of serum for a brief period) and dexamethasone induced synchronisation (Balsalobre et al. 1998; Balsalobre 2000). Following the Philpott method for HF culture (section 3.1.1) (Philpott et al. 1990), HFs are cultured in absence of serum and as such, the glucocorticoid dexamethasone was chosen for synchronisation. After an acclimatisation period (day 0) HFs were incubated for 30 minutes in hair follicle media supplemented with 100 nM dexamethasone (day 1). Following this synchronisation period the media was aspirated and replaced with fresh hair follicle media.

3.1.6 Hormone treatment

For chapter 6, a 50 mg/ml stock of thyroxine was made by diluting thyroxine (Sigma-Aldrich,) in 4 M ammonium hydroxide in methanol as per manufacturer's instructions. HFs were subsequently cultured with 100 nM of thyroxine (T4) diluted in HF media for 6 hours, 24 hours and 6 days with a parallel vehicle control (Tiede et al. 2010; Vidali et al. 2014). For cultures exceeding 24 hours the culture media was replaced every second day. After culturing for either 6 hours, 24 hours or 6 days HFs were either placed in 1 ml of RNAlater for RNA extraction and kept at 4°C or embedded in OCT Cryomatrix and snap frozen in liquid nitrogen for cryosectioning (**Fig. 3.1**). Snap frozen samples were stored at -80°C and stored and registered according the HTA requirements.

3.1.7 Skin organ culture

Four millimetre punch biopsies were taken from discarded abdominal or facial skin from surgical procedures. Punch biopsies were taken on day 0 and floated in hair follicle media in a 6 well plate (**Fig. 3.3**). After incubation over night to acclimate, skin biopsies were used for experimentation following the same protocols as to that of HFs.

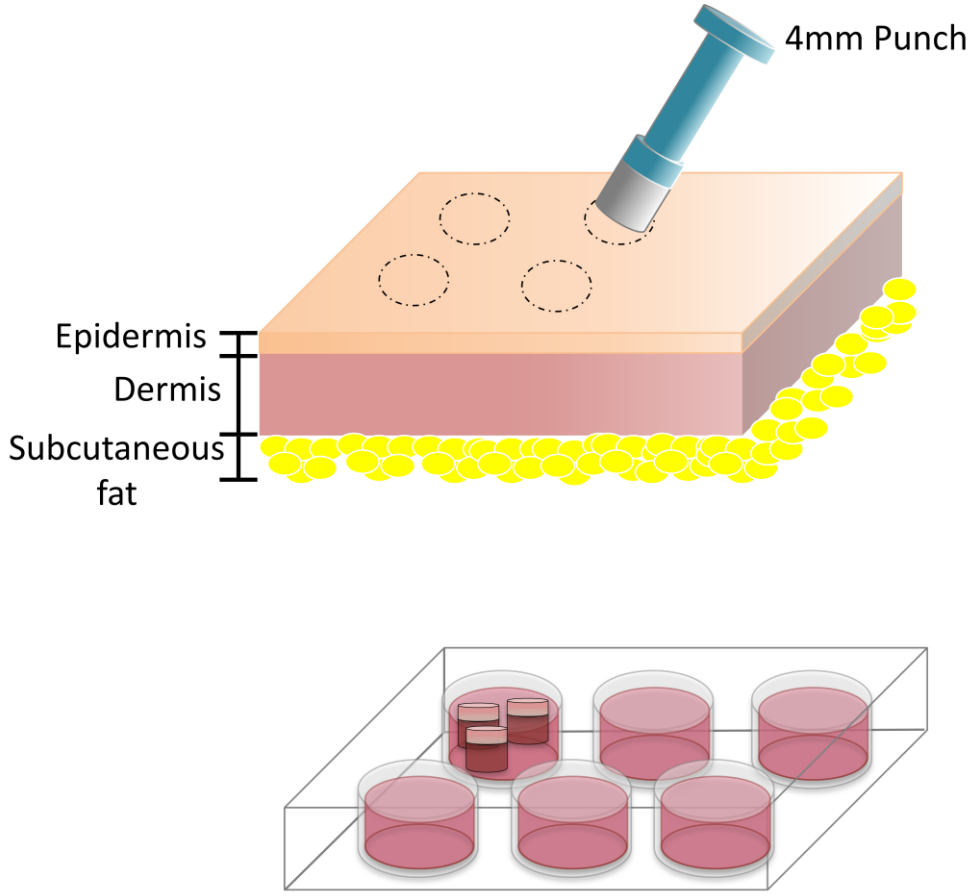

Figure 3.3: Human ex vivo skin organ culture model.
A representation of the human skin culture model used (Lu et al. 2007).

3.1.8 Human primary epidermal melanocyte culture.

Primary human epidermal melanocytes (donated by the University of Bradford), isolated from discarded skin biopsies, were seeded at a density of $1.5x10^6$/ml. Cells were maintained in a cell culture flask in melanocyte growth media (Promocell ®, (Heidelberg, Germany)) supplemented with Anti-Anti (Gibco) antibiotic and antimycotic at 37ºC with 5% CO_2. When cultures appeared 80% confluent they were passaged by incubation with 5 ml Trypsin/EDTA mix (1:1) at 37ºC for 5 minutes. Following this incubation period the flask was gently tapped to dislodge melanocytes and 5 ml of melanocyte growth media was added to neutralise the reaction. The suspended melanocytes were transferred to a 15 ml centrifuge tube and centrifuged for 5 minutes at 3000 RPM to pellet the cells.

The supernatant was gently removed with a pipette and melanocytes were re-suspended in melanocyte growth media. Following this melanocyte density was assessed using a haemocytometer. Melanocytes were re-seeded at a density of $1.5x10^6$/ ml in a cell culture flask or $1x10^4$ /ml per well on chamber slides (Thermo Scientific) for knock-down experiments.

To preserve melanocytes, cells were pelleted by centrifugation at a density of $1x10^6$/ ml and re-suspended in Recovery™ (Gibco) cell freezing media and gradually frozen at a rate of -1°C/minute over 24 hours using a Mr. Frosty™ freezing container (Thermo Scientific) in a -80°C freezer. Melanocytes were subsequently transferred to liquid nitrogen for long-term storage.

3.1.9 Small interfering RNA (siRNA) mediated gene knock-down.

Small interfering RNA mediated gene knock-down was performed on human HFs *in situ* following the methodology as previously described (Chen and Roop 2012). All reagents used for siRNA mediated gene knock-down experiments were purchased from Santa Cruz© (California, USA). To make 1 ml of the transfection mix 6µl of BMAL1 (sc-38165), PER1 (sc-38171) or a scrambled oligonucleotide (oligo) control (sc-37007) was mixed with 6µl of a Santa Cruz© transfection reagent (sc-29528) and made up to a total volume of 200 µl with transfection medium (sc-36868). This transfection mix was incubated in the dark for 30 minutes. During this incubation period HFs were washed with the transfection medium. After the incubation period the transfection mix was added to HFs and incubated at 37ºC (5% CO_2) for 6 hours. After this time the transfection mix was removed from the HFs and replaced with HF culture media (section 3.1.3). After 24 hours 5 HFs were placed in RNAlater® (Ambion, Connecticut, USA) for qRT-PCR or fixed in OCT for cryosectioning. HFs were also removed after 4 days in culture and fixed in OCT. This protocol was also used for gene knock-down on skin punch biopsies. Knock-down efficiency was checked by qRT-PCR and immunofluorescence.

3.1.10 Time series experiments

To assess clock gene rhythmicity over 24 or 48 hours, time series experiments were performed. After overnight incubation hair follicles were synchronised by dexamethasone (3.1.5) and placed in HF culture media. After synchronisation five HFs were placed in RNAlater™ (from control and treated if applicable) which is designated as time zero, the remaining HFs were incubated at 37°C. Subsequently 5 HFs were removed from culture and placed in RNAlater™ every 6 hours for up to 24 or 48 hours for RNA extraction.

3.2 Immunohistochemistry and immunofluorescence

3.2.1 Tissue processing

After experimentation HFs or skin sections were placed in OCT embedding medium (Thermo-scientific (Leicestershire, UK)), snap frozen in liquid nitrogen and stored at -80ºC (3 follicles per block). Subsequently 5µm thin cryosections of OCT embedded HFs or skin sections were cut using a cryostat (OTF5000, Bright (London, UK)) and fixed to microscope slides (Superfrost+, Thermo-scientific).

3.2.2 Sample preparation for high-resolution light microscopy (HRLM)

HFs were fixed at 4°C in half strength Karnovsky's fixative (2 g paraformaldehyde, 500µl of 1 M Sodium hydroxide, 5 ml of 50% gluteraldehyde and 20 ml of 0.2 M sodium cacodylate buffer) for 3 hours and placed in 2% Osmium tetroxide (in water) for 1 hour at 4°C. HFs were subsequently washed in 0.1M Sodium Cacodylate buffer and then dehydrated in ethanol. Finally HFs were embedded in a mix of propylene oxide/araldite (2:1 for 1 hour, 1:1 for 3 hours, 1:2 overnight and finally 100% araldite) then placed at 60°C for 3 days (Magerl et al. 2001; Peters et al. 2002; Bodó et al. 2007).

Once fixation was complete semi-thin (0.5µm) sections were cut on an ultra-tome (Reichert Jung Ultra cut-e, (Austria)) and counterstained with Toluidine-blue (5% in water) for 10seconds on a heated plate (approximately 60°C) (Magerl et al. 2001; Peters et al. 2002; Bodó et al. 2007).

3.2.3 Immunofluorescence protocols.

All immunofluorescence experiments were performed in a humidified chamber protected from light following the subsequent basic methodology. Additional details specific to each protocol are detailed in **Table 3.1**.

HF or skin cryosections were dried at room temperature for ten minutes and fixed at -20ºC in the appropriate fixative. Subsequently cryosections were washed and incubated in a blocking buffer for

30 minutes at room temperature in a humidified chamber. Following this cryosections were left over night at 4ºC. On the second day cryosections were washed and incubated with an Alexa Fluor® (life technologies, Paisley, UK) secondary antibody. Finally cryosections were washed and counterstained for 2 minutes with DAPI 4', 6-diamidino-2-phenylindole (DAPI) (1:1000 in phosphate buffered saline (PBS) and mounted in faramount mounting media (DAKO© Cambridge, UK) with a coverslip. Once dried, slides were kept at -20ºC. All HFs were imaged using a Biozero 8000 fluorescence microscope (Keyence, Osaka, Japan).

3.2.4 Melanin histochemistry (Masson-Fontana)

To stain the melanin content of HFs or skin, cryosections were fixed at -20°C in ethanol/acetic acid mix (2:1), washed in TBS and rinsed in dH_2O. Following this cryosections were placed in a 10% silver nitrate solution (in water) containing 1ml of ammonium hydroxide for 3 minutes in a microwave (low temperature). Cryosections were washed again in TBS and placed in 5% sodium thiosulphate solution in dH_2O. Finally cryosections were dehydrated in ethanol and placed in xylene before mounting (Kloepper et al. 2010; Samuelov et al. 2013).

3.2.5 PER1/BMAL1 double-immunostaining with cytokeratin 15 (K15).

For PER1 immunostaining cryosections were fixed in acetone for 10 minutes at -20°C. Subsequently sections were washed in Phosphate buffered saline (PBS) and pre-incubated in 10% normal goat serum (NGS) for 30 minutes at room temperature in a humidified chamber. Antibodies were diluted in PBS containing 10% NGS and PER1 (Rabbit-anti- PER1, table 3.1) diluted to 1:100 with K15 diluted to 1:200 (Mouse anti-K15, Abcam® (Cambridge, UK]). 15µl aliquots of the antibody mix was applied to cryosections and left at 4°C overnight. PER1 immunostains were optimised using human pineal gland, HaCat cells and human skin. Subsequently human skin was used in all experiments as a positive control and omission of the primary antibody as a negative control.

For BMAL1 immunostaining cryosections were fixed in methanol for 10 minutes at -20°C. Subsequently sections were washed in PBS and pre-incubated in 10% normal goat serum (NGS)

containing 1% bovine serum albumin (Sigma-Aldrich ® Dorset, UK) and 1% triton X (Sigma-Aldrich®) for 30 minutes at room temperature in a humidified chamber. Antibodies were diluted in PBS containing 10% NGS and BMAL1 (Rabbit-anti- BMAl1, table 3.1) diluted to 1:50 with K15 diluted to 1:200 (Mouse anti-K15, Abcam® (Cambridge, UK]). 15µl aliquots of the antibody mix was applied to cryosections and left at 4°C overnight. BMAL1 immunostaining was optimised using human pineal gland and skin. Subsequently human skin was in each experiment as a positive control and omission of the primary antibody as a negative control.

On day 2 for both BMAL1 and PER1 stains the same protocol was followed. First sections were washed in PBS and incubated with Alexa Fluor® goat-anti-rabbit 488 (Life technologies Ltd) for 45 minutes in a humidified chamber. Subsequently sections were washed in PBS and incubated for a second time with Alexa Fluor® goat-anti-mouse 594 for a further 45 minutes. Following this sections were washed again in PBS and counterstained with DAPI (1:1000 in PBS) and mounted with faramount aqueous mounting media.

3.2.5 Ki-67 - TUNEL immunohistochemistry for proliferation and apoptosis

To stain for apoptotic and proliferating cells the protocol as described by Kloepper *et al.* (2010) was followed. Sections were first dried at room temperature for 10 minutes and fixed in 4% para-formaldehyde in PBS (Sigma-Aldrich®). Sections were then washed in PBS and post-fixed in ethanol mixed with acetic acid (2:1) (both from Fisher Scientific UK Ltd, Loughborough, and UK). Samples were again washed and bathed in equilibration buffer (Apoptag ® TDT enzyme kit, Millipore©, Darmstadt, Germany) for 10 minutes. Following this, sections were incubated with the TDT enzyme in reaction buffer at 37°C for 1 hour (70%:30% respectively) (supplied in the Apoptag® kit). After this period the provided stop buffer was added for 10 minutes at 37°C. Samples were again washed and incubated with 10% NGS in PBS for 20 minutes. Sections were then incubated with ki-67 antibody (mouse anti- human Ki-67 (1:20) Dako Ltd, Cambridgeshire, UK) diluted in 10% NGS at 4°C overnight at 4°C. On day 2 samples were washed and incubated for 30 minutes at room temperature with a fluorescein-labelled anti-digoxigenin antibody (48% blocking

buffer with 52% secondary antibody) (Apoptag kit ®). After this period samples were washed in PBS and subsequently incubated with goat-anti- mouse Alexa Fluor® 594 diluted (1:200) in 2% NGS in PBS. Finally samples were washed with PBS, counterstained with DAPI (1:1000 in PBS) and mounted in faramount aqueous mounting media (DAKO Ltd) with a coverslip. (Kloepper et al. 2010)

3.2.6 Enzymatic activity for tyrosinase activity.

Frozen HF cryosections were stained for tyrosinase activity following the protocol as first described by Han *et.al* (2002) (Han et al. 2002). First HF cryosections were dried at room temperature for 10 minutes then fixed at -20°C in a 1:1 mix of methanol and acetone. Sections were subsequently washed for 5 minutes in PBS and then endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide (Thermo-scientific) diluted in PBS. Following this sections were pre-treated with avidin and biotin for 15 minutes each at room temperature using reagents from the vectastain ® avidin/biotin blocking kit (Vector laboratories, Peterborough, UK) to block endogenous activity. Next sections were incubated in 5% bovine serum albumin (BSA) (Thermo-scientific) diluted in PBS for 30 minutes at room temperature. After this sections were treated with the Biotinyl tyramide signal amplification reagent (TSA®) diluted in the supplied amplification buffer (1:50) from the Perkin and Elmer (Boston, USA) TSA® kit, as per the manufacturer's instructions, for 15 minutes. Subsequently sections were washed in PBS then treated with a streptavidin-conjugated Alexa Fluor™ 594 (Life technologies Ltd) at a dilution of 1:600 in PBS for 1 hour at room temperature. Finally sections were washed in PBS, counterstained for 1 minute with DAPI and mounted with a coverslip using farmount aqueous mounting media (DAKO Ltd).(Han et al. 2002; Kloepper et al. 2010; Samuelov et al. 2013)

Protein target	Fixative	Wash buffer	Controls	Blocking buffer	Primary antibody	Secondary antibody	Additional Comments
PER1 *(PER1-2A , Alpha diagnostics)*	Acetone	PBS	*Positive control:* Human skin *Negative control:* Omission of primary antibody	10% NGS in PBS	Rabbit anti-human PER1 1:200 dilution in 10% NGS	Goat anti-rabbit Alexa Fluor® 488 (1:200 in PBS)	
BMAL1 *(MOP31 [N1N3], Genetex, (California, USA))*	Methanol (1:1)	PBS	*Positive control:* Human skin *Negative control:* Omission of primary antibody	10% NGS in PBS with 1% BSA 0.1% triton x-100 (Sigma-Aldrich®)	Rabbit anti-human BMAL1 1:50 dilution in 10% NGS	Goat anti-rabbit Alexa Fluor® 594 (red) (1:200 in PBS)	Blocking step is 45 minutes
gp100 *(ab137708, Abcam® (Cambridge, UK))*	Acetone	PBS	*Positive control:* Human skin and fixed HF melanocytes *Negative control:* Omission of primary antibody	-	Rabbit anti-human gp100 1:200 dilution in 10% NGS	Goat anti-rabbit Alexa Fluor® 488 (1:200 in PBS)	As described by (Singh et al. 2010; Samuelov et al. 2013)

MITF *(Av37977* *Sigma-Aldrich®)*	Acetone/Methanol (1:1)	TBS	*Positive control:* Human skin and fixed HF melanocytes *Negative control:* Omission of primary	10% NGS in TBS	Rabbit Anti-human MITF 1:50 dilution in 10% NGS in TBS	Goat anti-rabbit Alexa Fluor® 594 (1:200 in TBS)	Primary antibody was incubated at room temperature over night
MITF-P *(SAB4503940* *Sigma-Aldrich®)*	Acetone/Methanol (1:1)	TBS	*Positive control:* Human skin and fixed HF melanocytes *Negative control:* Omission of primary	10% NGS in TBS	Rabbit Anti-human phospho-MITF(pSer180/79)	Goat anti-rabbit Alexa Fluor® 594 (1:200 in TBS)	Primary antibody was incubated at room temperature over night

Table 3.1: Immunofluorescence protocols.
Table 3.1 describes the protocols followed for Period 1 (PER1), BMAL1, gp100, Microphthalmia-associated transcription factor (MITF) (and phosphorylated MITF). All samples were washed in either phosphate-buffered saline (PBS) or tris-buffered saline (TBS). Samples were incubated in 10% normal goat serum (NGS) in either PBS or TBS where indicated. For BMAL1 1% bovine serum albumin (BSA) was also added to the pre-incubation step.

3.3 Quantitative immunohistomorphometry

3.3.1 PER1 and BMAL1 expression in the human HF.

To quantify the immunoreactivity of PER1 and BMAL1 on human HFs, a standard reference area was used on images taken at the same magnification. The exposure time was adjusted based on the negative control to eliminate any autofluorescence and the positive control was used to confirm a true staining pattern. Once set, the exposure time was not changed throughout the experiment. Where possible all images were taken in the same sitting. To measure the immunoreactivity three boxes of defined sizes were chosen to encompass maximum immunoreactivity of the bulb region and standardise this measurement between images and patients (**Fig. 3.4a**). When measuring the immunoreactivity in the bulge region, K15, a marker for the bulge stem cells was used to verify the location. A set reference area was then used to measure the immunoreactivity in this region.

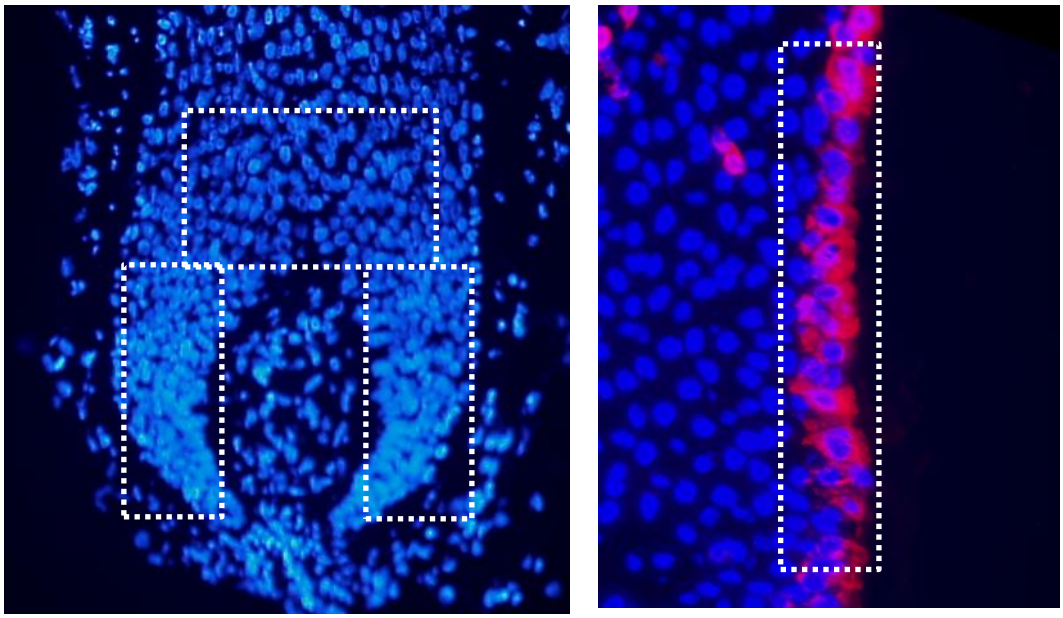

Fig. 3.4: Standardized references areas used for immunohistomorphometry.
(a) For standardisation three boxes were used in the bulb area (400x1000 pixels for left and right, 1000x500 for upper box). When analysing immunoreactivity the DAPI counterstain channel was not used. (b) When quantifying the immunoreactivity in the bulge area, the marker K15 for the bulge stem cells was used to confirm the bulge region then a reference box was used to quantify immunoreactivity (100x1000 pixels).

3.3.2 Ki-67/TUNEL stain for proliferative and apoptotic cells.

When quantitatively assessing the number of proliferative (ki-67 positive) cells and apoptotic cells (TUNEL positive) the methodology as described by Kloepper *et.al* (2010) was followed. The number of Ki-67 and TUNEL positive cells below Auber's line (the widest part of the DP) (**Fig. 3.5)** was counted. The percentage of proliferative and apoptotic cells was calculated against the total number of DAPI+ nuclei below this line (Kloepper et al. 2010).

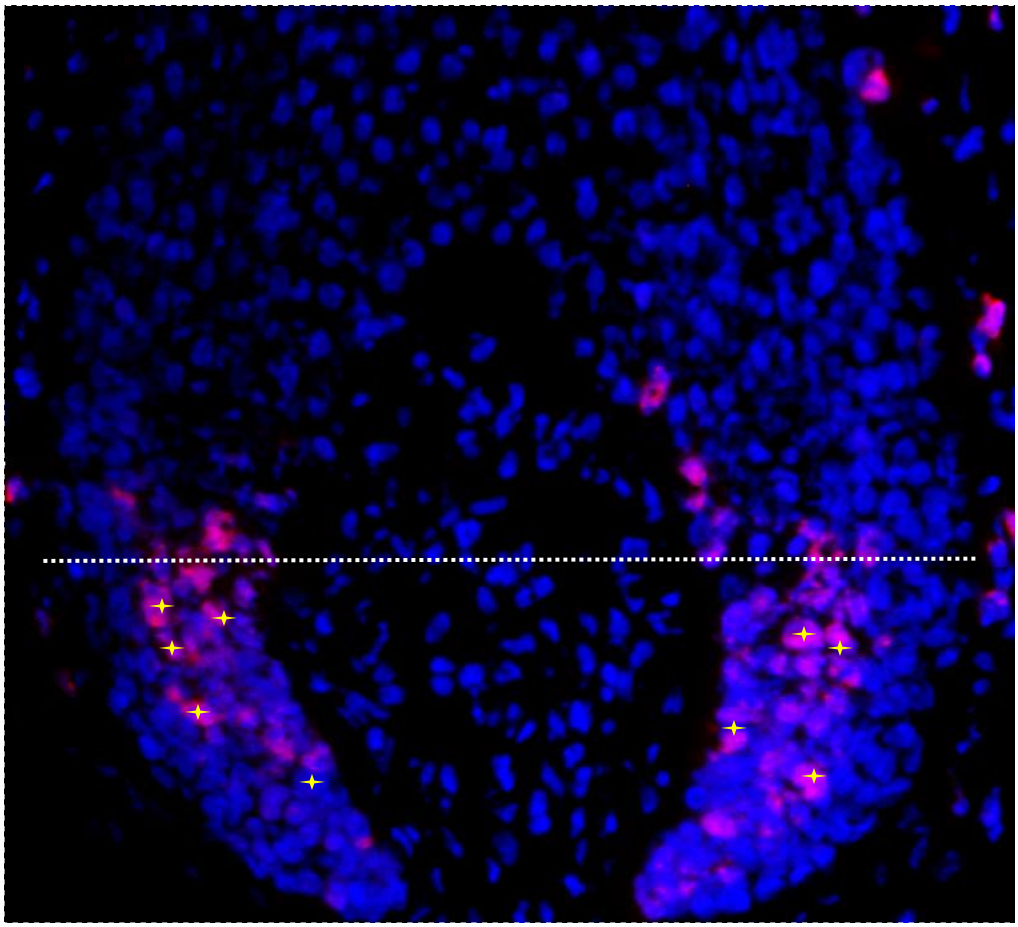

Fig. 3.5: Analysis of proliferative and apoptotic cells in the HF bulb.
The number of proliferative cells (Red) and apoptotic cells (green) were counted below Auber's line, the widest part of the HF as described by Kloepper *et. al* (2010).

3.3.3 Assessment of melanin content (Masson-Fontana).

To assess the melanin content of HFs the established methodology described by Kloepper *et.al* (2010) was used. In this methodology HFs stained for melanin content were imported into image J (NIH, MD USA). Using this software the image was converted to 8-bit and the image colours were inverted (Fig. 3.6). This allowed the melanin content to be observed with little interference from the haematoxylin counterstain. Once inverted, a standardized box size was defined incorporating the HFPU. The box size was kept constant for all images and all patients. (Kloepper et al. 2010; Samuelov et al. 2013)

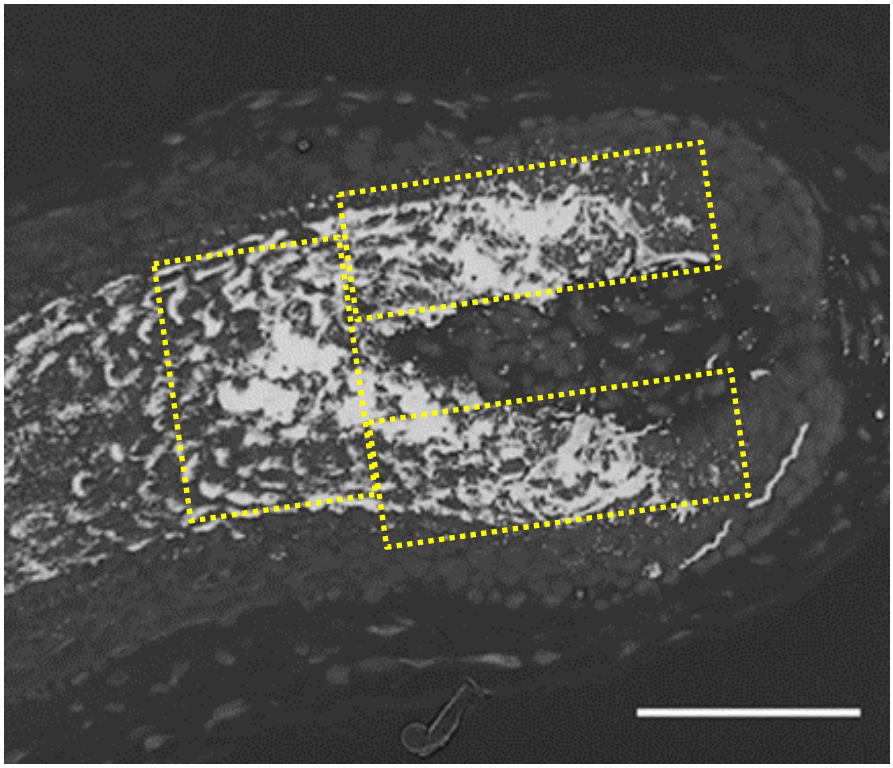

Fig 3.6: Analysing HF melanin content.
To analyse HF melanin content, the image colours were inverted and a standardised reference area incorporating the HFPU was defined (Kloepper et al. 2010; Samuelov et al. 2013).

3.3.4 Melanosome number.

To analyse melanosome number, semi-thins (0.5µm) sections taken at 1000x resolution were used which allowed their visualisation. When counting the melanosome number, a small reference area (**Fig. 3.7**) was chosen within the melanocytes or keratinocytes. This reference area was used in three different locations per melanocyte/keratinocyte to get an average number of melanosomes per cell. Using a set reference area reduced the likelihood of getting any anomalous readings that may have arose from the plane of sectioning which may have meant some melanocytes were much larger than others, thereby skewing the dataset (Magerl et al. 2001).

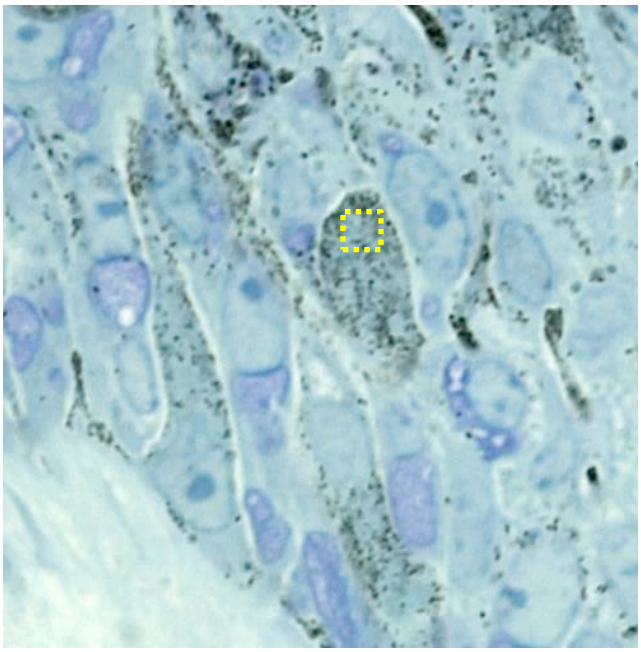

Fig. 3.7: Counting melanosome numbers.
To assess the changes in melanosome number, a small box was used as a reference area in each melanocyte. This box was used to measure the melanosome number three times per cell to get an average melanosome number (Magerl et al. 2001).

3.3.5 Assessment of immunoreactivity in the HFPU.

For immunofluorescence experiments assessing the HFPU (gp100, tyrosinase, MITF and MITF-P), the same reference areas as used for the melanin content (section 3.3.3) was used. This was chosen as these dimensions incorporate the entire HFPU including the melanocytes which are found adjacent to the DP. However, when imported into image J, these images were not inverted.

3.4 Quantitative- real time PCR (qRT-PCR)

3.4.1 Total-RNA extraction

Total RNA was extracted from HFs or skin biopsies using the RNeasy® microkit (Qiagen, Manchester, UK) according to the manufacturer's instructions. Tissue was removed from RNAlater™ and placed in RLT buffer (RNeasy® microkit) containing 0.1% β-mercaptoethanol (Sigma-Aldrich®). HFs/skin was disrupted with a pestle and homogenised (T-10 basic Ultra-turrax (IKA, Staufen, Germany)). Samples were subsequently centrifuged at maximum speed (13,000 RPM) for 30 seconds and the supernatant collected and mixed with 70% ethanol diluted in dH_2O to precipitate. The precipitated RNA was placed in an RNA binding column on top of a collection tube. Once bound the RNA was washed with 350 µl of RPE buffer from the RNeasy® microkit and centrifuged at maximum for 15 seconds. This was followed by the addition of 500 µl of RW1 buffer (RNeasy® micro kit), which was again centrifuged at maximum speed for 15 seconds. Next, 500 µl of 80% ethanol (in dH_2O) was added and the collection tube centrifuged at maximum for 2 minutes. In between each centrifugation step the eluent was disposed of and the collection tube reused. After the wash steps the binding column was dried by centrifugation for 5 minutes and RNA was eluted from the column with 15µl of dH_2O. Total RNA concentration was subsequently determined using a nanodrop (Thermo-scientific).

3.4.2 Reverse transcription

Complimentary DNA (cDNA) was reverse transcribed using the Tetro® cDNA synthesis kit (Bioline, London, UK). A mix of random oligonucleotides $(dT)_{18}$ (1µl), 10mM of a dNTP mix (1µl), 4µl of Reverse transcription buffer (5x concentrated, from kit) a further 1µl of RNase inhibitor and 1µl of Tetro Reverse transcriptase (200U/µl) making a total of 8µl per reaction. The extracted mRNA was diluted with DEPC-treated dH_2O to a concentration of 100ng/µl up to a total volume of 20µl. The samples were heated at 45°C for 30 minutes followed by 5 minutes at 85°C to terminate the reaction before cooling to 4°C. Samples were stored at -20°C.

3.4.3 Quantitative RT-PCR (qRT-PCR)

Quantitative RT-PCR was performed using the StepOnePlus™ system and then analysed using the comparative CT method. Before data generation the housekeeping genes *PPIA* and *GAPDH* expression levels were compared between control and treated samples and the gene showing the least amount of variation between samples was used for quantification.

For each reaction a total volume of 20µl per reaction was obtained by adding 10µl of Taqman® Fast Advance master mix (Life technologies Ltd, Paisley, UK), 5µl of nuclease free water, 1µl of a TaqMan probe set specific to the gene of interest (Table 3.2) and an additional 4µl of cDNA diluted in nuclease free water to a concentration of 5ng/µl. Quantitative-RT PCR experiments were performed using the thermocycling conditions as described in figure 3.4.

In every experiment, each gene and cDNA sample was performed in triplicate and experiments were performed on a single plate to reduce experimental error. Furthermore, each experiment was performed with a no transcript control (NTC) and a no amplification control (NAC). The NAC contained a reverse-transcription PCR product, which contained no reverse transcriptase and was used to check for genomic contamination in the experiment.

Relative expression was determined using the delta CT or delta delta CT method against PPIA, the gene that showed the least variation in all experiments, and results were confirmed against GAPDH. Relative expression was determined using Microsoft Excel™ and statistical analysis was performed using Graphpad prism® using the appropriate statistical test for each experiment.

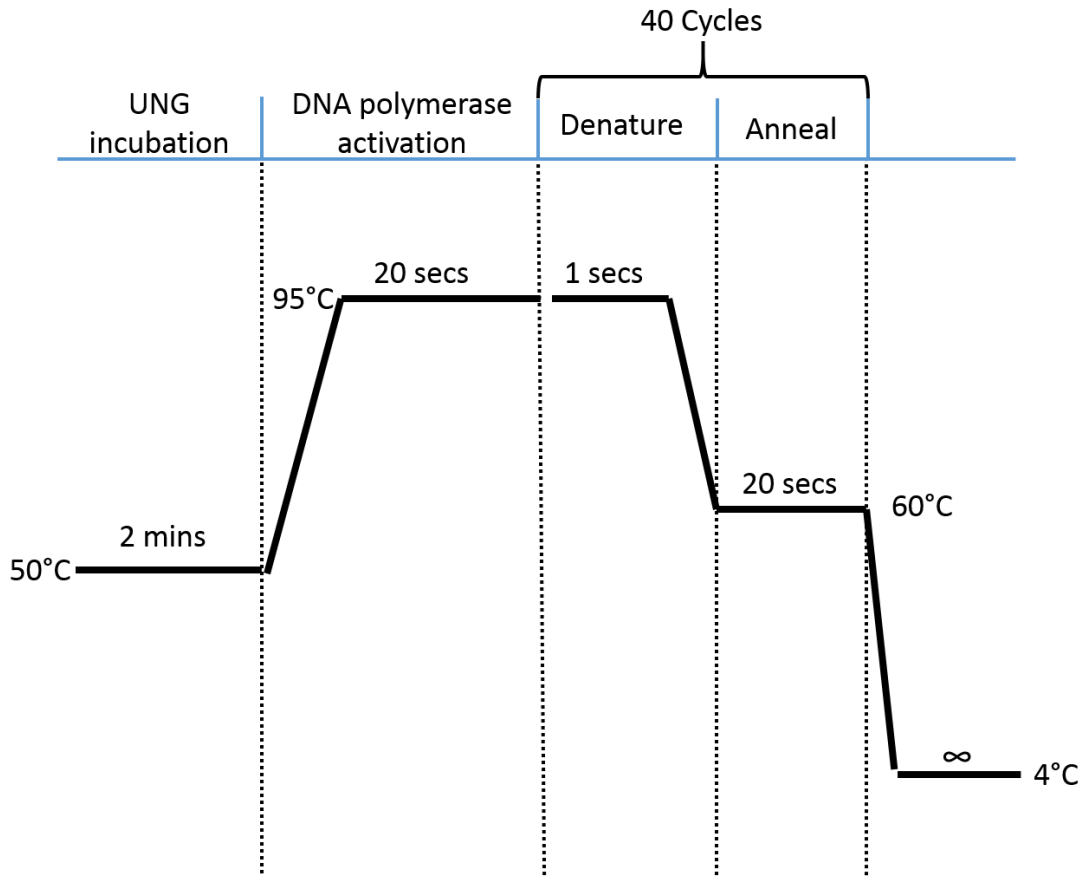

Figure 3.4: StepOnePlus™ thermocycler conditions.

Gene	Taqman® assay ID	Full gene name
PPIA	Hs99999904_m1	Peptidylprolyl isomerase A
GAPDH	Hs02758991_g1	Glyceraldehyde 3-phosphate dehydrogenase
GP100	Hs00173854_m1	Premelanosome protein (PMEL)
TYR	Hs00165976_m1	Tyrosinase
TYRP1	Hs00167051_m1	Tyrosinase related protein -1
TYRP2	Hs01098278_m1	Tyrosinase related protein-2
MITF	Hs01117294_m1	Microphthalmia-associated transcription factor
BMAL1	Hs00154147_m1	aryl hydrocarbon receptor nuclear translocator-like
PER1	Hs00242988_m1	Period homolog 1 (Drosophila)
CRY1	Hs01597804_m1	Cryptochrome-1
CLOCK	Hs00231857_m1	clock homolog (mouse)
CDKN1a	Hs00355782_m1	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
c-Myc	Hs00905030_m1	v-myc myelocytomatosis viral oncogene homolog (avian)
NR1D1	Hs00897531_m1	Nuclear receptor subfamily 1, group D, member 1

Table 3.2: Taqman® primer/probes used in qRT-PCR reactions

Chapter 4: A meeting of two chronobiological systems: circadian proteins Period1 and BMAL1 modulate the human hair cycle clock

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4.1 Authors

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Candidate's contribution

The candidate was involved in continuing and supplementing the research performed and initiated by Yusur Al-Nuaimi. The candidate was involved in performing the following experiments:

- 48 hour time course experiments (Figure 4.1)
- Establishing BMAL1 immunofluorescence and anagen catagen cultures related to BMAL1 (Figure 4.2a, b), performed all BMAL1 immunofluorescence experiments
- Change in PER1 immunofluorescence over 6 hours (Figure 4.2 f)
- Change in PER1 in anagen and catagen at 2 time points (Figure 4.3c.)
- Performed BMAL1 knock-down experiments with YA
- Subsequently performed all additional experiments with BMAL1 including staging, knock-down efficiency analysis (S4.4 c,d) and BMAL1 staging (Figure 4.5)

The candidate was also involved substantially in the writing of the manuscript, preparation of all images and with editing and performing the additional experiments and analysis requested during three rounds of revision, prior to acceptance.

Authors' contribution

YA- Was the initial author of this project and was involved in much of the experimental work, design and analysis and providing supervisory support throughout the project

TB and **BT** – Performed qRT-PCR for the data in Figure 4.3 and were involved in correcting the manuscript text.

IH – Was involved in the 24 hour time course experiments, RNA extraction for the PER1 knock-down experiments and performed the ki-67/TUNEL experiments for the PER1 knock-down experiments. IH was also involved in a co-supervisory function as well as in correcting and co-editing numerous versions of the manuscript.

MP – Performed the CLOCK *in situ* hybridisation experiment (Figure 2 g)

NF & BF– Provided human scalp hair follicles for experimentation

REBW- provided supervisory support to Y Al-Nuaimi and corrected manuscript text.

GB – Provided supervisory support to both JA Hardman and Y Al-Nuaimi

BG – Provided expertise in chronobiology suggesting essential experiments and edited the manuscript text.

JK – Acted as second reader for hair follicle staging and performed one of the PER1 knock-down experiments. J Kloepper also supervised Y Al-Nuaimi

RP- Acted as the main supervisor to this project designing the experimental strategy and contributing substantially to data interpretation and manuscript writing, edited the manuscript, and guided the revision strategy.

4.2 Abstract

The hair follicle (HF) is a continuously remodelled mini-organ that cycles 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 permits the study of the unknown controls of autonomous, rhythmic tissue remodelling of the HF which intersects developmental, chronobiological and growth-regulatory mechanisms. The hypothesis, that the peripheral clock system is involved in the hair cycle transition, i.e. the anagen-to-catagen transformation, was tested. 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*), with Period1 expression being hair cycle-dependent. Knock-down of either *BMAL1* or *Period1* in human anagen HFs significantly prolonged. This provides the first evidence that peripheral core clock genes modulate human HF cycling, and are an integral component of the human hair cycle clock. Specifically, our study identifies BMAL1 and Period1 as novel therapeutic targets for modulating human hair growth.

4.3 Introduction

The hair follicle (HF) is a highly dynamic mini-organ that undergoes a cyclical remodelling process, called the hair cycle (Kligman 1959; Paus et al. 1999b; Stenn and Paus 2001; Schneider et al. 2009). In the hair cycle the HF cyclically undergoes massive cell death and subsequently regenerates owing to its rich endowment with various stem cell populations (Lavker et al. 2003; Cotsarelis 2006b; Fuchs 2009; Plikus et al. 2011, 2012; Plikus 2012). It 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. 2.3**). 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 (Stenn and Paus 2001; Halloy et al. 2002; Al-Nuaimi et al. 2010, 2012; Murray et al. 2012).

While numerous molecular players are known to impact on HF cycling, the basic controls of this oscillatory mechanism ("hair cycle clock") remain unknown (Paus and Foitzik 2004; Plikus et al. 2008; Lin et al. 2009; Schneider et al. 2009). Investigating these controls is of major clinical relevance as the vast majority of hair growth disorders can be attributed to altered HF cycling; particularly the anagen-catagen transition (Paus and Cotsarelis 1999; Cotsarelis and Millar 2001; Paus and Foitzik 2004; Arck et al. 2006; Paus 2006; Lu et al. 2007; Schneider et al. 2009).

There is growing consensus that the regulatory mechanisms governing the human hair cycle are based on an intra-follicular oscillatory system (Robinson et al. 1997; Paus and Foitzik 2004; Kwon et al. 2006; Lin et al. 2009; Al-Nuaimi et al. 2010; Plikus et al. 2013). One such candidate is the circadian clock, a molecular oscillatory system with a 24-hour periodicity **(Fig. 2.3)**. 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, although the exact mechanisms of this synchronisation are not fully understood (Schibler and Sassone-Corsi 2002; Lowrey and Takahashi 2004; Dardente and Cermakian 2007; Bass 2012; Brown et al. 2012; Feng and Lazar 2012; Ota et al. 2012; Plikus et al. 2013). With the recent focus on peripheral clock activity on tissue functions, separate from the SCN, chronobiology research has entered into the field of peripheral tissue physiology (Nagoshi et al. 2004; Dardente and Cermakian 2007; Spörl et al. 2011; Albrecht 2012; Ota et al. 2012; Tonsfeldt and Chappell 2012). Since clock dysfunction may cause tissue pathology (Lee 2005; Chen-Goodspeed and Lee 2007; Takahashi et al. 2008; Sahar and Sassone-Corsi 2009; Geyfman and Andersen 2010; Geyfman et al. 2012b; Lee et al. 2013; Takita et al. 2013; Chen et al. 2014) 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; Tanioka et al. 2009; Spörl et al. 2011; Plikus et al. 2013), and murine and human skin express clock genes (Zanello et al. 2000), the molecular clock and/or clock-controlled genes (CCGs) may be implicated in human hair growth/cycle control **(Fig 2.4)** (Lin et al. 2009; Geyfman and Andersen 2010; Geyfman et al. 2012b), particularly as deletion of core clock genes delayed anagen onset (Lin et al. 2009). In addition, clock genes impact the cell cycle activity, and apoptotic genes (Fu et al. 2002; Matsuo et al. 2003; Lee 2005; Chen-Goodspeed and Lee 2007; Takahashi et al. 2008; Sahar and Sassone-Corsi 2009; Geyfman et al. 2012b), key processes during HF cycling (Al-Nuaimi *et al.*, 2012; Paus and Foitzik, 2004; Schneider *et al.*, 2009; Stenn and Paus, 2001). Furthermore, Clock genes co-ordinate the activation of murine HF stem cells (Janich et al. 2011). Finally, plucked scalp hair shafts also permit one to study the human peripheral circadian clock.

On this basis, we hypothesised that clock genes, (Lowrey and Takahashi 2004, 2011; Lee 2005; Dardente and Cermakian 2007; Saini et al. 2011; Spörl et al. 2011; Albrecht 2012; Bass 2012;

Tonsfeldt and Chappell 2012) may function as molecular components of the human “hair cycle clock” (Paus and Foitzik, 2004). To elucidate the role of the peripheral clock, in absence of the central clock in human HFs we have addressed the following questions.

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 and hair growth *in vitro*?

4.4 Results

4.4.1 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 in murine and human skin and plucked human hair shafts (Brown et al. 2008; Akashi et al. 2010; Geyfman et al. 2012b; Sandu et al. 2012), human anagen scalp HFs expressed CLOCK, BMAL1 and PER1 mRNA and protein (**Fig. 4.1, 4.2, 4.3b, S4.1**). In addition, human anagen scalp HFs transcribed the CCGs, *c-Myc, NRD1* and *CDKN1a* (**Fig. 4.1**).

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 2000), HFs were sampled every 4 hours over a 24-hour period **(Fig. 4.1b and c)** or every 6 hours for 48 hours (**Fig.4.1a**). All three core clock genes and all tested CCGs (*NR1D1, c-Myc and CDKN1a* (*P21))* were expressed in the HFs of three separately tested patients, and showed circadian variation in their transcription patterns (**Fig. 4.1 a, b and c**). Furthermore, Figure 4.1a shows that circadian rhythmicity was maintained over 48 hours. Despite the expected inter-individual variation, all patients showed a similar rhythmicity over the test period documenting that isolated human HFs exhibit peripheral molecular clock activity independent from central clock inputs.

Following this, HFs were cultured until half entered catagen, with half remaining in anagen (taking between 4 and 14 days). The time course was then repeated confirming there was circadian rhythmicity of clock gene expression; therefore showing the peripheral molecular clock was still active in both anagen and catagen HFs after 4 or more days in organ culture **(Fig. 4.3a).**

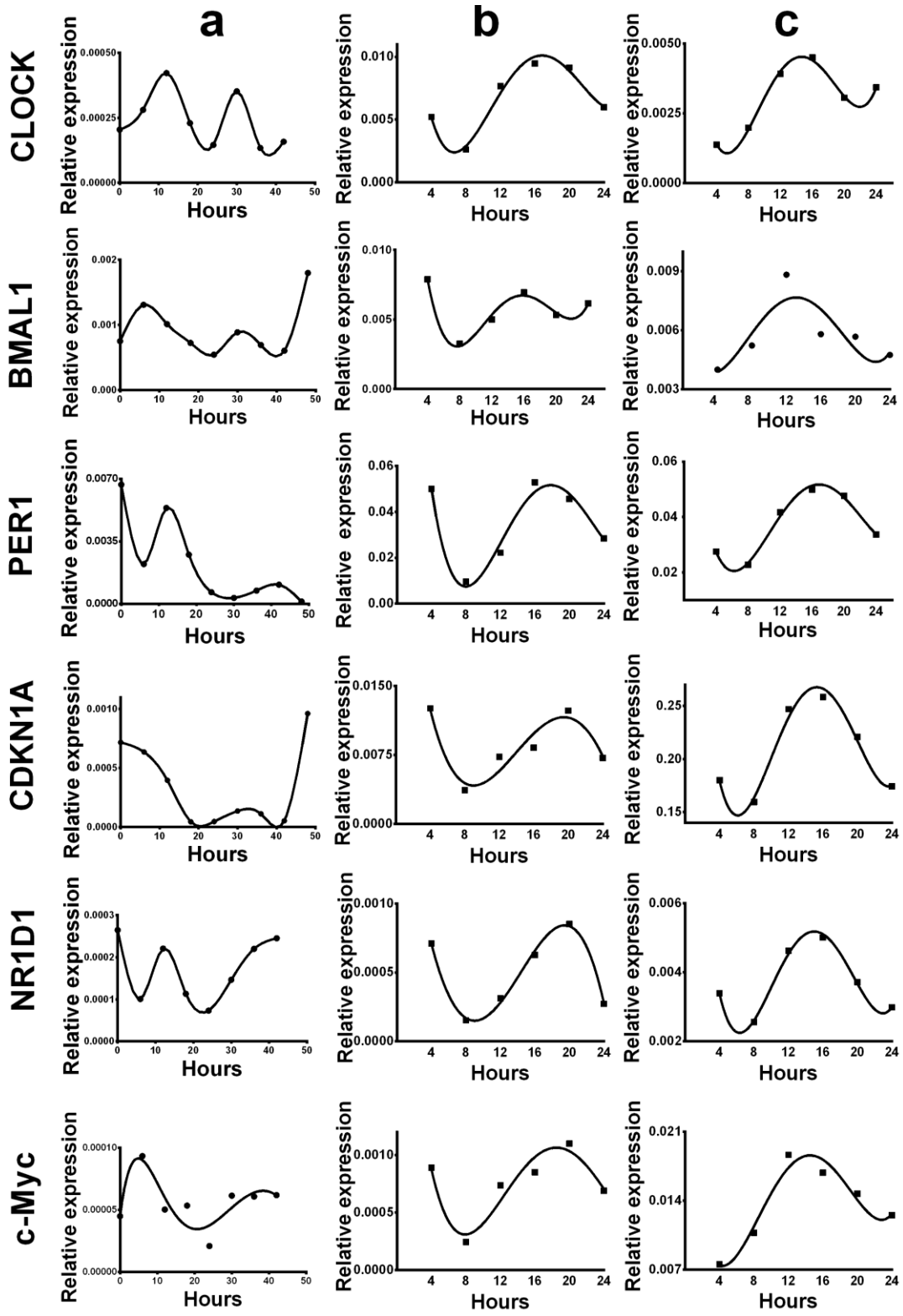


Fig. 4.1: Circadian expression profiles of clock transcripts CLOCK, BMAL1, PER1 and clock controlled genes NR1D1, C-MYC and CDKN1A in isolated human anagen HF.

Transcript levels of the above candidates was quantified using qRT-PCR in whole hair follicles synchronised with dexamethasone and sampled for either 48 Hours (a) or 24 hours (b and c) post-synchronisation. Data shown are the mean relative expression levels of 15 hair follicles each from 3 different male individuals (a, b and c) compared to house-keeping gene PPIA (black dots). All subjects showed circadian rhythmicity of all genes lasting 24 hours (b and c), which was further maintained for the full 48 hour time course (a). Data was not grouped due to recognized inter-individual variations between subjects (Akashi et al. 2010).

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

To better understand the functional role of the molecular clock in the human HF, clock protein expression was analysed by immunohistochemistry. BMAL1 showed strong immunoreactivity in the matrix keratinocytes (MK) of human anagen and catagen HFs **(Fig. 4.2a and b)**. BMAL1 protein was also located in the outer root sheath (ORS), dermal papilla (DP) and connective tissue sheath **(Fig. 4.2a)**. Unlike BMAL1, PER1 protein immunoreactivity was restricted to the epithelium where it was most prominent in the ORS **(Fig. 4.2 c, d and f)**. CLOCK RNA was also restricted mainly to the HF epithelium, being more prominent in the ORS than in the inner root sheath (IRS) **(Fig. 4.2h)**.

4.4.3 Intrafollicular PER1 gene and protein expression is hair cycle-dependent

To probe, whether clock gene/protein expression in organ-cultured human scalp HFs is hair cycle-dependent, anagen VI and catagen HFs was compared. This showed that the mRNA steady-state levels (**Fig. 4.3b**) and the intrafollicular PER1 protein expression (**Fig. 4.2d**) were significantly higher in catagen HFs compared to anagen VI HFs. In order to check whether the observed increase in PER1 expression was hair cycle-dependent and did not result from diurnal expression changes, intrafollicular PER1 immuno-reactivity was compared at two different time points (9 a.m., 3 p.m.) in anagen and catagen HFs. This showed that there was no net change in diurnal PER1 expression and that irrespective of the time of day; PER1 expression was always higher in catagen than in anagen HFs (**Fig. 4.2f**). Furthermore, in synchronised HFs, the amplitude of *PER1* mRNA levels differed significantly between anagen and catagen (**Fig. 4.3b**). While minor amplitude differences between anagen and catagen HFs were also seen for *CLOCK* and *BMAL1*, these did not reach significance (**Fig. 4.2b and 4.3b**). Taken together this shows that expression of at least one core clock gene product, PER1, is robustly hair cycle-dependent.

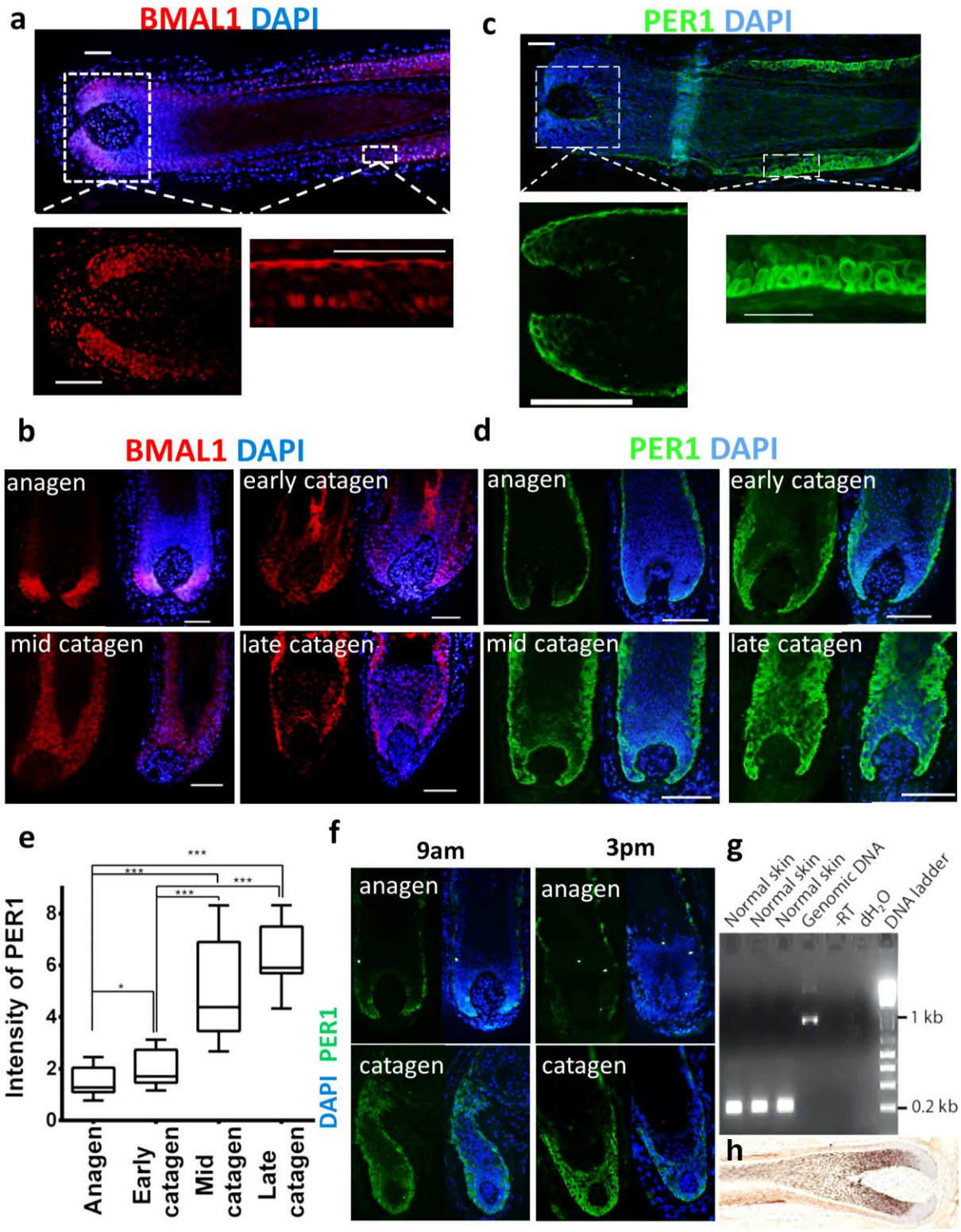


Fig. 4.2: CLOCK, BMAL1 and PER1 expression in human hair follicles (HF).
(a) BMAL1 protein expression was found in the cell nuclei with high intensity in the hair matrix, dermal papilla, connective tissue sheath and the outer and inner root sheaths (ORS and IRS), and did not showed significant hair cycle-dependent expression changes **(b). (c)** PER1 protein expression was mainly cytoplasmic localising to the MKs and ORS. **(d)** PER1 showed statistically significant hair-cycle expression changes as human HFs progress from anagen VI through catagen (quantitative immunohistomorphometry, image J) **(e).** Mann-Whitney test (Holm-Bonferroni correction) *p<0.05, ***P<0.001). **(f)** Diurnal expression changes were considered however, PER1 showed no net change in protein expression over 6 hours. **(g)** CLOCK mRNA levels were found by RT-PCR demonstrating it is expressed in the skin. **(h)** Utilising *in situ* hybridisation CLOCK expression was found to be localised to the ORS. (Bar = 50µm). [**See appendix 1 page 224-6 for individual panels.]**

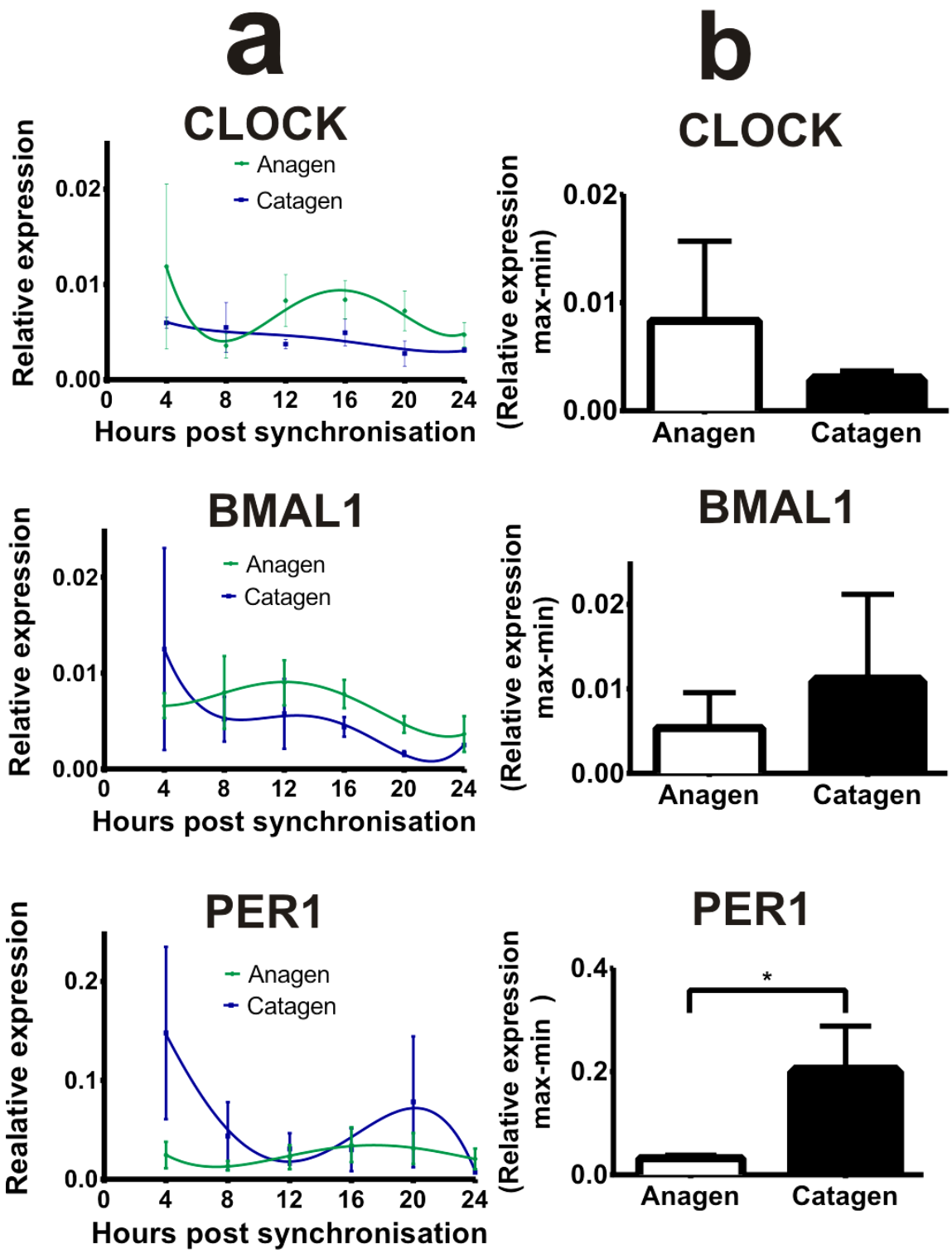


Fig. 4.3: Time series expression of clock mRNA in anagen and catagen human hair follicles (HFs). Human anagen VI HFs were cultured until half of them had spontaneously entered catagen (4-14 days); a time-course experiment was then performed. **(a)** Quantitative RT-PCR (5 male patients) against house-keeping gene *PPIA* showed that not only was circadian rhythmicity maintained beyond 4 days but that *CLOCK* expression was significantly higher in anagen than in catagen ($p=0.046$). **(b)** On qualitative assessment there was an apparent difference in waveforms; therefore differences in amplitude between anagen and catagen were quantified (averaging maxima and minima expression) showing there was a statistically higher amplitude of *PER1* mRNA in catagen HFs ($p<0.05$, Student's T-test, ±SEM).

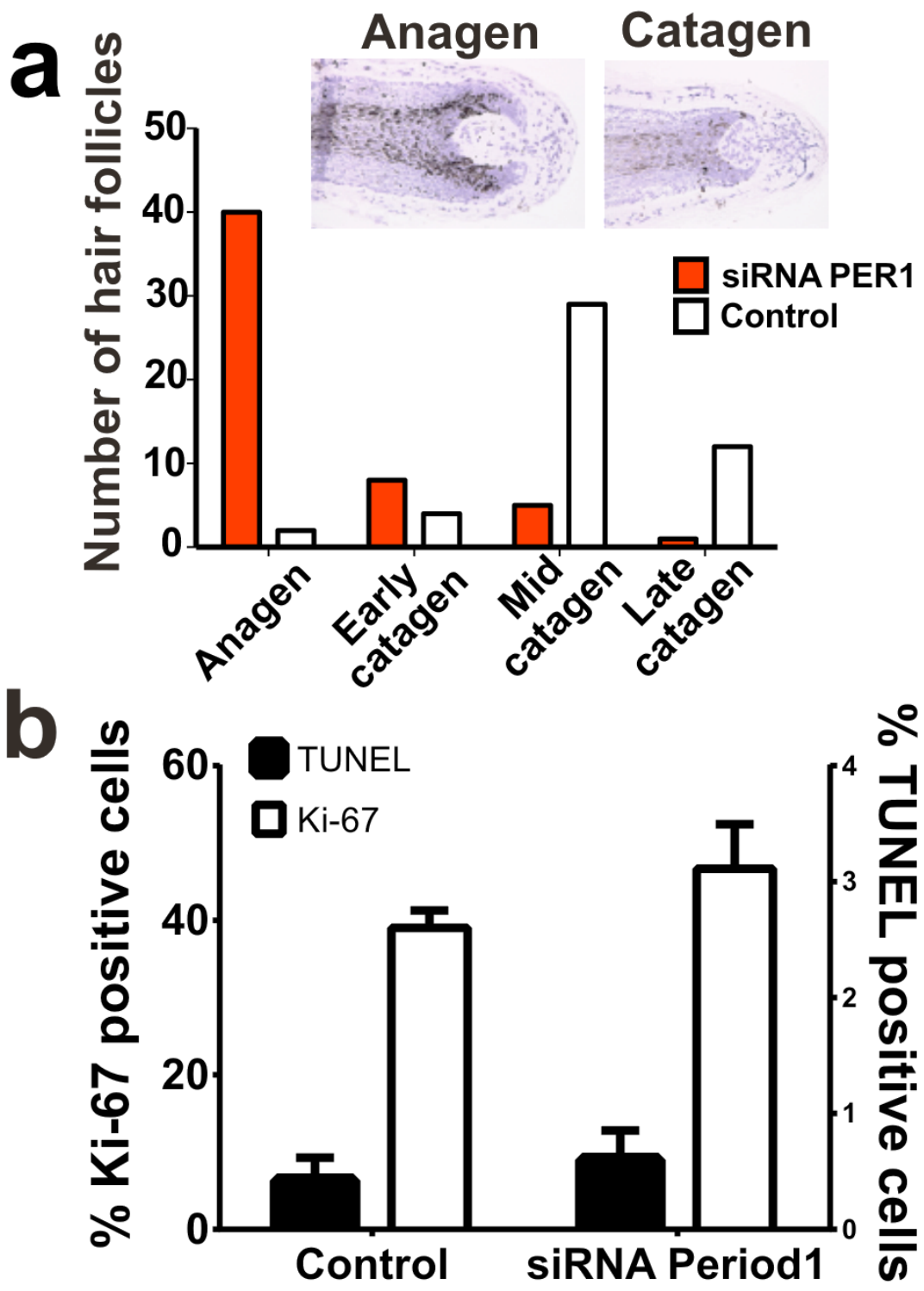


Figure 4.4: Effects of PER1 knock-down in human hair follicles (HF).
(a) 96-hours post-PER1 knock-down, cycle stages were determined by morphology. A significantly higher number of HFs remained in anagen in silenced HFs ($p<0.05$, Fisher's exact test). **(b)** PER1 knock-down in HFs also increased proliferation 24-hours following transfection (assessed by Ki-67/TUNEL) compared to a control. However, this was not significant (Mann-Whitney, $p=0.2$). Error bars ± SEM. Results from four patients (3 male/1 female).

4.4.4 PER1 silencing in human HFs significantly prolongs anagen

Therefore, the functional consequences of reducing *PER1* gene activity on human HF cycling by intrafollicular gene knock-down was investigated (Samuelov et al. 2012b) by transfecting anagen VI HFs with *PER1* siRNA. Successful PER1 knock-down in human anagen HF organ culture was demonstrated at the mRNA and protein level (**Fig. S4.2a & b**). Given that PER1 expression was low in anagen VI 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 (40 HFs) than in the scrambled oligo-treated control group (11 HFs) (**Fig. 4.4a**). This observation was confirmed in 4 separate experiments from different individuals (**Fig. 4.4a**). Hair cycle stage was confirmed by Ki-67/TUNEL staining (Kloepper et al. 2010). Although there was a slight trend towards an increased number of proliferating (Ki-67 positive) cells in the MKs of PER1-silenced anagen VI HFs compared to a scrambled oligo control, this was not statistically significant (**Fig 4.4b**). Nevertheless, this identifies PER1 as a novel catagen-inducing signal in human cycle control, whose silencing prolongs the duration of anagen.

4.4.5 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, which achieved knock-down on the mRNA and protein level (**Fig. S4.2c & d**). This experiment was necessary as BMAL1 is essential for the core clock oscillations as it induces PER1 and its deletion eliminates clock activity (see **Fig. 2.4)**, thus leading to disruption of the intrafollicular peripheral clock (Balsalobre 2000; Bunger et al. 2000; Lee et al. 2013).

96-hours after BMAL1 knock-down a significantly greater proportion of silenced HFs (8 HFs) remained in anagen VI than in the control group (2 HFs) (**Fig. 4.5a**). Although BMAL1-silencing slightly modulated hair MK proliferation/apoptosis, this did not reach significance (**Fig. 4.4b**). Pilot data from an additional CLOCK knock-down experiment (1 patient) also demonstrated anagen prolongation (**Fig. S4.3a**). Taken together, this suggests that the molecular clock as a system, rather than individual clock components, controls the human “hair cycle clock”.

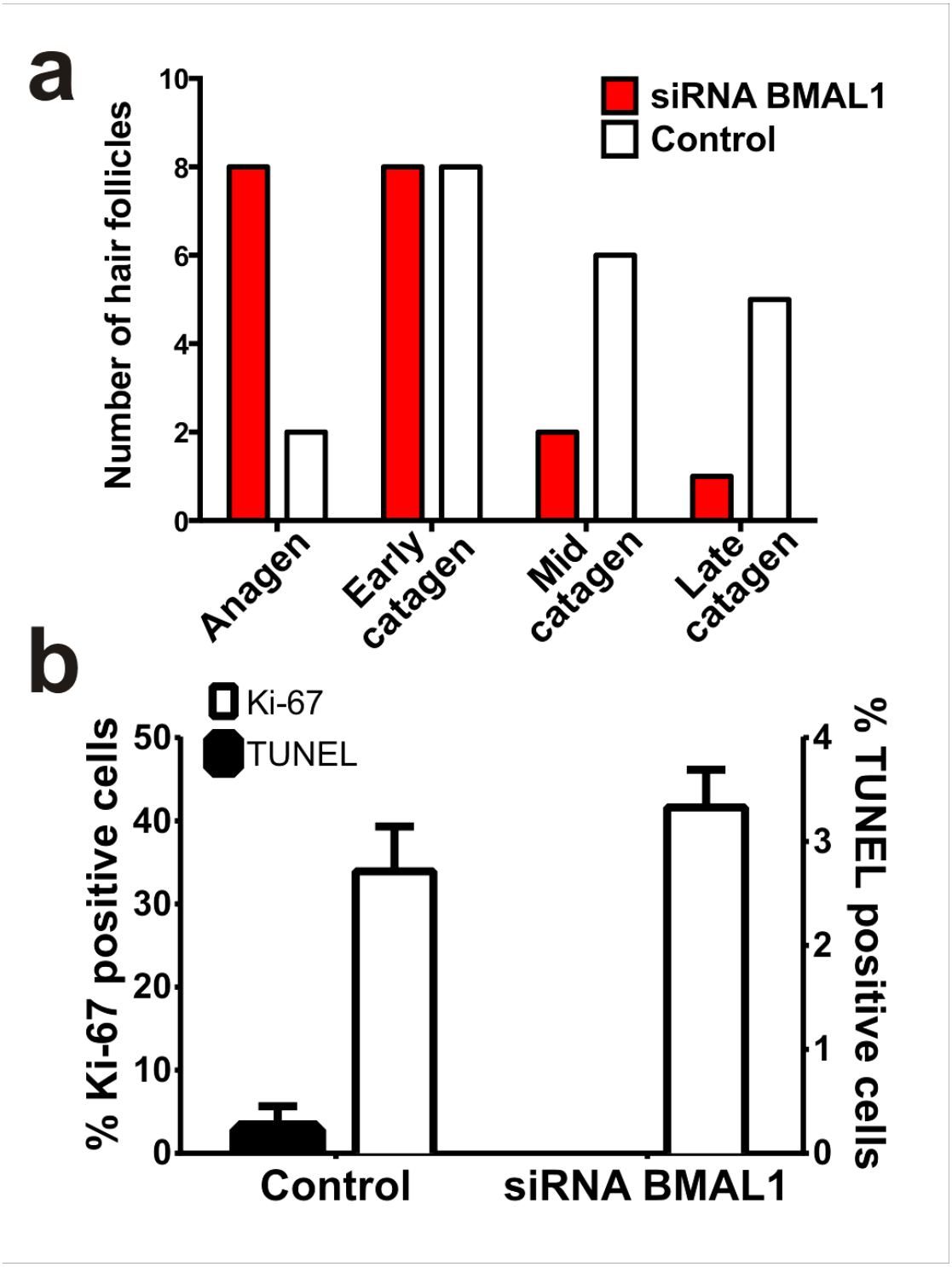


Fig. 4.5: Effects of BMAL1 knock-down in human hair follicles (HF).
(a) 96-hours post-BMAL1 knock-down, cycle stages were determined by morphology. A significantly higher number of HFs remained in anagen in silenced HFs ($p=0.028$, Fisher's exact test). **(b)** BMAL1 knock-down in HFs also increased 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, $p=0.29$). Error bars ± SEM. Results from three patients (2 male/1 female).

4.5 Discussion

Following 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 circadian activity is present after culture of periods exceeding 4 days (**Fig. 4.3a**) and, in addition that PER1 and BMAL1 produce anagen-terminating signals implicating their role in HF cycling (**Fig. 4.4 and 4.5**).

Our findings correspond to a growing body of evidence that clock genes regulate physiological processes such as the cell cycle (Matsuo et al. 2003; Khapre et al. 2011) metabolism(Bass, 2012; Geyfman *et al.*, 2012b), tumour growth (Fu et al. 2002; Chen-Goodspeed and Lee 2007; Yang et al. 2009), seasonal rhythms (Hazlerigg and Loudon 2008), reproductive cycle (Ware et al. 2012), age-related pathologies such as Alzheimer's disease (Hatfield et al. 2004; Bedrosian and Nelson 2012), and diseases including diabetes mellitus and depression (de Bodinat et al. 2010; Etain et al. 2011). That CLOCK, PER1, BMAL1 and all CCGs observed show circadian rhythmicity lasting a minimum of 48 hours, beyond any transient effect of the dexamethasone synchronisation (Balsalobre 2000), implicates their role in modulating the hair-cycle. Thus, the autonomous oscillations of PER1 and BMAL1 observed in human scalp HFs support the importance of "circadian" clock functions in controlling local peripheral tissue physiology (Geyfman and Andersen 2010; Janich et al. 2011; Plikus et al. 2011, 2013; Geyfman et al. 2012b). Moreover, they suggest that the core peripheral clock is an integral component of the elusive "hair cycle clock" (Paus and Foitzik 2004; Al-Nuaimi et al. 2012). While murine *in vivo* work had already implicated clock gene activity in the control of murine HF cycling (Lin et al. 2009), our study shows that clock genes/proteins are expressed in human HFs showing circadian rhythmicity and that the central clock is dispensable for clock-controlled hair cycle modulation.

As silencing both PER1 and BMAL1, had the same hair growth effects strongly suggests the importance of the core peripheral clock in human hair cycle control. This is corroborated by our CLOCK knock-down pilot data (**Fig. S4.3a**). While PER1 has many non-circadian roles in tumour suppression, cardiovascular disease and Alzheimer's disease (Lee 2006; Rosenwasser 2010; Bedrosian and Nelson 2012), as does BMAL1 in oxidative damage homeostasis and mitochondrial function (Knuever et al. 2012; Razorenova 2012), it could be argued that the effects observed are non-circadian and largely reflect a stress response. However, that PER1, BMAL1 and CLOCK silencing all showed anagen-prolonging effects, and that deletion of BMAL1 eliminates molecular clock activity (Bunger et al. 2000; Lee et al. 2013), suggests that the catagen delay is caused by the peripheral clock system.

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*. It was necessary to next look at the diurnal effects of PER1 expression. Results show that PER1 expression did not show a change in expression changes over 6 hours, enough time to observe such changes but too short for spontaneous catagen entry. That PER1 expression was always higher in catagen regardless of the time of day shows that PER1 expression is regulated in a hair cycle-dependent manner.

In contrast to previous work, our study is focused on the human system and unlike previous human work clearly excludes the central clock. With our organ-culture model we are able to exclude any possible side-effects of a global knock-out, species differences and central inputs (Lin et al. 2009; Akashi et al. 2010). It is a strength and limitation of this methodology that we can only draw conclusions on a functional role of the intrafollicular clock system in regulating the anagen-catagen transformation of human HFs *ex vivo*. That recent microarray analyses of synchronized murine HFs show peripheral clock gene activity in other cycle transformation stages supports a role for the

peripheral clock in influencing the 'hair cycle clock' (Lin et al. 2009; Geyfman and Andersen 2010; Geyfman et al. 2011; Plikus et al. 2013) (Geyfman and Andersen, 2010; Geyfman *et al.*, 2012a; Lin *et al.*, 2009; Plikus *et al.*, 2013) and suggests if we were able to track the catagen-telogen transition or telogen-anagen transition in human HFs, we would see a similar effects.

In human HFs, BMAL1 shows strong expression in the MKs. As BMAL1 has been linked with cell-cycle control, this may be how it influences the hair cycle (Matsuo et al. 2003; Sahar and Sassone-Corsi 2009; Geyfman and Andersen 2010). As BMAL1 shows consistent protein expression throughout the anagen-catagen transformation (**Fig. 4.2b**), differing from the murine system where *BMAL1* mRNA and protein expression peaked in late anagen, (Lin et al. 2009; Plikus et al. 2013) potential species-specific differences in the peripheral core clock on HF cycling may exist. The significant inter-individual variations observed match earlier human work (Akashi et al. 2010); However, our data show that the clock genes will continue to oscillate in human HFs in absence of the SCN.

Although the mechanisms through which PER1 and BMAL1 exert their hair growth-modulatory effects remain to be dissected, they follow the established concept that clock genes and CCGs control cell cycling (Lowrey and Takahashi 2004; Miller et al. 2007; Geyfman and Andersen 2010; Plikus et al. 2013). Recognised hair cycle-regulatory genes, c-Myc (Bull et al. 2001, 2005) and p21 (Mitsui et al. 2001; Ohtani et al. 2007), are key cell cycle regulators and are 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 D1(Fu et al. 2002), a 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 by impacting the cell cycle and apoptotic machinery of MKs, similar to role of CCGs Nr1d1 and Dbp in the murine MKs (Lin et al. 2009; Plikus et al. 2013). Furthermore, PER2 mutations in humans causes familial advanced sleep phase syndrome and are implicated in tumorigenesis and phase shifts in cyclin D1 and P21 (Gu et al. 2012), while CLOCK mutations in humans leads to altered sleeping phenotypes (Wager-Smith and Kay 2000). Whilst to the best of our knowledge there is no report of altered hair growth in such

patients, subtle hair growth or cycling abnormalities may have missed; therefore, future screening in such patients would provide definitive *in vivo* confirmation of our study in the future.

There is an increasingly appreciated link between the long-term effects of the clock in age-related pathologies. For example, reduction in circadian amplitude and response to external cues are linked with Alzheimer's severity, potentially by increased amyloid-beta peptide accumulation (Hatfield et al. 2004; Rosenwasser 2010). This suggests that long-term clock disruptions lead to pathologies surpassing circadian boundaries (Rosenwasser 2010). Our data raise the possibility that circadian clock outputs exert long-term cumulative effects. Specifically clock protein accumulation within human HFs during anagen may represent one mechanism in which 24-hour rhythms impact the "hair cycle clock" and thus human HF cycling. However, with the work in peripheral clock biology in its infancy, validation of such a hypothesis would require further experimentation.

Evidently, a translational aspect of our 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; Knorr et al. 2009; Patzelt et al. 2011; Gu et al. 2012) 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 CCGs may 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, our study supports the peripheral core clock in modulating the anagen-catagen transformation of human HFs under clinically relevant *in vitro*-conditions. We show that BMAL1, PER1, and likely CLOCK form an integral component of the human "hair cycle clock". These clock genes therefore are novel targets for the therapeutic modulation of human hair growth. Moreover, we demonstrate that HF organ culture offers an instructive, clinically relevant model for preclinical

peripheral clock research in a complex, oscillating human mini-organ where two chronobiological systems meet.

4.6 Materials and methods

4.6.1 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 HF 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, snap-frozen in liquid nitrogen or first embedded in Shandon Cryomatrix (Fisher Scientific) prior to snap freezing.

4.6.2 Human HF organ culture

Human scalp HFs in anagen stage VI of the hair cycle (**Fig. 2.3**) were micro-dissected and organ-cultured under serum-free conditions in the presence of insulin and hydrocortisone as described (Philpott et al. 1990, 1994) **(Section 3.1.5).** Under these conditions human anagen HFs continue to produce a pigmented hair shaft and eventually spontaneously enter a catagen-like state (Sanders et al. 1994; Kloepper et al. 2010). The telogen hair cycle phase cannot be captured in human HF organ culture.

4.6.3 24-hour and 48-hour time series experiment

Circadian rhythmicity of core clock genes and selected CCG expression was investigated in human anagen HFs from three male subjects (**Supplementary table S4.2** a, b, c). Micro-dissection and organ culture was started within 2h post-surgery (time window of 09:30-13:00 GMT), and HFs were incubated for a 24 hours equilibration period. Clock activity was then synchronized (100nM dexamethasone, 30 minutes) (Balsalobre *et al.*, 2000), after which HFs were harvested every 4 hours for 24 hours (patients b and c) or every 6 hours for 48 hours (patient a) and 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 S4.2**); once half the HFs were in anagen and the remaining follicles had entered catagen, the samples were synchronised. Both anagen and catagen HFs were collected every 4 hours. For subjects D and E this was repeated however, all HFs entered catagen (**Supplementary table S4.2**). This took between 4 and 14 days for the HFs to enter the correct stage. The HFs were maintained in RNAlater until processed for qRT-PCR.

4.6.4 Quantitative immunohistomorphometry

Immunohistochemistry or immunofluorescence microscopy (IHC/IF) staining for localisation and quantification of clock proteins (CLOCK, BMAL1, PER1) *in situ* was performed on human scalp (8µm) skin or isolated HFs (6 µm) [See **Supplementary Table S4.1** (Ackermann et al. 2007)]. Primary antibodies were incubated overnight at 4°C. Sections were washed in 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; van Beek et al. 2008; Kloepper et al. 2010). 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).

4.6.5 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) (4 subjects) or *BMAL1* siRNA (FsiRNA (h): sc-38165) (3 subjects) 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 S4.3** for details). A pilot knock-down of a CLOCK supported this data (1 subject) **(Fig. S4.3a)**.

4.6.6 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) (**table 3.2**).

4.6.7 *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).

4.7 Declarations

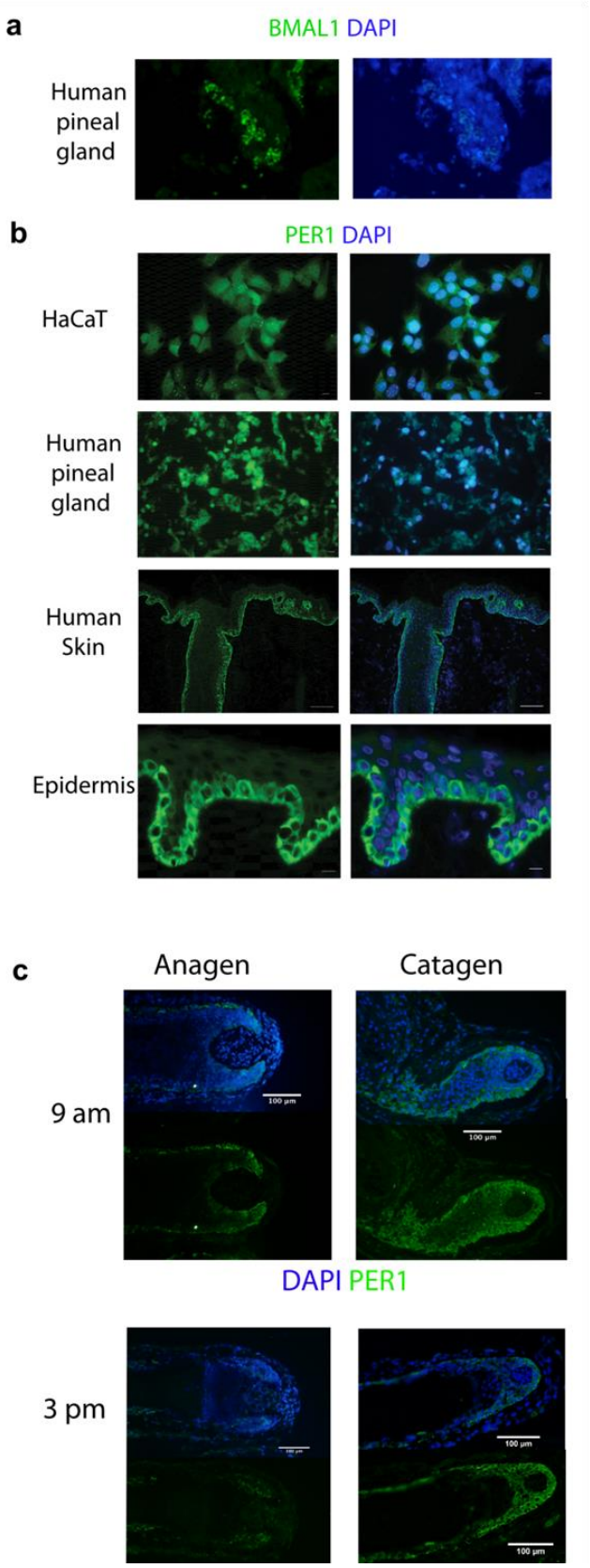
4.7.1 Conflict of Interests

The authors state no conflict of interest

4.7.2 Acknowledgements

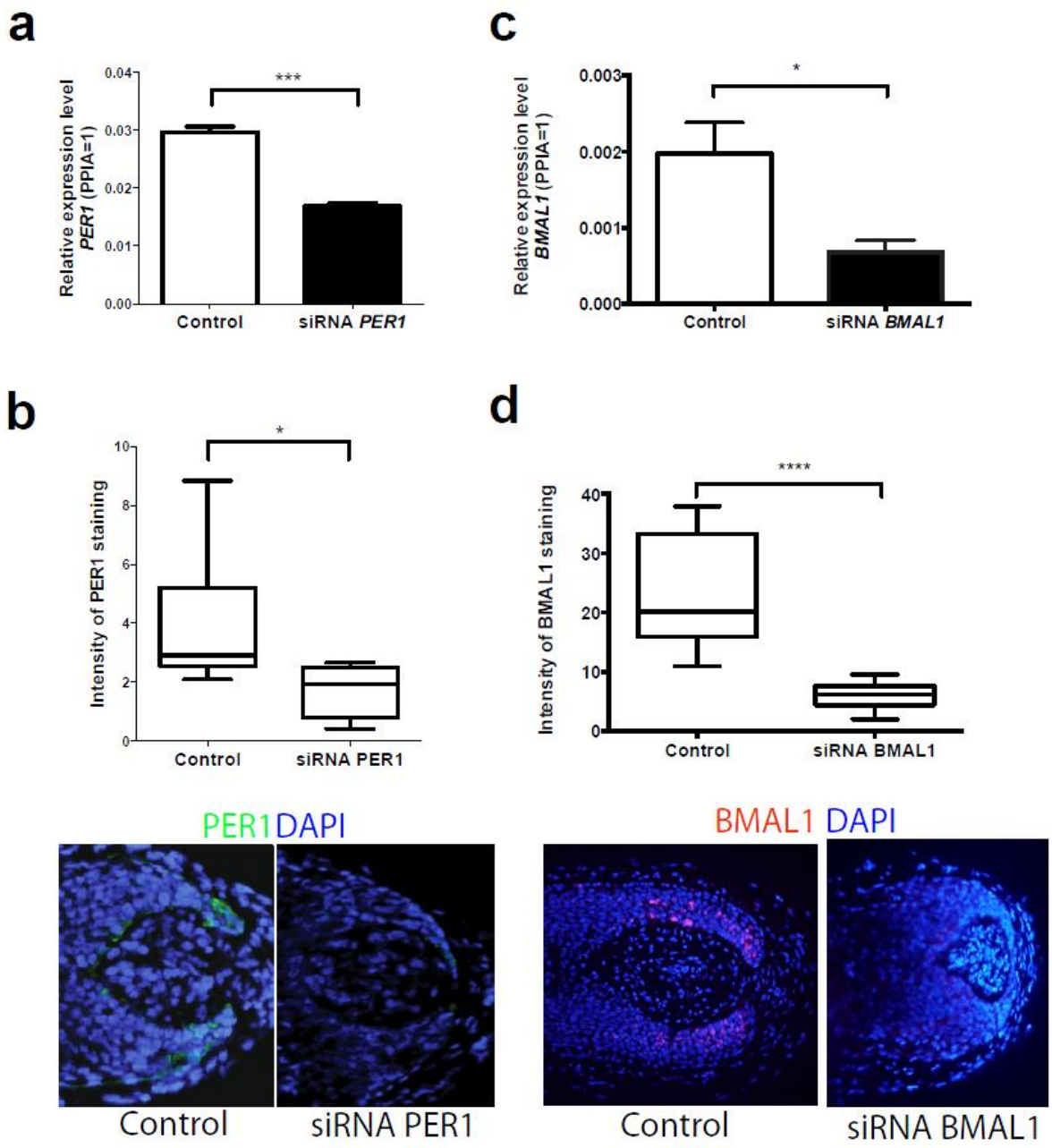
Drs. Stephan Tiede, Koji Sugawara, Eniko Bodo, Erzsébet Gáspár and Natalia Maier and the lab technicians in Luebeck are gratefully acknowledged. We also thank Professor Bogi Andersen, Dr. Mikhail Geyfman (University of California), Dr Qing Jun Meng, Professor Christopher E.M. Griffiths and Professor Hans Westerhoff (University of Manchester) for expert advice. Lastly, we are most grateful to our plastic surgery colleagues who generously provided human skin samples for this study, namely Dr. W. Funk (Munich).

4.8 Supplementary information



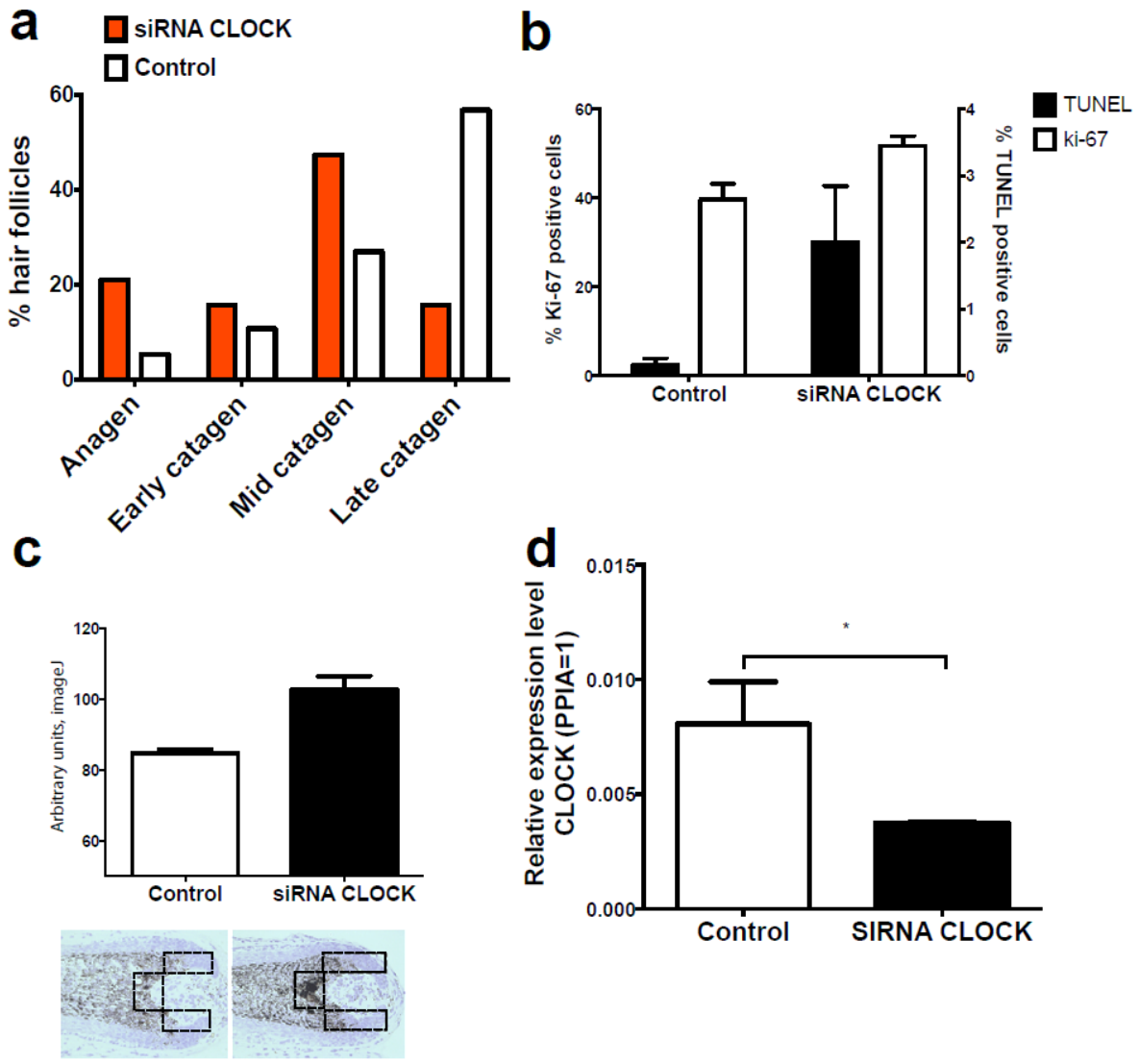
Supplementary fig. S4.1: Immunofluorescent staining of BMAL1 and PER1 in positive control tissue and skin cryosections.

(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 Fig. S4.2: 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 Fig. S4.3: 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*.

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	(45)
BMAL1	Rabbit anti-human MOP3, 1:50 (MOP31 [N1N3] Genetex)	Goat anti-rabbit fluorochrome 1:200 (Jackson Immunoresearch)		Human pineal gland	(94)
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	(45, 94)

Supplemental Table S4.1: Protocol summaries for clock protein immunofluorescence and immunohistochemistry experiments.

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 S4.2: Subject samples for 24 hour synchronised time series experiment.

GENE	SIRNA PROBES USED	SUBJECT AGE	NO. OF HFS	LOCATION	DURATION OF KNOCK-DOWN
PERIOD1	Period1 siRNA (h): sc-38171 and control siRNA (FITC conjugate) (h): sc-36869	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 (FITC conjugate) (h): sc-36869	24 yr., male	40	Scalp, occipital	24 hrs.
		31 yr., female	40	Scalp, temporal	96 hrs.
		37 yr., male	40	Scalp, occipital	96 hrs.

Supplementary Table S4.3: siRNA transfection experiments.

Chapter 5: The peripheral clock regulates human pigmentation

This chapter has been accepted for publication in the Journal of Investigative Dermatology pending minor amendments (September 2014).

5.1 Authors

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Candidate's contribution

The candidate performed all experiments except Fig 5.1b (PER1 knock-down, Masson-Fontana experiment) which was performed by YA, performed all analysis and statistics and authored the manuscript.

Authors' contribution

DJT: Provided expertise on melanocytes/melanogenesis, technical expertise on HRLM and provided epidermal melanocytes for culture.

ISH: Provided general technical knowledge, corrected manuscript drafts and gave co-supervisory input to JAH.

NF & BF: Provided all occipital scalp HFs used throughout the experiment.

YA: Performed the PER1 knock-down experiment used for the initial melanin stain (Masson-Fontana) and proof read/edited the manuscript.

BG: Provided expertise on the molecular clock system and edited the manuscript.

RP: Acted as the main supervisor to JAH for this project, designing the experimental strategy and contributing substantially to data interpretation and manuscript writing, and edited the manuscript

5.2 Abstract

While the molecular 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). Since both human HFs and epidermal melanocytes express clock genes and proteins, and given that core clock genes (*PER1, BMAL1*) help to regulate human HF cycling, we investigated whether peripheral clock activity impacts on human HF pigmentation. We found that partial silencing of *BMAL1* or *PER1* in cultured human HFs induced a significant increase in the HF melanin content (Masson-Fontana histochemistry). Furthermore, tyrosinase expression and activity *in situ* as well as *TYRP1* and *2* mRNA levels, gp100 protein expression, melanocyte dendricity and the number gp100+ HF melanocytes were all significantly increased in *BMAL1* and/or *PER1*-silenced HFs. These complex clock-regulated changes in melanocyte biology were not HF-specific, since *BMAL1* or *PER1* silencing also increased the epidermal melanin content, gp100 protein expression and tyrosinase activity in organ-cultured human skin. These *in situ* pigmentation effects reflect, at least in part, a direct modulation of melanocytes, since *BMAL1* and/or *PER1* silencing in isolated human epidermal melanocytes increased tyrosinase activity and *TYRP1* and *2* expression. Mechanistically, *BMAL1* knockdown reduces *PER1* transcription, and *PER1* silencing induces phosphorylation of the master regulator of melanogenesis, MITF, thus stimulating human melanogenesis and melanocyte activity *in situ* and in vitro. These data provide the first evidence that the peripheral molecular clock operates as a cell-autonomous modulator of human pigmentation and may be targeted as a novel therapeutic strategy for the clinical management of pigmentation disorders.

5.3 Introduction

Despite its high regeneration potential and the relative aging resistance of the HF, it is the HF pigmentary system that routinely shows the first signs of aging (canities or greying), 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 ROS accumulation and reduction of 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 greying (Arck et al. 2006; Wood et al. 2009) and clock genes have been linked with ROS homeostasis (Geyfman et al. 2012b; Lai et al. 2012; Lee et al. 2013; Avitabile et al. 2014), investigating the role of the molecular clock in HF pigmentation may enhance our understanding of how canities and pigmentation disorders develop, highlight potential novel 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 Fig. S1), which produce melanin in the growth stage (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 the outer root sheath (ORS) (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 specialised organelles, melanosomes (Medes 1932; Chávez-Béjar et al. 2013). Once mature, melanosome 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 Fig. S5.1).

Many different factors have been implicated in the control of HF melanocytes and melanogenesis (Hearing 1999; Ancans et al. 2001; Slominski et al. 2004; Lin and Fisher 2007; Schallreuter et al. 2008; Simon et al. 2009; Hirobe 2011; Singh et al. 2012; Samuelov et al. 2013; Smith et al. 2013) (see **Supplementary Table S2.2**) including the rate-limiting enzyme tyrosinase and 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 which ultimately control the hair cycle-dependent activation and deactivation of intrafollicular melanogenesis are yet to be uncovered.

It is the cyclic growth, regression and pigmentation activity that makes the HF an informative model system to dissect how oscillating molecular systems, i.e. circadian rhythmicity controlled by clock gene activity (Schroeder and Colwell 2013; Partch et al. 2014; Robinson and Reddy 2014), interact with local tissue physiology (Al-Nuaimi et al. 2010, 2014; Geyfman and Andersen 2010; Al-Nuaimi 2011; Plikus et al. 2013). The clock and the HF were first linked in mouse where it was shown that core clock genes regulate the murine hair cycle (Lin et al. 2009) and cell cycle progression in the murine hair matrix *in vivo* (Plikus et al. 2013). In humans clock gene and that clock gene transcription and protein expression was reduced in human melanoma samples (Lengyel et al. 2013a, 2013b).

Whilst previous reports demonstrate 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 functionally impacts on melanogenesis or other 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 a central clock inputs (Al-Nuaimi et al. 2014), suggesting that the molecular clock is an integral component of the as yet elusive "hair cycle clock". As melanogenesis is tightly coupled to melanogenesis, this highlights the peripheral molecular clock (supplementary fig. S5.2) as a plausible candidate for the

timing mechanism involved in the on/off switch of HF pigmentation. Moreover, serotonin and melatonin, both of which 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 metabolised in human skin and are implicated in the control of the human hair cycle and pigmentation (Slominski et al. 2005b, 2008). In addition, POMC and CRH, key regulators of melanogenesis that lead to the production of α-MSH 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 knock-down impacts on the HFPU in micro-dissected, organ-cultured human scalp HFs, using a wide range of 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.

5.4 Results

5.4.1 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, we began by studying whether any changes in HF pigmentation seen after *BMAL1* or *PER1* silencing, which down-regulates the intrafollicular expression of multiple core clock target genes (Al-Nuaimi et al. 2014), simply reflect the anagen-prolonging effect of clock silencing. To answer this question, we compared the intrafollicular melanin content by quantitative Masson-Fontana histochemistry (Kloepper et al. 2010; Gáspár et al. 2011; Samuelov et al. 2013) of anagen stage VI HFs only and thus showed maximal melanogenesis activity.

In both *BMAL1* and *PER1* silenced human HFs there was a significant increase in melanin content ($p=0.014$ and $p=0.015$ respectively) (Fig. 5.1a-c). This was further investigated by high resolution light microscopy (HRLM) (Supplementary Fig. S5.4a), which independently confirmed a significant increase in melanosome associated melanin content in multiple HFs (siRNA group= 12 HFs, control group = 10 HFs, each derived from 3 different patients). These data document that the activity of core clock components influences melanin content in a hair cycle-independent manner and suggest that the peripheral molecular clock may be involved in modulating HF melanogenesis.

5.4.2 Knock-down of core clock proteins increases melanosome synthesis in HF melanocytes and their transfer to HF keratinocytes

Next, we asked whether the melanin increase observed histochemically was influenced by an increase in the melanosome number and/or the transfer of melanosomes from HFPU melanocytes to precortical hair matrix keratinocytes. The number of melanosomes in both cell types was counted by HRLM in multiple HFs from three patients in a standardised reference area within selected HF melanocyte and keratinocyte (Supplementary Fig. S5.4b).

This demonstrated that both knock-down groups had significantly more melanosomes in both HFPU melanocytes and in precortical hair matrix keratinocytes when compared to a scrambled-oligo control 24 hours post knockdown (Figure 5.1d & f) ($p<0.001$ for both). After 4 days a significantly higher melanosome number in PER1 silenced HFs was still observed ($p<0.002$); however, this was no longer the case for BMAL1 knock-down HFs, which showed no difference from the control (Figure 5.1e & g). On the other hand, in both clock-silenced groups, the number of melanosomes in HF keratinocytes was significantly higher than the control HFs (Figure 5.1e & g) ($p<0.001$). Thus, peripheral clock disruption increased both intrafollicular melanosome synthesis and melanosome transfer within human anagen VI HFs *in situ*.

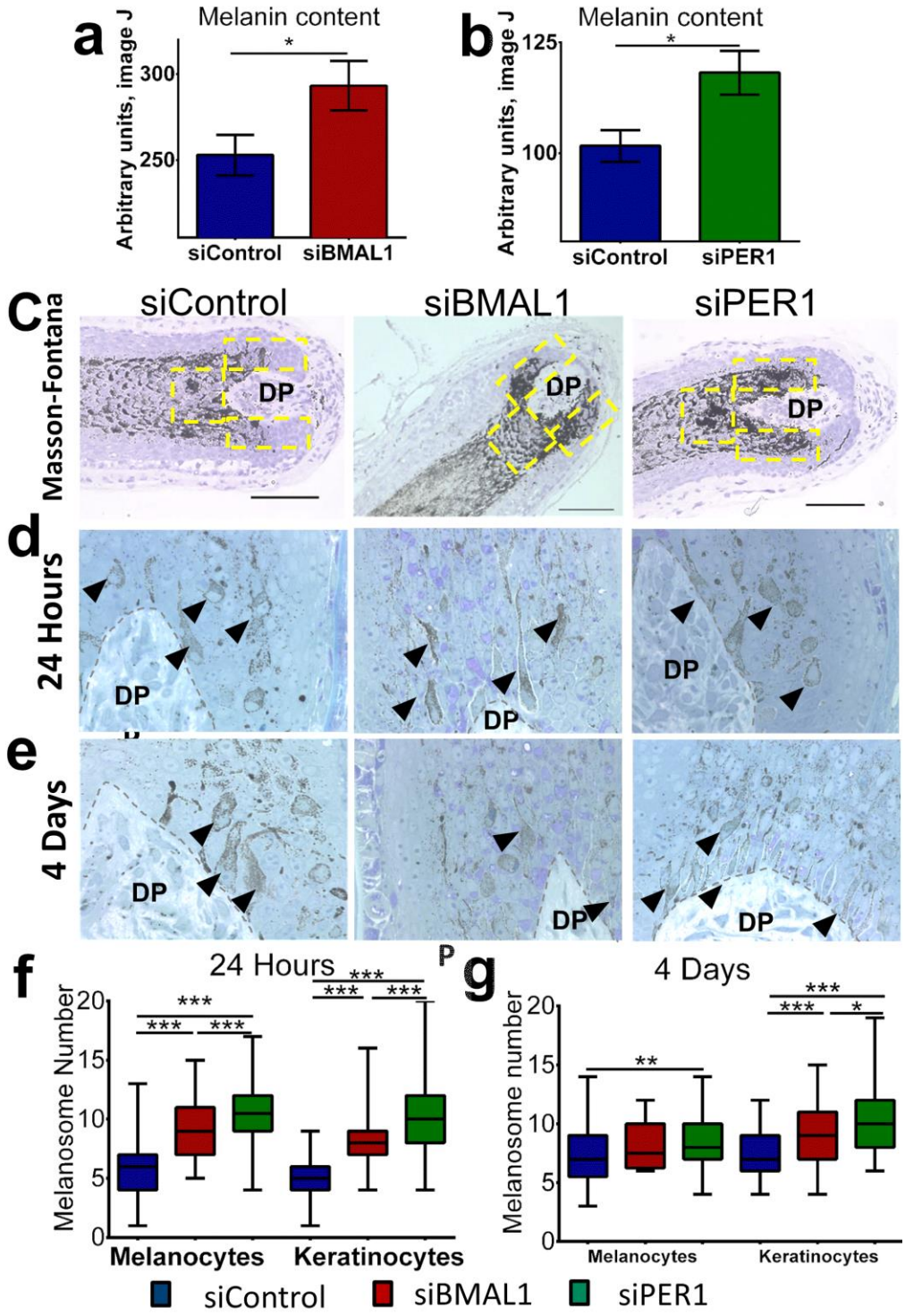


Fig. 5.1. Partial Silencing of BMAL1 or PER1 increases melanin content of human hair follicle (HF) and melanosome number.

Masson Fontana staining was utilised to detect pigmentary changes. In both the *PER1* (a) and *BMAL1* (b) knock-down groups there was a higher melanin in the reference areas chosen (c) (scale=50µm), n=22). Subsequently the number of melanosomes in melanocytes and keratinocytes were counted from HRLM images at 24 hours and 4 days (d, e) (x3600 magnification) and quantified (f, g) (n=9, 3 patients). It was found that *PER1* silencing increases the melanosome number in melanocytes and keratinocytes at both time points. *BMAL1* silencing increased the melanosome number in both melanocytes and keratinocytes at 24hours. (Mann-Whitney (a & b), ANOVA (f & g) with Holm-sidak post-hoc test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, error bars are ± SEM). (scale bar= 50µm). **[See Appendix 2*: page 228* for individual panels]**.

5.4.3 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), an activity indicator of differentiated melanocytes (Ohbayashi et al. 2012; Chang et al. 2014). Quantitative immunohistomorphometry showed that silencing PER1 increased both gp100 immunoreactivity in individual melanocytes ($p<0.001$) (Fig. 5.2a, e) and their dendricity (Fig. 5.2d, e, Supplementary Fig. S5.3). 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 of melanocyte dendricity at day 4 (Figure 5.2d). Although the *PER1* siRNA group also showed a tendency to have more dendrites than the BMAL1 siRNA treated group, this was not statistically significant at day 4.

These data demonstrate that the silencing-induced reduction of 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 melanosomes that had been detected in these cells by HRLM (Fig. 5.1d, e).

5.4.4 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 both differentiated, melanotic and undifferentiated, amelanotic 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 5.2 b & e) when compared to 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-immunostains were 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 Fig. S5.3). Therefore, this suggests the increase in melanocyte number by *PER1* knock-down was not due to melanocyte proliferation.

5.4.5 *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 knock-down, the activity of the rate-limiting enzyme of melanogenesis, tyrosinase, was assessed using an *in situ* enzyme activity assay (Han et al. 2002, Gaspar 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 5.2c & 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 melanogenesis.

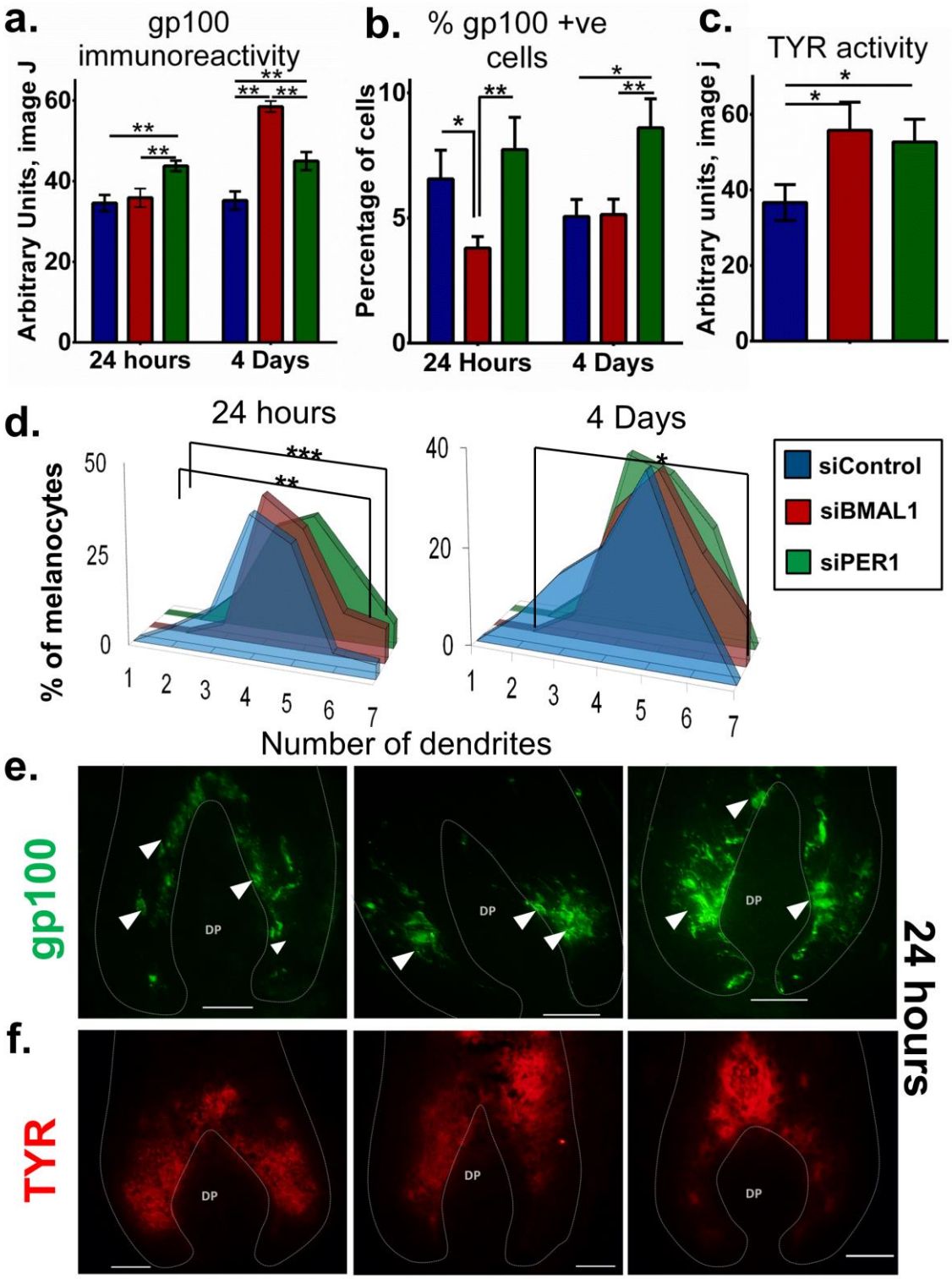


Fig. 5.2. Silencing core clock members increase gp100 immunoreactivity, melanocyte dendricity and Tyrosinase activity in human HFs.

(a and e) Immunohistomorphometry of the melanocyte marker gp100 shows that gp100 staining is enriched in the si*PER1* group at 24 hours and 4 days; however, immunoreactivity of individual melanocytes did not increase in the si*BMAL1* HFs until 4 days post-knock-down. (b and e) Gp100 analysis further demonstrated that silencing *PER1* increases melanocyte number at both time point yet *BMAL1* silencing seems to decrease melanocyte number at 24 hours recovering by day 4. (d.) Again utilising gp100 it was discovered that silencing either *BMAL1* or *PER1* increases dendricity at 24 hours with the increased dendrite number still being observed at day 4 for the *BMAL1* knockdown group. (c and f) Finally, by utilising a tyrosinase activity assay it became evident that this too was significantly increased in both knockdown groups significantly. (n=27) (one way ANOVA, Holm-Sidak post-hoc test, * $p<0.05$, ** $p<0.01$, ***$p<0.001$, error bars and ±SEM). (scale bar= 50µm). **[See appendix 2: *page 229* for individual panels.]**

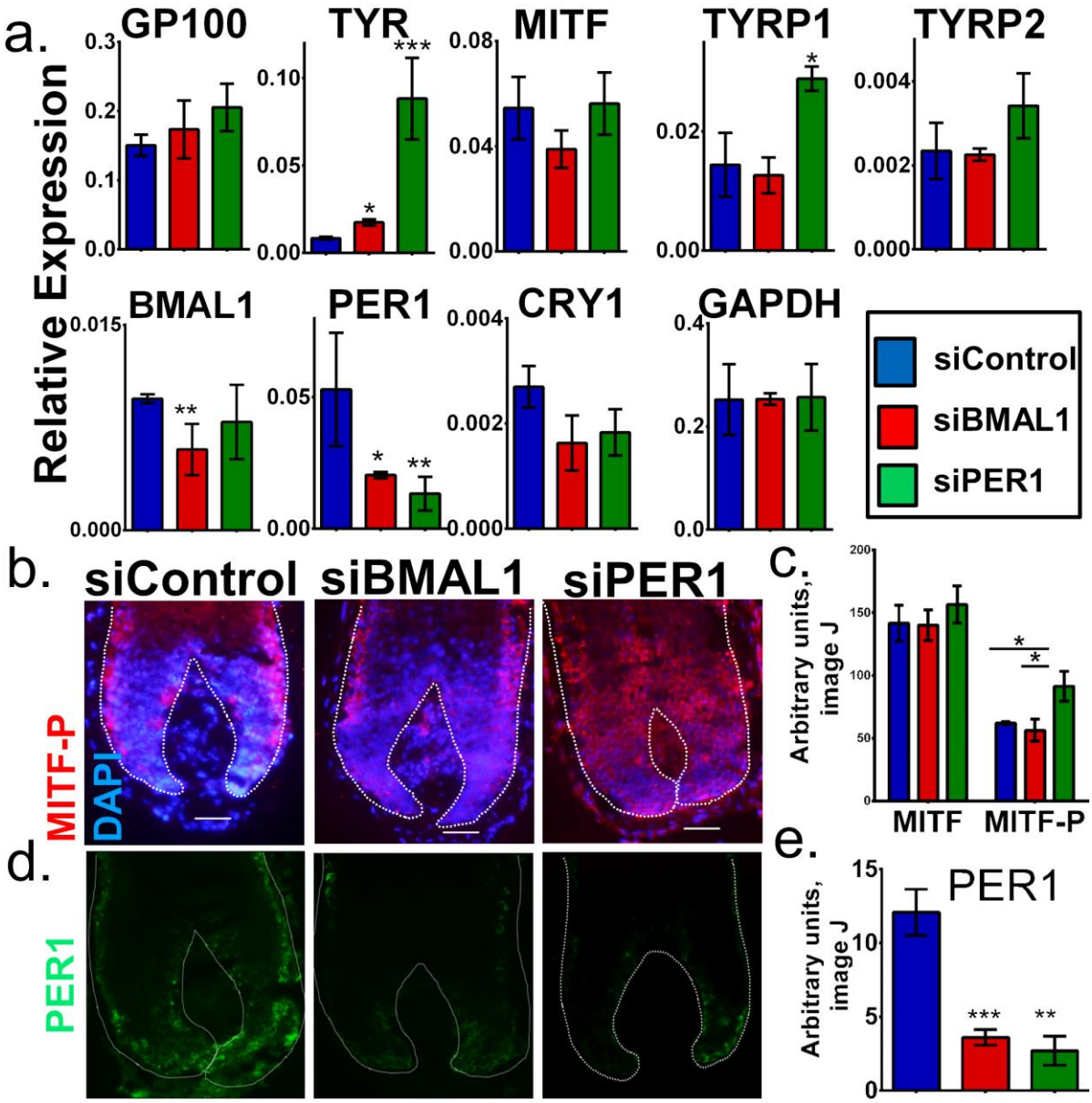


Fig. 5.3. Silencing of core clock genes increases tyrosine metabolism.
To elucidate how *BMAL1* and *PER1* affect melanogenesis, qRT-PCR was performed on clock members (*PER1*, *BMAL1*, *CRY1*) and key genes involved in melanogenesis (*gp100*, *MITF*, *Tyrosinase (TYR*), *TYRP1&2*) against housekeeping gene *PPIA* (*GAPDH* was used to confirm results). Significant transcript level changes were observed for tyrosinase in both knock-down groups (a) with PER1 silencing also increasing *TYRP1*. Although *MITF* transcript levels didn't change the levels of phosphorylated-MITF increased in the PER1 knock-down group (b & c), suggesting that silencing *PER1* increases MITF activity leading to the observed increase in melanogenesis. As *BMAL1* leads to the transcription of *PER1*, the role of *BMAL1* silencing on PER1 protein and mRNA was assed finding that both were reduced (d &e). (One way ANOVA, Holm-Sidak post hoc test, * $p<0.05$, ** $p<0.01$, ***$p<0.001$ error bars are ±SEM, n=15 from 3 patients). (scale bar= 50µm). [**See appendix: *page 230* for high-resolution panels**]

5.4.6 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) was investigated by qRT-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 (non-significantly) 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 (Fig. 5.3a), in line with the observed increase in tyrosinase activity *in situ* (Fig. 5.2c, f). The melanogenesis-promoting enzyme, *tyrosinase-related protein 1 (TYRP1)* (Cheli et al. 2010; Ghanem and Fabrice 2011), a key MITF-target gene (**Fig 2.5**), was also significantly increased when compared to the oligo control group, yet only in the *PER1*-silenced HFs (Fig. 5.3a). Together with the tyrosinase activity assay, this suggests that an up-regulation 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.

5.4.7 Silencing core clock members increases 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 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 (*siBMAL1* $p<0.001$, *siPER1* $p=0.004$), yet with higher gp100 immunoreactivity in *BMAL1*-silenced group (Fig. 5.4a & b). Furthermore, tyrosinase

activity was significantly increased in both *BMAL1* and *PER1* silenced HFs (Fig. 5.4c). However, there was no observable difference in dendricity (Supplementary Fig. S5.1d).

5.4.8 *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 was assessed *in situ*. 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 mirrored the *MITF* transcription data, showing no change between each knock-down group and oligo-treated control HFs (Figure 5.3b). In contrast, *PER1* silencing increased MITF-phosphorylation when compared to both the oligo control and *BMAL1*-silenced HFs (p=0.016 / 0.019) (Fig. 5.3b & 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

5.4.9 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 up-regulated 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 Figs. 5.1 f, g; 2b, 3a). Since BMAL1 directly stimulates the transcription of PER1 (**Fig. 2.5**) we also assessed how *BMAL1* silencing affected *PER1* gene and protein expression. Quantitative RT-PCR demonstrated that silencing *BMAL1* significantly reduced PER1 transcript levels (Figure 3a) and protein expression (Fig. 5.3d & e). This suggests that the pigmentary events observed in the *BMAL1* siRNA-treated HFs group may be caused at least in part by a significant reduction in PER1 activity. As other clock genes (*CRY1*) were not affected by *BMAL1* silencing (Fig. 5.3a), this highlights a key role for PER1 in melanocyte control.

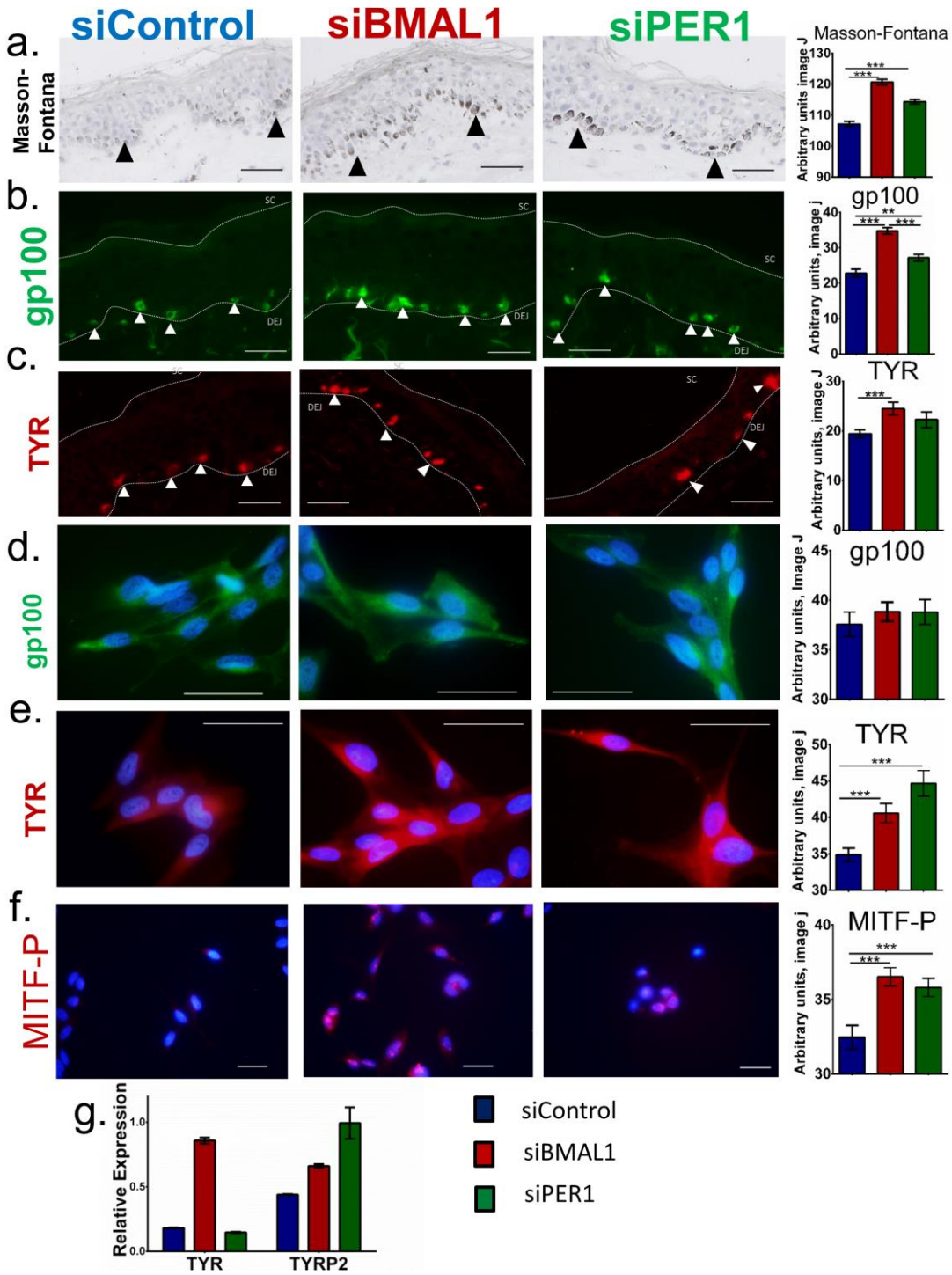


Fig. 5.4 Knock-down of clock proteins affects human skin pigmentation and cultured primary epidermal melanocytes.

To assess whether the observed results are specific to HF melanocytes or a feature of all melanocyte populations and to identify if the effects observed are specific to melanocytes, clock members *PER1* and *BMAL1* were silenced in skin biopsies and cultured primary melanocytes. Immunohistomorphometry demonstrated a significant increase in melanin content in skin (a) and gp100 immunoreactivity in both knock-down 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 BMAL1 and PER1 siRNA treated groups. Similarly MITF-P was significantly increased in knock-down HFs (f). (g) After knock-down in primary melanocytes there was also an increase in *tyrosinase (TYR*) and *TYRP1* in *BMAL1* silenced HFs with *TYRP1,2* increasing in the *PER1* knock-down (One way ANOVA, Holm-Sidak post hoc test, * p< 0.05, **p<0.01, ***p<0.001, error bars= ±SEM) (scale bar= 50µm). **[See appendix: *page 231-3* for individual panels].**

5.4.9 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 (e.g. via keratinocytes) or operate directly within human melanocytes. To answer this question, clock genes were silenced in isolated, cultured primary human epidermal melanocytes, i.e. in the absence of epithelial cell input.

This showed that both tyrosinase activity (Fig.5.4e) and MITF phosphorylation (Fig. 5.4f) were significantly increased in siRNA-treated melanocytes compared to control melanocytes (both $p<0.001$), with very few nuclei showing MITF-P immunoreactivity in the scrambled-oligo control group. Also, by qRT-PCR both tyrosinase and *TYRP2* mRNA steady state-level was increased in *BMAL1*-silenced melanocytes, whereas in the *PER1*-silenced melanocytes both *TYRP1* and *TYRP2* increased but *tyrosinase* transcription did not (Fig. 5.3a).Gp100 immunoreactivity and dendricity did not change significantly after clock silencing in cultured human epidermal melanocytes (Fig. 5.4d, Supplementary S1e).

5.5 Discussion

Here, we provide the first 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 knock-down 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 (Fig.1 a-c) confirms 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 biology and pigmentation. By demonstrating that clock gene (partial) silencing increases not only melanin synthesis, *tyrosinase* and *TRP1* gene expression and tyrosinase activity in the anagen HFs (Fig. 3a), but also the melanosome number in both melanocytes and keratinocytes (Fig.1 d-g) implying there was an increase in 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 (Fig. 2a). Moreover, phosphorylation of MITF, the “master regulator” of melanogenesis biology (Vachtenheim and Borovansky 2010) is significantly up-regulated after *PER1* knockdown (Fig. 3b & 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* knock-down (Fig. 2a & b), but recover in number and show

increased gp100 protein expression at day 4; in contrast, *PER1* silencing rapidly and lastingly up-regulates both the HF melanocyte number and gp100 protein expression. Since 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 survival 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 knock-down 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 (Fig. 4b & 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 melanocyte respond to DNA-damaging UVR stimulation by increasing dendricity, hormone production and pigment, as well as acting as “sensory” cells within the epidermis (Slominski et al. 2004; Plonka et al. 2009; Singh et al. 2010; Iyengar 2013). As both clock and melanocytes respond to UV light 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 *TYRP*2 transcript levels, which increased after clock silencing, suggesting that clock activity directly regulates the enzymatic

production of melanin (Supplementary Fig. 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), e.g. 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).

While 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 Fig. S2) and BMAL1 knockdown significantly decreased *PER1* mRNA and protein levels (Fig. 3a, d & 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 might exert a similar effect as Clock knock-out mice which show up-regulated 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.

As melanin is a major ROS-scavenger (Herrling et al. 2008; Plonka et al. 2009; Wood et al. 2009) and that both BMAL1 and PER1 are linked with ROS homeostasis (Avitabile et al. 2014). 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 up-regulation in activated MITF by PER1 silencing appears to play a key role in mediating the observed pigmentary phenomena. Unfortunately, this concept cannot be convincingly tested (e.g. by silencing or antagonizing MITF in the presence of *BMAL1* or *PER1* siRNA), 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 (Fig. 3b & c) by dampening clock gene activity. One plausible possibility is that this operates via the MAPK pathway, namely ERK 1/2 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 BMA1 or PER1 activity may up-regulate ERK1/2 activity and thus stimulate pigmentation. Since 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 were 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, e.g. 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.

5.6 Materials and methods

5.6.1 Human HF and skin samples

Discarded human scalp hair follicles and abdominal skin was obtained with informed, written consent following the 'Declaration of Helsinki Principles'. All tissue was received and stored with ethical and institutional approval from the University of Manchester and stored and carefully regulated in a human tissue biobank.

5.6.2 In vitro silencing of *BMAL1* and *PER1* in human HFs

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

5.6.3 Masson Fontana stain for melanin

HF or skin cryosections were fixed in ethanol:acetic acid (2:1) at -20°C. After fixation slides were washed in TBS and treated with in a 10 % silver nitrate solution with water (Fisher scientific, (Loughborough, UK)) 1ml of ammonium hydroxide solution (Thermo-scientific, London, UK) for 3 minutes in a microwave on low power. Sections were washed in 5% sodium thiosulphate (Sigma-Aldrich, Suffolk, UK) solution in water for 1 minute before being washed in dH_2O. Subsequently sections were counterstained with Haematoxylin, dehydrated and mounted.

5.6.4 HF fixation for high resolution light microscopy (HRLM)

After gene knock-down 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 0.5µm semi-thin sections were counterstained with toluidine blue. Images were taken using a 600x magnification (60x digital zoom) on a Keyence Biozero 8000 microscope (Osaka, Japan).

5.6.5 Gp100 immunofluorescence

OCT-embedded cryosections (5 µm) were fixed in acetone and stained for gp100 (PMEL, Silver locus protein) (Singh et al. 2008, 2010) by immunofluorescence microscopy using an Alexa Fluor® 488 (Life, Paisley, UK) goat-anti-rabbit secondary antibody following previously described protocol (22, 60).

5.6.6 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 normal goat serum HF/skin sections were incubated at room temperature overnight with primary antibody directed against MITF or phosphorylated (i.e. transcriptionally active MITF [MITF-P]) (Sigma-Aldrich, Dorset, UK) (27). Subsequently HFs were incubated with a goat-anti-rabbit Alexa Fluor® 594 secondary antibody for 45 minutes and counterstained with DAPI before mounting (**see materials and methods table 3.1).**

5.6.7 Tyrosinase activity *in situ* assay

Tyrosinase activity *in situ* was assessed by an immunofluorescence enzyme histochemistry technique using a TSA™ indirect kit (Perkin and Elmer®) (Han et al. 2002) and quantitative immunohistomorphometry (Gaspar et al. 2011, Samuelov et al. 2013).

5.6.8 Immunohistomorphometry

Immunoreactivity was quantified on images from multiple cryosections (6 µM) and measured with image J (NIH) software using defined set reference areas, as described before (Kloepper et al. 2010, Gaspar et al. 2011, Samuelov et al. 2013). For individual cell measurements (Figure 2a), measurements were corrected for cell size and background.

5.6.8 Total RNA extraction and qRT-PCR

RNA was extracted from 5 human HF or one human skin biopsies per group, using an RNeasy® micro kit (Qiagen). RNA was used to synthesise cDNA using a Tetro cDNA Synthesis kit (Bioline). Quantitative PCR was performed using a StepOne™ real time PCR system (Applied Biosystems) and the probes listed in *Chapter 3 materials and methods* (**table 3.2**). Steady state transcript levels were normalized to that of two housekeeping genes (PPIA, GAPDH) which was least regulated by clock gene silencing.

5.6.10 Human epidermal melanocyte culture

Isolated human scalp epidermal melanocytes were seeded at a density of 1.5x106 /ml and cultured in melanocyte growth media (Promocell, Heidelberg, Germany). For knock-down 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 knock-down (see Chapter 3. Material and Methods)

5.6.11 Statistical analysis

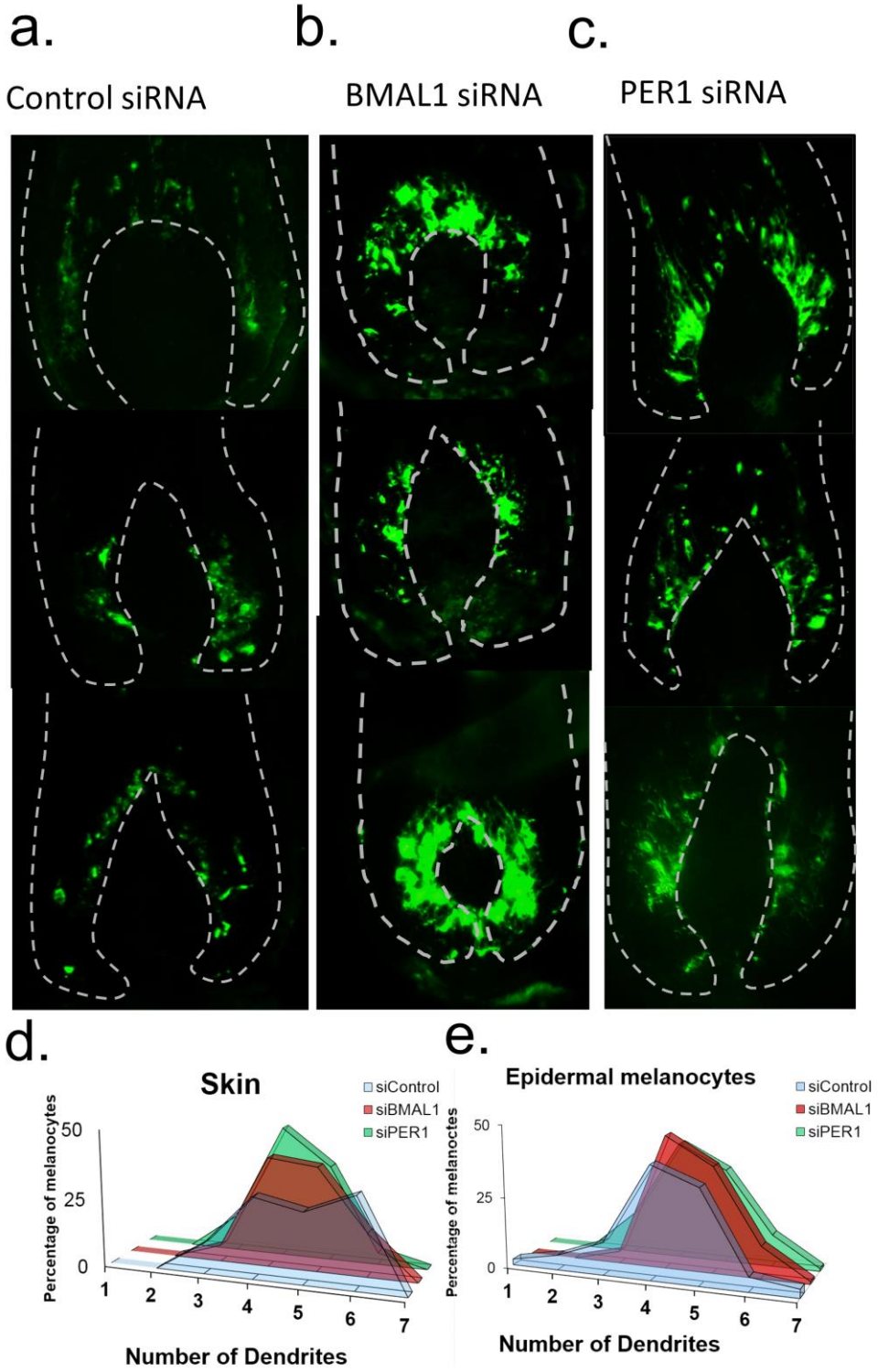
All statistical analysis was performed in Graphpad Prism® (La Jolla, CA, USA). All data was assessed for a normal distribution before statistical analysis using a D'Agostino-Pearson omnibus normality test. A one way ANOVA was used for all parametric datasets using a Holm-Sidak post hoc test. For melanin content (**Fig 5.1 a & b**) a Mann-Whitney test was used.

5.7 Declarations

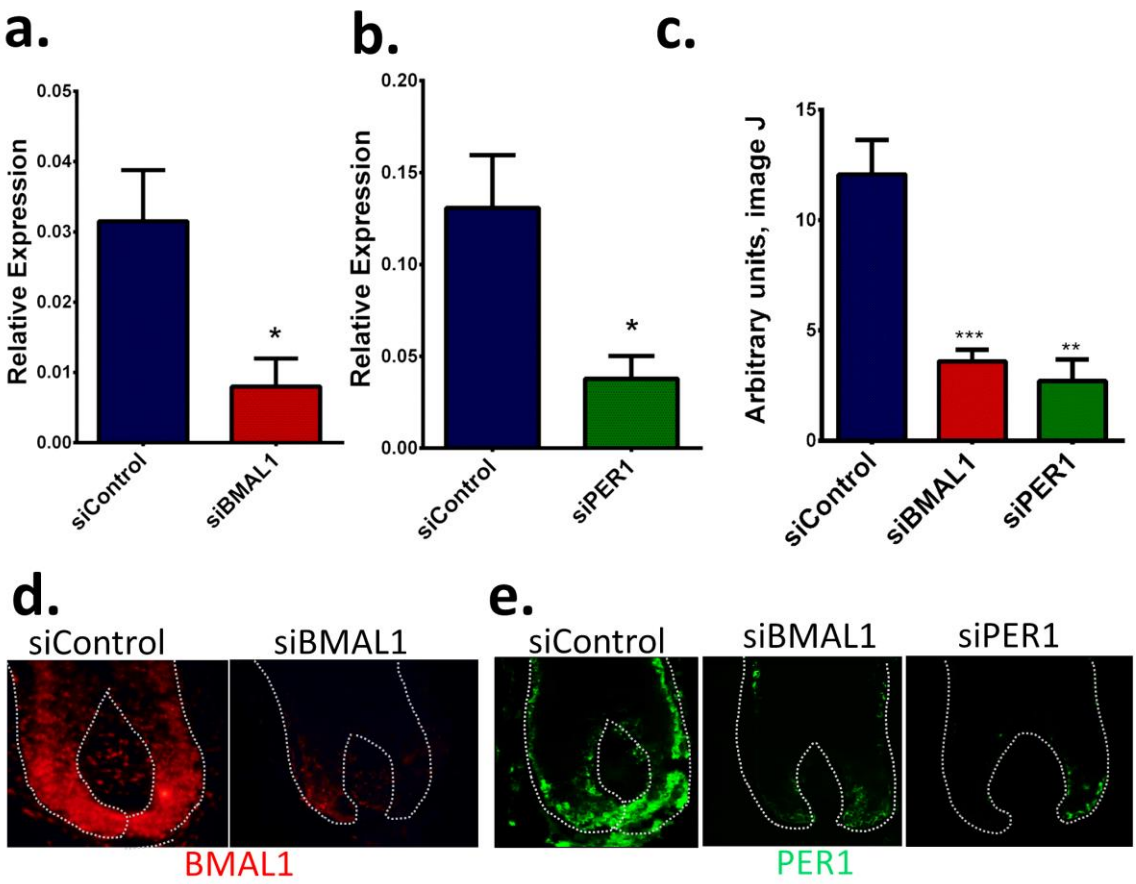
5.7.1 Acknowledgements

Dr Stephen Sikkink (University of Bradford) is gratefully acknowledged for providing primary melanocytes for cell culture as well as cell culture advice. We also are most grateful to Dr Ardeshir Bayat (University of Manchester) for kindly providing human skin samples.

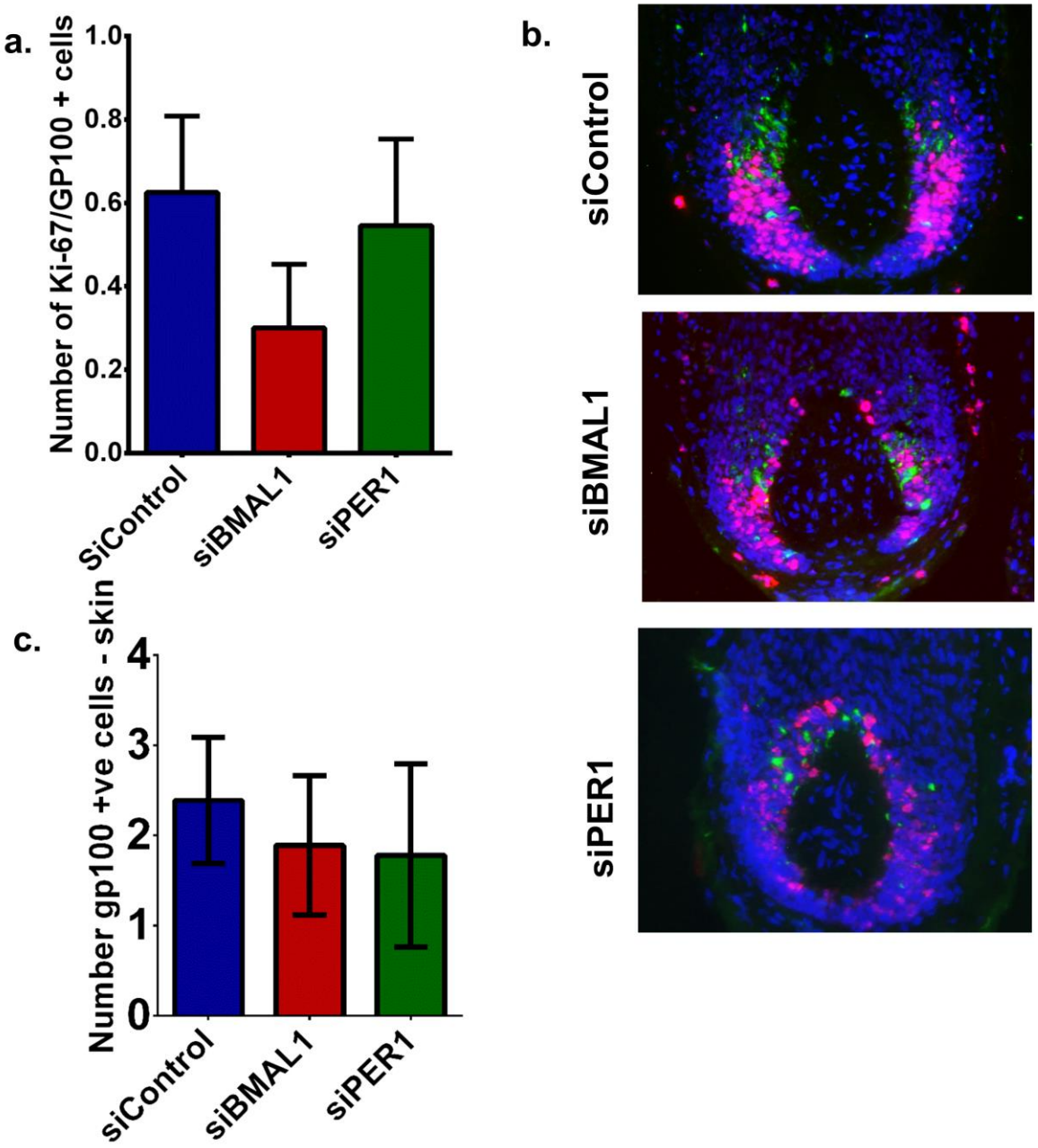
5.8 Supplementary Information



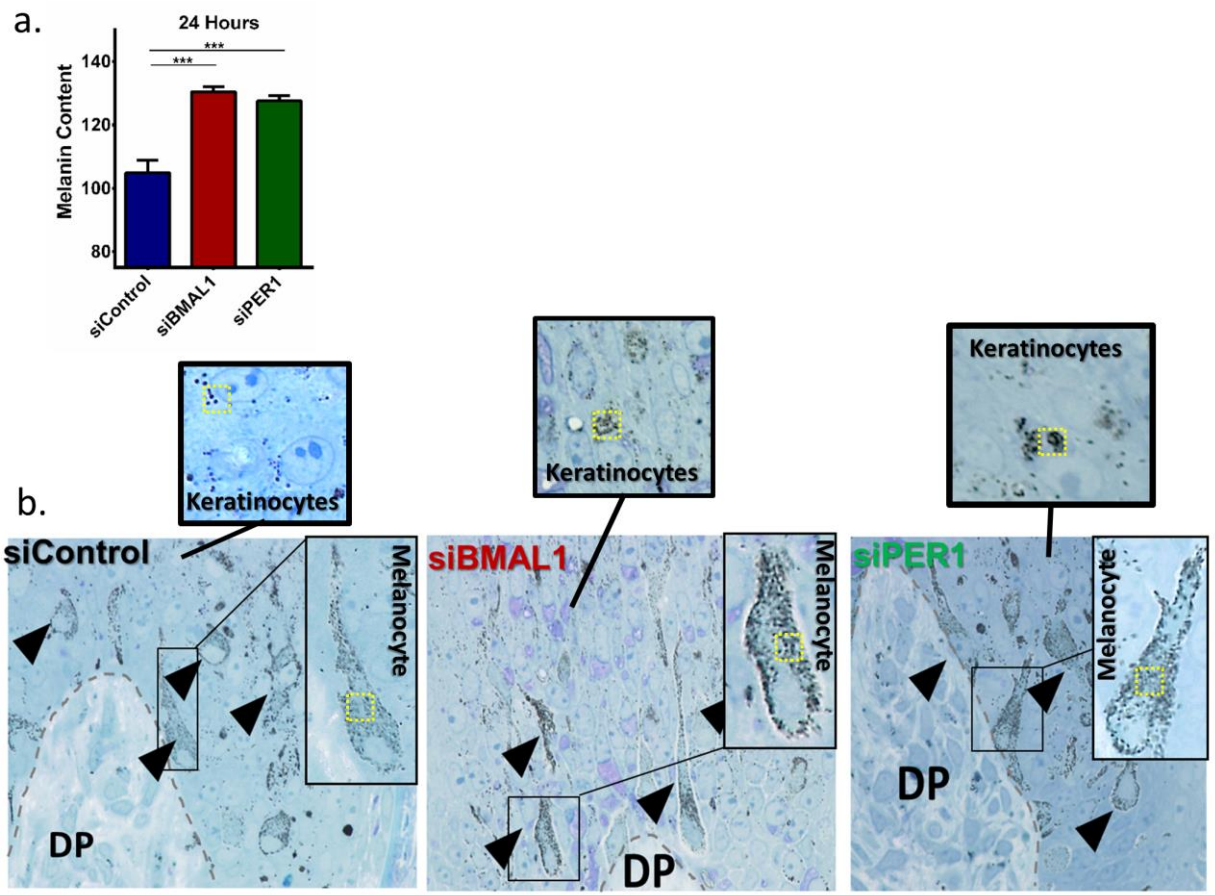
Supplementary Fig. S5.1. Dendricity analysis.
During analysis it became apparent that melanocytes in the clock silenced groups had a tendency to have a larger number of dendrites. Figure S3 a-c show multiple images from different hair follicles from different patients demonstrating qualitatively the increased number of dendrites in each knock-down group (gp100 positive cells). When counted in skin and primary melanocytes there was no difference in dendricity between groups (**S3d & e**) (n=96-98 per group). (scale bar= 50µm)



Supplementary Fig. S5.2. siRNA mediated gene knock-down efficiency analysis.
To verify the knock-down was successful it was necessary to check the steady state mRNA levels of either *BMAL1* or *PER1* in the respective knock-down group **(a, b)**. **(a, b)** Both groups showed a significantly reduced steady-state mRNA level of both clock genes 24 hours post-knockdown. To further validate the knock-down protein levels were assessed by immunofluorescence (d, e) for either BMAL1 or PER1 and quantified by immunohistomorphometry (d, e). The protein levels of BMAL1 were significantly decreased (c, d) in the BMAL1 silenced group as were the levels of PER1 in the PER1 silenced group (c, e). Furthermore, as BMAL1 directly leads to the transcription of PER1, the protein levels of PER1 in the *BMAL1* knock-down group were also assessed. Mirroring *PER1* silencing, the levels of PER1. Results are pooled from multiple patients. (One way ANOVA, Holm-Sidak post hoc test, $p<0.05$, $**p<0.01$, $***p<0.001$, *error bars are ± SEM).*



Supplementary Fig. S5.3. GP100 and Ki-67 dual stain for proliferative melanocytes.
To identify whether melanocytes are proliferative after silencing molecular clock members a dual stain was establish for gp100 for melanocytes and Ki67 for proliferative cells. Immunofluorescence identified there was little to no proliferation in most gp100 positive cells. The difference between each group was found to be non-significant. (c) The number of gp100 cells in a reference area was also counted on skin sections however, no difference in melanocyte number was found *(Error bars are ± SEM,* One way ANOVA, Holm-Sidak post hoc test*).*



Supplementary Fig.S5.4. High Resolution Light Microscopy additional analysis.

Melanosome associated melanin content from HRLM images was assessed to confirm the Masson-Fontana analysis (**Fig. 1a & b**) (**supplementary Fig. S6b**). There was a significant increase in melanin content in supporting previous observations. When counting the number of melanosomes per melanocyte it was necessary to use a reference area in each melanocyte to normalise the analysis (**Supplementary Fig. S6 a**). This was repeated three times per melanocyte. Additional images demonstrate the reference area used in melanocytes and keratinocytes. (One way ANOVA, Holm-Sidak post hoc test *$p<0.05$, **$p<0.01$, ***$p<0.001$, error bars are ± SEM).*

ANTIBODY	SPECIES	DETAILS
GP100	**Goat – Anti - Rabbit**	Catalogue number: ab137078 Supplier: Abcam®
MITF	**Goat – Anti - Rabbit**	Catalogue number: Av37977 Supplier: Sigma-Aldrich
MITF-phosphorylated	**Goat – Anti - Rabbit**	Anti-phospho-MITF ($pSer^{180/79}$) Catalogue number: SAB4503940 Supplier: Sigma-Aldrich®
Tyrosinase	**n.a.**	TSA™ Indirect kit Catalogue number: NEL70001KT Supplier: Perkin and Elmer®

Supplementary Table S5.1: Antibodies/ kits used for immunofluorescence stains.

PATIENT ID	AGE	SEX	EXPERIMENT
MSHB0400	63	Female (occipital scalp)	siControl/siBMAL1/siPER1 treated hair follicles: RNA analysis, high resolution light microscopy, Immunofluorescence. 24 hours and 4 day time points
MSHB0405	37	Males (occipital scalp)	siControl/siBMAL1/siPER1 treated hair follicles: RNA analysis, high resolution light microscopy, Immunofluorescence. 24 hours and 4 day time points
MSHB0406	36	Males (occipital scalp)	siControl/siBMAL1/siPER1 treated hair follicle: RNA analysis, high resolution light microscopy, Immunofluorescence. 24 hours
MSHB0416	42	Males (occipital scalp)	siControl/siBMAL1/siPER1 treated hair follicles: RNA analysis, high resolution light microscopy, Immunofluorescence. 4 Day
MSHB0449	53	Males (occipital scalp)	siControl/siBMAL1/siPER1 treated hair follicles: RNA analysis, high resolution light microscopy, Immunofluorescence. 24 hours and 4 day time points
MSHB0474	n/a	Female (skin)	siControl/siBMAL1/siPER1 treated abdominal skin: RNA analysis, high resolution light microscopy, Immunofluorescence. 24 hours and 4 day time points
MSHB0485	n/a	Female (skin)	siControl/siBMAL1/siPER1 treated abdominal skin: RNA analysis, high resolution light microscopy, Immunofluorescence. 24 hours and 4 day time points

Supplementary Table S5.2. Patient information.

Chapter 6: Thyroxine differentially modulates the peripheral clock: Lessons from the human hair follicle.

This chapter has been submitted to *PLoS One* and has been accepted for publication pending additional experiments (September 2014).

6.1 Authors

Jonathan A. Hardman, Nilofer Farjo, Bessam Farjo, Iain S. Haslam, Ralf Paus

Candidate's contribution

The candidate performed all experiments and analysis, created all figure and authored the manuscript.

Authors' contribution

NF & BF: Provided human hair follicles for all experiments.

ISH: Provided co-supervision to JAH, technical support for laboratory work and corrected the manuscript.

RP: Acted as the main supervisor to JAH for this project, designing the experimental strategy and contributing substantially to data interpretation and manuscript writing, and edited the manuscript

6.2 Abstract

The human hair follicle (HF) exhibits peripheral clock activity, with knock-down of certain clock genes prolonging active hair growth (anagen). Similarly, thyroid hormones prolong anagen in cultured human HFs and are recognized as key regulators of the central clock that controls circadian rhythmicity. Therefore, we asked whether thyroxine (T4) also influences peripheral clock activity in the human HF. After 6 and 24 hours we found a significant reduction in the transcript and protein levels of core clock members *BMAL1* and *PER1* in T4 (100 nM) treated HFs, while circadian rhythmicity of intrafollicular clock and cell cycle genes was maintained. Contrary to short term cultures, after 6 days, transcript and/or protein levels of all core clock genes (BMAL1, PER1, clock, CRY1, CRY2) were up-regulated in T4 treated HFs. *BMAL1* and *PER1* mRNA was also up-regulated in the HF bulge, the location of HF epithelial stem cells, with PER1 protein levels increasing in T4-treated keratin 15 (K15)+ HF progenitor cells. Intriguingly, intrafollicular K15 mRNA expression itself oscillated in a circadian manner, and was modulated by T4 administration. Together this provides the first direct evidence that T4 modulates the expression of the peripheral molecular clock. Thus, patients with thyroid dysfunction may also show a disordered peripheral clock, which raises the possibility that short term, pulsatile treatment with T4 might permit one to modulate circadian activity in peripheral tissues as a target to treat clock-related disease.

6.2 Introduction

There is an increasing appreciation for the role of the biological clock and its molecular components in maintaining tissue homeostasis (Geyfman et al. 2012b; Lai et al. 2012; Lee et al. 2013; Plikus et al. 2013; Avitabile et al. 2014). It is now understood that most peripheral tissues exhibit functional, oscillating molecular clock activity which is synchronised by a central master regulator, the suprachiasmatic nucleus (SCN) of the hypothalamus (Karatsoreos et al. 2011; Engelberth et al. 2014; Orozco-Solis and Sassone-Corsi 2014). When normal molecular clock activity is altered, e.g. during nightshift work, psycho-emotional stress or through poor diet, normal tissue homeostasis is disrupted triggering or aggravating disease, including metabolic syndrome, Alzheimer's disease, hypertension, diabetes, and cancer (Kondratov and Antoch 2007; Baron and Reid 2014; Kalsbeek et al. 2014; Mazzoccoli et al. 2014; Robinson and Reddy 2014; Sheikh-Ali and Maharaj 2014). Moreover, clock knock-out mice have an increased number of age-related pathologies, including reduced bone density and life span (Kondratov et al. 2006; Geyfman and Andersen 2010; Kondratova and Kondratov 2012). Finally, it is well-established that the pharmacological effects of drug administration on peripheral tissue function can be dependent on the circadian timing of drug administration (Giebultowicz 2004; Innominato et al. 2014; Ortiz-Tudela et al. 2014). Therefore a greater understanding of molecular clock regulation may pave the way for the development of novel therapies aimed at correcting clock dysfunction and maintaining normal tissue function.

Due to the complexity of circadian biology, research has primarily utilised *in vitro* cell culture models (Zanello et al. 2000; Sandu et al. 2012; Lippert et al. 2014), which cannot capture the complex interactions between difference cell types found within tissues, or animal models (Beasley and Nelson 1982; Lin et al. 2009; Geyfman et al. 2012b; Plikus et al. 2013; Al-Nuaimi et al. 2014). Moreover, it is insufficiently understood how intrinsic oscillatory behaviours found in peripheral human tissues, separate from the SCN, are regulated. Thus, it remains a major challenge for translational chronobiological research to identify clinically relevant, SCN-independent regulators of the human peripheral clock.

The human hair follicle (HF) is an ideal model system for biological research in areas ranging from molecular biology, stem cell biology to systems biology and chronobiology (Al-Nuaimi et al. 2010, 2014; Purba et al. 2014). The HF is a skin appendage which undergoes life-long cyclic transformations from an active growth phase (anagen) to a destructive phase (catagen) and a phase of relative quiescence (telogen) (Stenn and Paus 2001; Schneider et al. 2009). The molecular clock is now appreciated in hair cycle control both in mice, where clock activity is highly compartmentalised in anagen HFs (Plikus et al. 2013), with mice lacking the core clock protein *Bmal1* having a delayed onset of anagen (Lin et al. 2009), and humans, where clock genes/proteins expression has been shown in both human skin and plucked human hair shafts (Akashi et al. 2010).

Yet, a functional role for the peripheral molecular clock in human HF physiology has only recently been identified: *ex vivo*, human HFs not only maintain circadian rhythmicity of core clock gene (*CLOCK, BMAL1* and *PER1*) transcription in organ culture, but PER1 protein expression is also highly hair cycle dependent, increasing as HFs entered catagen (Al-Nuaimi et al. 2014). Functionally, knockdown of *PER1* or *BMAL1* prolongs HF anagen, implicating PER1, BMAL1 and clock target genes in the regulation of anagen-catagen switching during the human HF cycle (Al-Nuaimi et al. 2014). For this reason, human HF organ culture is a tractable and clinically relevant research model for understanding how the peripheral clock is regulated.

In the current study, we have examined the role of the thyroid hormone (TH), thyroxine (T4), a frequently administered hormone in clinical medicine, as a regulator of the peripheral clock (Mitchell et al. 2009). THs were selected as, on the one hand, HFs are known to be modulated by THs via signalling through the TH receptor beta (Billoni et al. 2000; van Beek et al. 2008; Tiede et al. 2010), which prolongs anagen, stimulates matrix proliferation and inhibits apoptosis in the human HF. Furthermore, T4 up-regulates the stem cell marker keratin 15 *in situ* after short-term application (van Beek et al. 2008; Tiede et al. 2010), increases mitochondrial activity and biogenesis and transcription of the clock gene, *BMAL1* (Vidali et al. 2014), mimicking some of the effects of clock knock-down in human HFs (Al-Nuaimi et al. 2014).

On the other hand, THs are known to influence the central clock (Kalsbeek and Fliers 2013; Yoshimura 2013; Kalsbeek et al. 2014). For example, the thyroid gland influences clock circadian

oscillations (Beasley and Nelson 1982) and is essential for seasonal rhythms and mating season timing in mammals and shows diurnal expression patterns (Dardente 2012; Dardente et al. 2014). Moreover, thyroidectomy alters circadian activity and blunts daily oscillations of PER2 (Amir and Robinson 2006) and T4 has been implicated in regulating metabolism (Dardente et al. 2014), a process regulated by the molecular clock (Sahar and Sassone-Corsi 2009; Bass 2012; Buxton et al. 2012). However, direct evidence that T4 modulates the peripheral clock in human tissues *in situ* is still missing.

Thus, T4 is a potential clinically relevant candidate for regulating peripheral clock activity, namely in the human HF. As the human HF is an easily accessible model system with robust and functional peripheral clock activity (Al-Nuaimi et al. 2014), we investigated whether T4 modulates peripheral clock activity in the human system in a physiologically and clinically relevant human mini-organ.

6.4 Results

6.4.1 Thyroxine is a gender-independent modulator of human HF cycling

First, we asked whether the previously reported anagen-prolonging effect of T4 on micro-dissected, organ-cultured human scalp HFs is robust and gender-independent, (van Beek et al. 2008) using occipital scalp HFs from male patients instead of female fronto-temporal HFs used in the van Beek *et al*. (2008) study. This showed that T4 administration over 6 days resulted in a significantly higher percentage of HFs in anagen (41%) compared to the vehicle control (26%) (p=0.007) (**Supplementary Fig. S6.1a**). Conversely, in the control group a higher percentage of HFs tended to be in early catagen (46%) compared to treated HFs (26%) (p=0.003) (**Supplementary Fig. S6.1a)**. This demonstrates that T4 is a potent, gender-independent modulator of human HF cycling *in vitro*, thus confirming T4 as a robust modulator of human HF cycling.

6.4.2 Intrafollicular clock gene and protein expression is significantly reduced by thyroxine treatment

Next, we investigated whether T4 treatment impacts on intrafollicular clock gene expression. Quantitative-RT-PCR of HFs treated with T4 for 24 hours demonstrated that there was a significant reduction in gene mRNA steady-state levels for the core clock genes (Partch et al. 2014; Robinson and Reddy 2014), *CLOCK*, *BMAL1* and *PER1* ($p < 0.001$)(**Fig. 6.1a**).

To compliment this we evaluated whether the protein levels were similarly reduced. This was assessed at two time points (6 and 24 hours) to eliminate any effects caused by a shift in circadian rhythmicity. Immunohistomorphometry demonstrated that at 6 hours there was a significant reduction in PER1 (p=0.014) and BMAL1 (p=0.18) protein levels when compared to vehicle controls (**Fig. 6.1 b-e**). This reduction was maintained after 24 hours for PER1 protein levels (p=0.16) (**Fig. 6.1c & e**) and while there was a tendency for BMAL1 protein level to be reduced, this was not statistically significant. Together these data show that intrafollicular clock gene and protein expression in human anagen scalp HFs is reduced by T4 treatment.

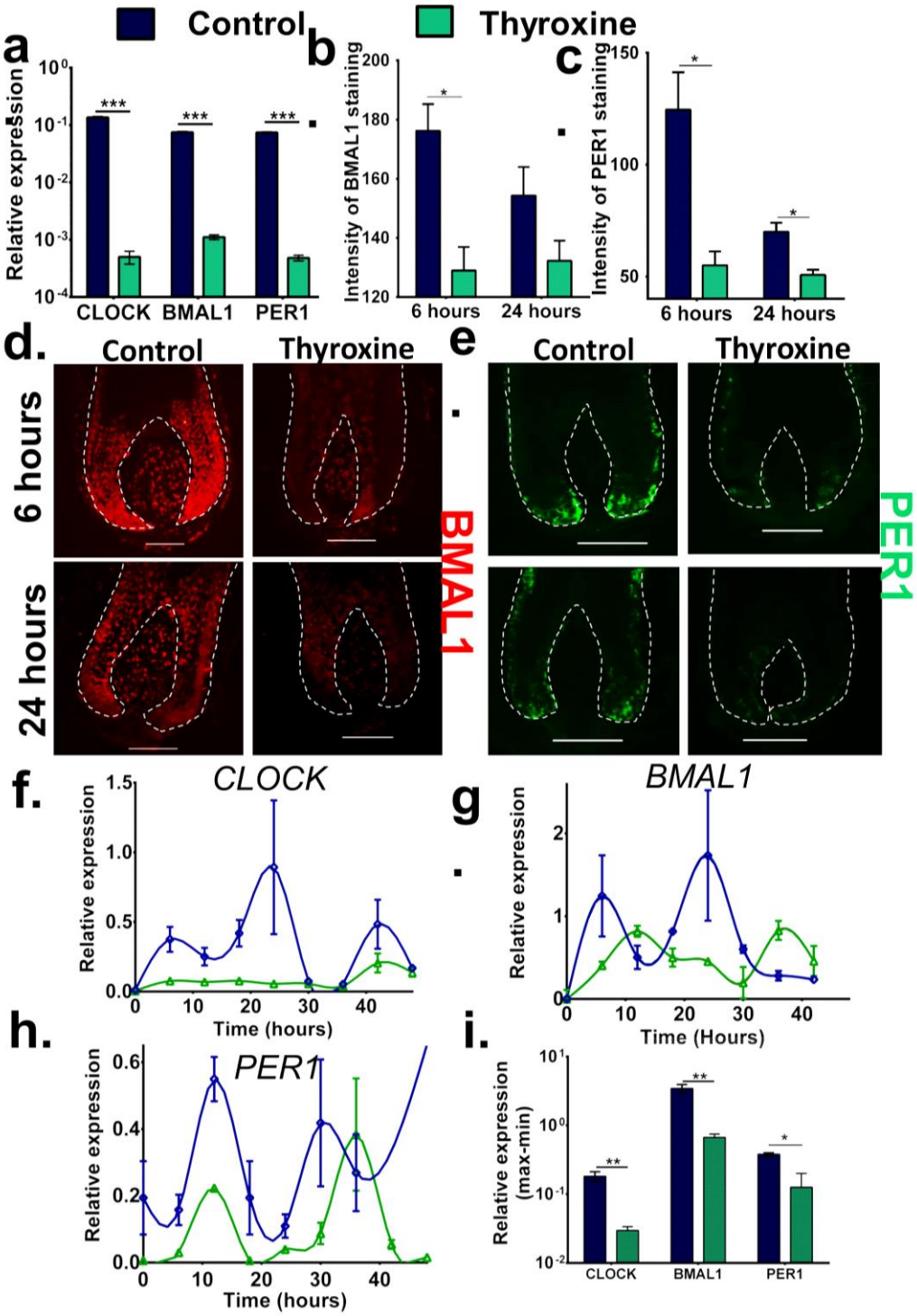


Fig. 6.1: BMAL1 and PER1 protein and transcript levels are reduced by thyroxine treatment.
To assess whether T4 affects clock gene/protein expression HFs treated with T4 for 6 and 24 hours were stained for either BMAL1 or PER1 and transcript levels were assessed. (a) qRT-PCR demonstrated that CLOCK, BMAL1 and PER1, were significantly down regulated by T4 expression (24 hours). (b-e) PER1 and BMAL1 protein were also significantly down-regulated at 6 hours with PER1 was also significantly down-regulated after 24 hours; however; whilst BMAL1 showed a tendency to decrease this was not significant. To assess the influence of T4 of on circadian expression of core clock genes, synchronised and treated HFs were sampled every 6 hours for 48 hours. (f-h) Quantitative-RT-PCR of clock transcripts shows that whilst clock rhythmicity is maintained, the amplitude of expression is reduced by T4 treatment, significantly for *CLOCK* and *BMAL1* (k). ($p < 0.05$ *, ** $p < 0.01$, *** $p < 0.001$, Student's Ttest ± SEM). (scale bar= 50µm). **[See appendix 3: *page 214* for individual panels.]**

6.4.3 Thyroxine dampens clock gene expression

To investigate whether the decrease in clock gene/protein expression was due to a loss of HF synchronisation and/or circadian rhythmicity, HF clock gene expression was assessed every 6 hours for 48 hours, comparing the amplitude of *CLOCK, BMAL1* and *PER1* gene expression in vehicle- or T4-treated male anagen VI HFs. These time course experiments demonstrated that over a 48 hour time period the amplitude of clock gene expression was indeed dampened, yet maintained circadian rhythmicity in gene expression (**Fig. 6.1 f-h**). To check for statistical significance the maxima and minima values were calculated for each patient (**Fig. 6.1 i**). *CLOCK* (p=0.006), *BMAL1* (p=0.005) and *PER1* (p=0.03) were all significantly decreased in T4 treated HFs. These data imply that whilst circadian rhythmicity was maintained, the clock gene transcript levels were lower suggesting T4 and may prolong anagen by reducing the overall amplitude of clock gene expression in human HFs *in situ.*

6.4.4 Long-term T4 treatment up-regulates clock gene mRNA and PER1 protein level

To assess how long-term administration of T4 affects intrafollicular clock activity, transcript level and protein level of core clock genes *BMAL1* and *PER1* were assessed after 6 days. This showed that, in contrast to HFs cultured for 6 and 24 hours, transcript levels of core clock genes (*CLOCK* [p=0.030]*, PER1* [p=0.029]*, BMAL1* [p=0.013]*, CRY1* [p=0.010] and *CRY2* [p=0.014]) were significantly up-regulated by T4, as assessed by qRT-PCR (**Fig. 6.2 a**).

Next, to evaluate whether this regulation occurred only at the level of transcription or translated also onto the protein level, human HF sections were immunostained for BMAL1 and PER1 after 6 days of HF organ culture in the presence of T4. Quantitative immunohistomorphometry showed that there was a significant increase in PER1 protein expression in the human HF (**Fig. 6.2 b & c**) after 6 day treatment with T4 (p=0.017). In contrast, BMAL1 protein expression tended to be lower in T4 treated HFs, yet this was not statistically significant (p=0.10) (**Fig. 6.2 b & d**). These findings suggest that selected intrafollicularly expressed clock genes are up-regulated by long-term T4 treatment on both the transcript and protein level.

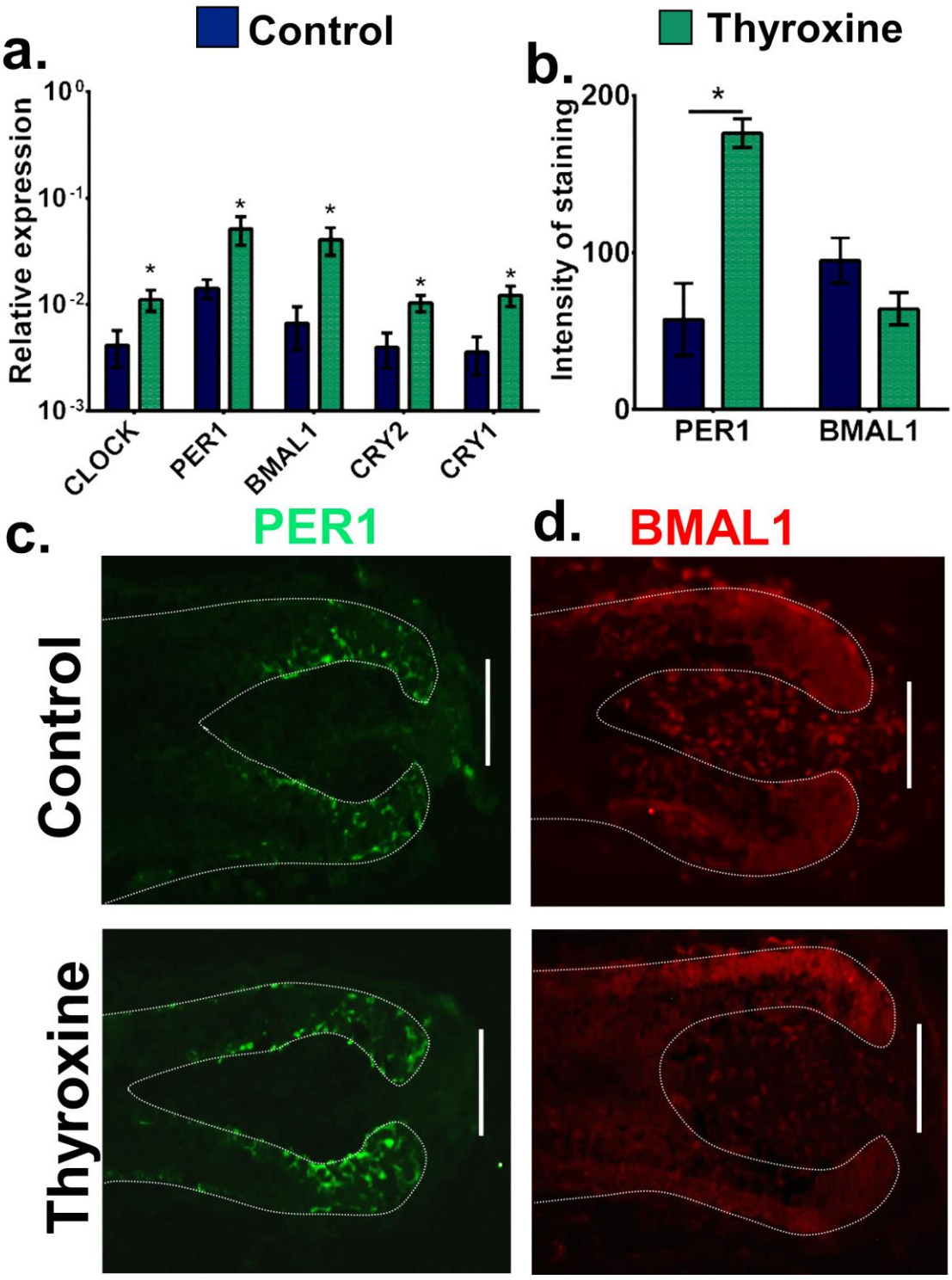


Fig. 6.2: 6 day T4 treatment increases Clock transcript levels and PER1 protein.
The effects of T4 on clock gene expression were assessed on HFs treated for 6 days by immunofluorescence and qRT-PCR. (a) All core clock genes were significantly up-regulated at 6 days by T4 treatment. The Protein expression of PER1 was also up-regulated after 6 days (b & c) however, BMAL1 expression remained unchanged (b & D). (Student's Ttest, * $p < 0.05$, ± SEM). (scale bar= 50µm). **[See appendix 3*: page 235* for individual panels.]**

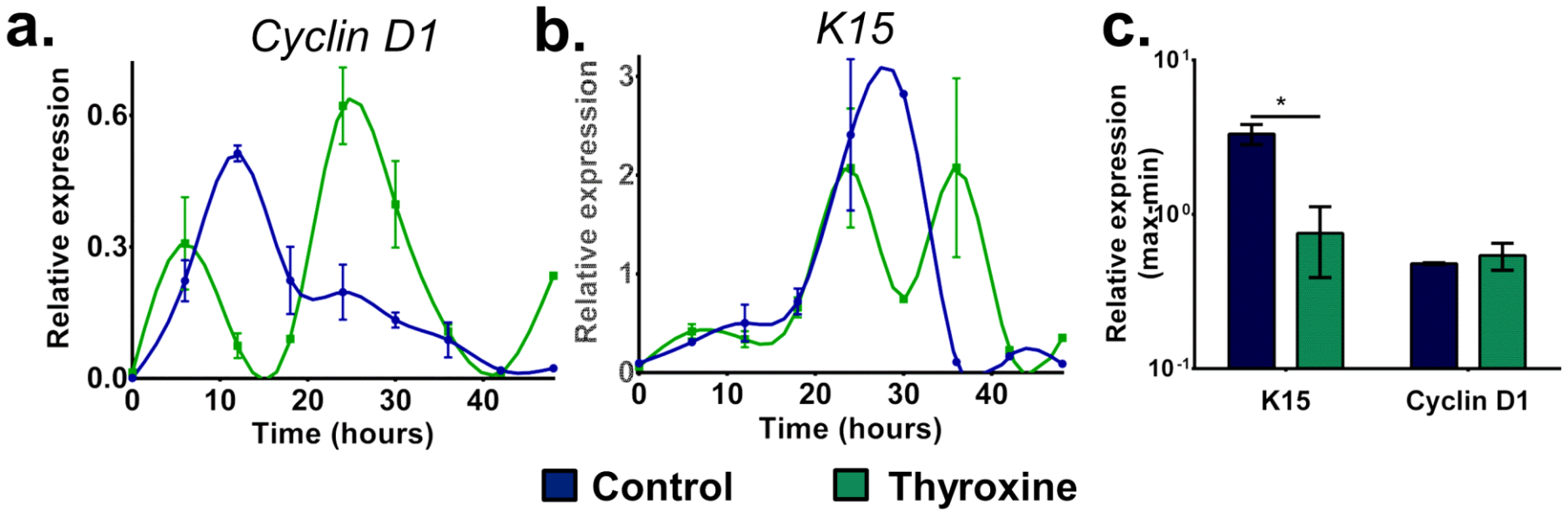


Fig. 6.3: K15 and Cyclin D1 transcript levels oscillate over 48 hours.
To probe is T4 modulation is functionally relevant, transcript levels of *Cyclin D1* and stem cell marker *K15* was assessed over 48-hours. Both *K15* (a) and *Cyclin D1* expression oscillates with *K15* being significantly decreased by T4 (c.). (Student's test *$p < 0.05$, ** $p < 0.01$, ±SEM).

6.4.5 Thyroxine modulation of peripheral clock activity via cyclin D1?

In order to probe potential mechanisms of the how T4 modulation of clock activity impacts on HF biology, we next investigated the effects of T4 stimulation on cyclin D1, a key cell cycle-regulatory gene that is well-recognized and as a regulator of human hair matrix keratinocyte proliferation (Xu et al. 2003; Rana et al. 2014). (**Fig. 6.3 a & c**). In line with a previous report from murine HFs that has implicated that cyclins show circadian expression patterns in hair matrix keratinocyte proliferation (Plikus et al. 2013), cyclin D1 showed a strong diurnal expression pattern over the 48 hour time course. While there was no significant difference in the cyclin D1 expression level between treated and control HFs, on day 2 (24hours to 48 Hours) the circadian rhythmicity of cyclin D1 remained at a high amplitude in T4 treated HFs, whilst this tended to decrease in the control group. This suggests that the intrafollicular "T4-peripheral clock connection" revealed may act on the cyclin D1-controlled G2-M cell cycle checkpoint (Sancar et al. 2010) of proliferating human hair matrix keratinocytes *in situ*.

6.4.6 K15 expression shows T4-regulated circadian rhythmicity *in situ*

Previously, we had shown that keratin 15 (K15) promoter activity, gene and protein expression in human scalp HFs is up-regulated by short-term TH treatment (Tiede et al. 2010). Given the recognized impact of THs on intrafollicular K15 expression and the key role of K15 in human epithelial stem cell biology (Cotsarelis 2006a; Purba et al. 2014), the peripheral clock modulation by T4 might also be functionally relevant for human HF stem cell biology, provided that one can show that a) K15 expression in human HFs displays circadian oscillations, and b) that this rhythmicity is modulated by T4. However, it is as yet unknown whether there are circadian variations in K15 expression in the HF, or in any other tissue.

Therefore, we first asked whether human HFs show circadian oscillations in their K15 transcription pattern. It was found that K15 transcript levels show circadian rhythmicity over a 48 hour time period (**Fig. 6.3 b and c**). To the best of our knowledge, this represents the first evidence that K15 expression is regulated by the peripheral clock, namely in human anagen scalp HFs. Importantly, T4 treatment significantly reduced the average K15 mRNA expression amplitude in human HFs ($p=0.13$) (**Fig. 6.3 b & c**). While the functional significance of K15 expression for human epithelial

progenitor cell survival and function remains poorly understood (Purba et al. 2014), the fact that T4 modulates the circadian rhythmicity in the expression of this key stem cell marker already suggests that the T4 modulation of the intrafollicular peripheral clock is functionally relevant for human HF biology.

6.4.7 PER1 and BMAL1 transcript levels are increased in bulge stem cells.

This observation raised the question; do epithelial HF stem cells express clock genes *in situ* and is this expression is regulated by T4? Therefore, we finally asked whether the bulge region of human HFs expresses clock genes and whether this expression is regulated by T4.

First, it was necessary to establish whether K15+ epithelial progenitor cells in the bulge/isthmus region of the HF epithelium cells express clock genes *in situ.* Using a dual stain for either PER1 and BMAL1 we were able to show that the stem cells do express both BMAL1 and PER1 (**Fig. 6.4 a**).

To analyse this, following culture with T4, human anagen VI HFs were first bisected and the upper (distal) part of the HF epithelium (well above the bulb), which contained all the bulge-associated HF stem cells, was analysed by qRT-PCR. This demonstrated that not only did the bulge stem cells express clock genes, but also that their expression was significantly increased following treatment with T4 (*BMAL1* (p=0.001) *PER1* (p=0.04) (**Fig. 6.4 b**). However, quantitative immunohistomorphometry of PER1 and BMAL1 protein demonstrated that T4 did not significantly change BMAL1 protein levels in K15+ cells *in situ*, although there was a tendency towards an increase in the level of PER1 immunoreactivity (also not significant) (**Fig. 6.4 d,e**). This may suggest that T4 regulates clock gene expression in human epithelial progenitor cells primarily at the level of transcription.

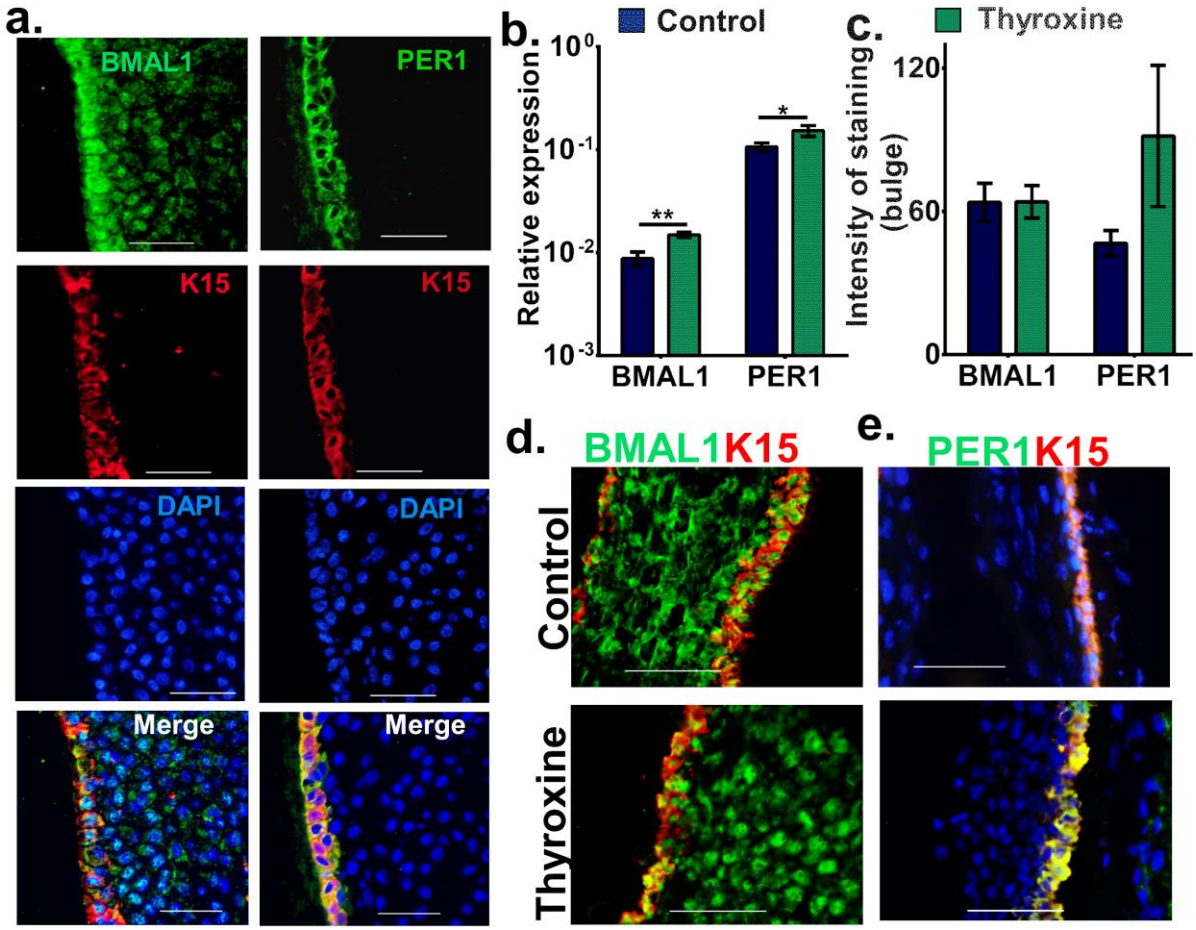


Fig. 6.4: K15+ stem cells express BMAL1 and PER1 protein.
(a) After establishing that K15 positive cells express BMAL1 and PER1 the role T4 on clock gene expression in the k15+ bulge stem cells was assessed by qRT-PCR and quantitative immunohistomorphometry. Transcript levels of BMAL1 and PER1 were up-regulated after 6 days (b), however, whilst PER1 showed a tendency to increase neither BMAL1 nor PER1 protein levels increased significantly. (scale bar= 50µm). **[See appendix 3: *page 236-8* for high-resolution panels].**

6.5 Discussion

Here we present the first evidence that T4 is a modulator of peripheral clock activity in a model human (mini-)organ in the absence of central clock inputs. We show that T4 differentially modulates clock gene activity, with short-term stimulation significantly reducing intrafollicular transcript and protein levels of core clock members *BMAL1* and *PER1*, while circadian rhythmicity of intrafollicular clock and cell cycle gene expression is maintained. In contrast, longer-term T4 stimulation up-regulates transcript and/or protein levels of all core clock genes (*BMAL1, PER1, CLOCK, CRY1, and CRY2)*. The effect of T4 on intrafollicular clock activity is likely to have a functional impact on HF biology, since it modulates the circadian rhythmicity of intrafollicular cyclin D1 expression and of keratin 15, *BMAL1* and *PER1* expression in the bulge, the niche for HF epithelial stem cells.

That T4 reduces the gene and protein levels of BMAL1 and PER1, two core clock genes whose knock-down promotes hair growth and prolongs anagen (Al-Nuaimi et al. 2014) raises the possibility that the anagen-prolonging effects of T4 in human scalp HFs (van Beek et al. 2008) are, at least in part, mediated by reducing the intrafollicular activity of these clock genes. However, this hypothesis could not be definitively tested as it is not yet possible to *selectively* up-regulate BMAL1 and/or PER1 expression and activity in a human organ (e.g. by engineered overexpression).

This study documents that peripheral clock activity is hormonally regulated in the human HF. However, in order to convincingly support this claim, it must be demonstrated that any differences observed were not simply caused by normal diurnal changes in clock gene expression patterns. Therefore, intrafollicular clock activity was synchronised by dexamethasone treatment (Balsalobre 2000). This allowed us to conclusively demonstrate that the observed changes in HF clock gene and protein expression were genuinely caused by T4, rather than by constitutive circadian oscillations.

The regulation of the peripheral clock by T4 reported here is not surprising, as T4 has long been associated with regulation of the molecular clock. For example, the thyroid gland influences clock circadian oscillations (Beasley and Nelson 1982), and triiodothyronine (T3), which is intracellularly metabolised from T4 and is considered to represent the main biologically active TH (Dardente 2012), is needed for development of the central circadian clock (Chu et al. 2012). Moreover, thyroidectomy dampens *PER2* oscillatory activity in rats (Amir and Robinson 2006), while T4 shows distinct diurnal expression patterns, along with thyrotropin and triiodothyronine (T3) (Tonsfeldt and Chappell 2012), and is essential for seasonal rhythms and mating season timing (Dardente 2012; Dardente et al. 2014). Despite this, our data is the first to demonstrate the direct role of T4 in modulating peripheral clock activity in human tissues *in situ.*

As cyclin D1 is a key player in cell-cycle progression, namely during the G2/M transition (Sancar et al. 2010) and since the circadian clock is tightly coupled to cell-cycle progression (Griniatsos et al. 2006; Gérard and Goldbeter 2012; Plikus et al. 2013) the effect of T4 treatment on cyclin D1 was investigated (Tiede et al. 2010). Although there was no significant difference in the mean amplitude of expression; on the second day of the time course experiment T4 treated HFs clearly showed a higher amplitude of cyclin D1 expression. That T4 is able to prolong the rhythmic expression of cyclin D1 suggests that the anagen-prolonging effect of T4 may be mediated by maintaining robust, extended circadian expression of cyclin D1. Indeed, a reduction in *Bmal1* increases proliferation and cyclin D1 expression in both murine cells and tumours and decrease apoptosis (Zeng et al. 2010)and furthermore, a reduction of *BMAL1* and *PER1* in human is also associated with an increase in cyclin D1 expression and cell progression (Rana et al. 2014). Previous work has suggested that cyclin D1 may also mediate the transition of bulge stem cells to their more differentiated, rapidly proliferating progeny (transient amplifying cells) in the suprabasal ORS of human HFs (Xu et al. 2003). On this background, the T4-mediated reduction in clock gene expression may lead to prolonged rhythmic expression of cyclin D1; this, in turn, may prolong the duration of anagen. It should be noted that in the time course experiments there is often a shift in cycling peaking at different times of day, this is to be expected as thyroid hormones have been shown to be able to lengthen circadian periodicity (Beasley and Nelson 1982).

As long-term T4 treatment prolongs anagen (van Beek et al. 2008) (**Fig. 6.1**) while high levels of clock proteins promote catagen (Al-Nuaimi et al. 2014), we asked whether the reduction in clock gene and protein levels was maintained by long-term T4 stimulation. Contrary to expectations, this was not observed, since transcript levels of all the core clock genes investigated were significantly up-regulated. However, this effect only translated to the level of protein expression for PER1. This suggests that the activity of the molecular clock as a system needs to be up-regulated in order to induce catagen, while up-regulation of just one of its protein components is insufficient. Alternatively, the increase in clock transcript levels could result from events in selected intrafollicular cell subpopulations outside of the hair matrix. Interestingly, long-term T4 treatment also increased clock gene expression in bulge epithelial stem cells (**Fig. 6.4**). Moreover, long-term TH receptor stimulation may induce complex gene regulation cascades that impact on intrafollicular clock gene expression and reverse the effects of a short-term pulse of T4 treatment. One potential mechanism is via T4 conversion to T3 which in turn leads to production NCOR1. NCOR1 is believed to associate with the accessory clock loop involving REV-ERBα with ultimately represses BMAL1, CLOCK and therefore PER1 production (Aninye et al. 2014).

The evidence provided here that T4 modulates not only the central, but also the peripheral clock, namely in human HFs, has important clinical implications. Our observations suggest that patients suffering from thyroid dysfunction are likely to also show a disordered peripheral clock and that this may contribute to the overall pathology associated with abnormally low or high serum TH levels. Furthermore, our study raises the intriguing possibility that, in diseases associated with clock dysfunction, such as hypertension and type 2 diabetes (Kondratov and Antoch 2007; Kalsbeek et al. 2014; Robinson and Reddy 2014), short-term, pulsatile treatment with T4 might permit one to modulate circadian activity in diseased peripheral tissues in a therapeutically beneficial manner, therefore providing a novel strategy for differential endocrine “peripheral clock therapy”.

6.6 Materials and Methods

6.6.1 Human hair follicle collection and culture

Discarded human HFs from hair transplant surgery were obtained with full written consent adhering to the 'declaration of Helsinki principles'. Human tissue was stored in a registered biobank following human tissue act guidelines and stored with ethical and institutional approval from the University of Manchester.

6.6.2 Human organ culture.

Human scalp HFs were dissected on the day of surgery from surrounding tissue and cultured at 37°C with 5% CO_2 in a minimal media of William's E media (Gibco®, Life technologies) supplemented with 2mM of L-glutamine (Gibco®), 10ng/ml hydrocortisone (Sigma-Aldrich), 10µg/ml insulin (Sigma-Aldrich) and 1% penicillin/streptomycin mix (Gibco) (Philpott et al. 1990). On the following day HFs were treated with 100nM of dexamethasone for 30 minutes to rest HF clock activity at 8:30am so all HFs had synchronised activity. At 9am HFs were cultured in minimal media (William's E media with clinically physiologically relevant levels of T4 (100nM) (Tiede et al. 2010) (Sigma-Aldrich, Dorset, UK) with a parallel control. HFs were either snap frozen in OCT or RNAlater® (Ambion, Connecticut, USA) 6 hours, 24 hours or 6 days later. For 6 day culture HFs were taken at 9am and 3pm to exclude that any differences in protein level were caused by any diurnal expression changes.

6.5.3 Time course experiments

For time course experiments HF clock activity was synchronised and HFs for 30 minutes with dexamethasone. Following synchronisation HFs were cultured with 100nM T4. HFs were removed from culture immediately after synchronisation (0 hours) and then subsequently sampled every 6 hours for 48 hours. Five HFs were taken from control and treated at each time point which were pooled for RNA extraction. Results are from three patients for 0-24 hours and two patients 0-24 hours.

6.6.4 RNA extraction and qRT-PCR.

HF RNA was extracted using an RNeasy® micro kit (Qiagen, Manchester, UK). Complimentary DNA was synthesised from this using a Tetro cDNA synthesis kit (Bioline, London, UK) and used for qRT-PCR at 10ng/µl per well. Quantitative-PCR was performed using the StepOne™ real time PCR system (Applied Biosystems, Warrington, UK) using Taqman® (Applied Biosystems) fast Advance master mix and probes listed in supplementary table S1. Results were performed in triplicate for each experiment. For experiments looking at the bulge clock gene expression HFs were bisected with a scalpel above the HF bulb separating the proliferating bulb and the upper ORS containing the bulge region. RNA was extracted from each region separately.

6.6.5 Immunofluorescence.

Human HF cryosections (5µm thick) were fixed at -20°C in acetone (PER1, rabbit-anti human PER1 (Santa Cruz)) stain or methanol (BMAL1, rabbit-anti human (Genetex)). HFs were washed in PBS and pre-incubated with 10% normal goat serum. For dual stains with K15, the protocol for either PER1 or BMAL1 was followed with the addition of mouse- anti K15 (Abcam®, Cambridge, UK). Clock proteins were stained green using goat- anti rabbit Alexafluor®488 (Life technologies, Paisley, UK) secondary antibody. K15 was visualised in red using goat-anti-mouse Alexafluore®594. HF sections were subsequently imaged using a Keyence Biozero fluorescence microscope (Osaka, Japan).

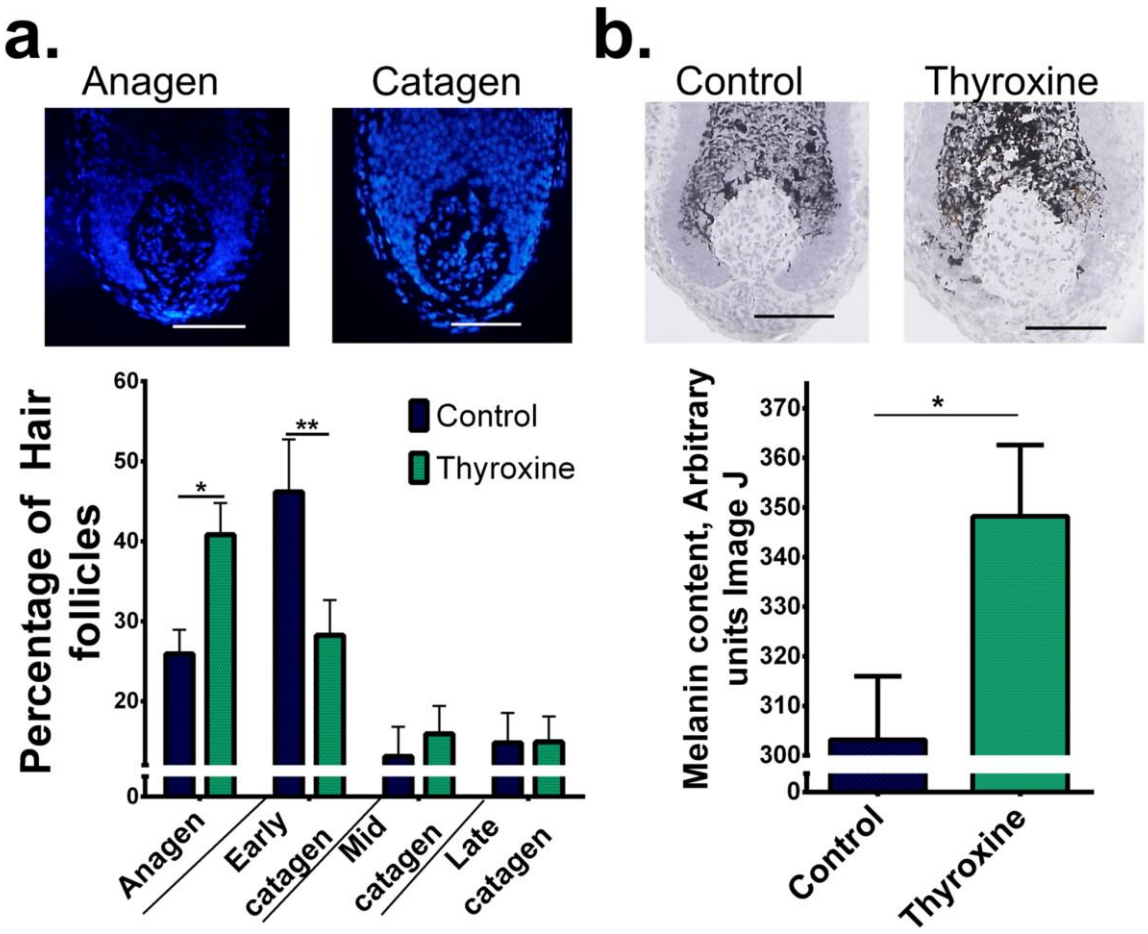
6.5.6 Statistical analysis

Data was assessed for normality using a D'Agostino-Pearson test in Graphpad prism 6. For normal data to test for significance a student's Ttest was used. Where the data did not follow a normal distribution a Mann-Whitney test was used. Data was corrected for multiple comparisons using the Holm-Sidak equation.

Acknowledgements

We would like to acknowledge Weiping Li and Talveen Purba for technical help with the time course experiments.

6.7 Supplementary information



Supplementary Fig. S6.1: Confirmation of the role of thyroxine (T4) on hair follicle (HF) physiology. Thyroxine has been shown to promote melanin production in HFs and prolong anagen. To confirm this HFs were cultured and the percentage remaining in anagen (a.) was assessed by morphological criteria and melanin content was assessed by Masson-Fontana. Results demonstrated that in the treated group a significantly higher percentage of HFs remained in anagen (a.) and had a higher melanin content (b.) confirming previous results (error bars = ±SEM, * $p<0.05$, ** $p<0.001$, Student's Ttest).(scale bar= 50µm).

Gene	Taqman® assay ID	Full gene name
PPIA	**Hs99999904_m1**	Peptidylprolyl isomerase A
GAPDH	**Hs02758991_g1**	Glyceraldehyde 3-phosphate dehydrogenase
CLOCK	**Hs00231857_m1**	Circadian-locomotor output cycle kaput (clock homolog (mouse)
BMAL1	**Hs00154147_m1**	aryl hydrocarbon receptor nuclear translocator-like
PER1	**Hs00242988_m1**	Period homolog 1 (Drosophila)
CRY1	**Hs01597804_m1**	Cryptochrome-1
CRY2	**Hs00901396_m1**	Cryptochrome-2
K15	**Hs00267035_m1**	Cytokeratin 15
CCND1	**HS00765553_m1**	Cyclin D1

Supplementary Table S6.1: A list of Taqman® advance probes used for qRT-PCR.

Chapter 7: Conclusions and future perspectives

While the molecular clock had been linked to the murine HF cycle at the beginning of this thesis project (Lin et al. 2009), it was unclear in the literature whether this was relevant to the human HF. A previous thesis project in the lab had already provided preliminary evidence that PER1 shows hair cycle-dependent expression, that human HFs express selected clock genes rhythmically over a 24 hour cycle, and that silencing PER1 might alter human HF cycling in vitro (Al-Nuaimi 2011). The current thesis project, therefore, continued this work, helped to generate the first definitive evidence linking the peripheral molecular clock and the human hair cycle, and expanded the research linking the chronobiology of the human HF into the area of human pigmentation and the endocrine control of intrafollicular clock activity.

This project began by asking whether the human HF had a functional, oscillatory molecular clock. It uncovered that the human HF does indeed express several clock and clock-controlled genes other than *PER1*, (*BMAL1*, *cyclin D1, c-Myc,* and *Nr1d1*) as well as PER1 and BMAL1 protein, in a circadian manner. Furthermore, it confirmed that the core clock protein PER1 shows strong hair cycle-dependent expression, which increases as human HFs enter catagen. In contrast to this, BMAL1 protein expression is not significantly regulated during the human anagen-catagen switch. Moreover, this project demonstrated that circadian rhythmicity of clock transcripts is maintained over 48 hours, thus providing evidence that the HF does have peripheral molecular clock activity, which is independent of the central clock. The latter is of particular importance as previous work in both mice and human (Lin et al. 2009; Akashi et al. 2010) had not distinguished between the synchronising central clock and intrinsically oscillating peripheral clock activity. This discovery is in line with the hypothesis that intrafollicular changes in peripheral clock activity contributes to the “hair cycle clock” (Paus and Foitzik 2004) of human scalp HFs.

This hypothesis was further supported by the demonstration that not only does silencing of *PER1*, but also knock-down a second core clock gene, *BMAL1*, impacts on human HF cycling *in vitro*: The current thesis project uncovered definitive evidence that core clock silencing significantly prolongs anagen in the absence of central clock inputs, therefore demonstrating that the molecular clock has a major a functional role in human HF biology. Given that abnormalities in HF cycling can lead to both undesired hair growth or hair loss (Paus and Cotsarelis 1999, Schneider et al. 2009), this

finding is translationally important as it identifies a new molecular target through which human hair growth might be clinically manipulated. Thereby reducing the psychosocial stress associated with hair loss or unwanted hair growth (Blume-Peytavi 2013).

Because melanogenesis is tightly coupled to the hair cycle (Slominski et al. 1994, 2005a) and as cultured human melanocytes express clock genes and proteins (Zanello et al. 2000; Sandu et al. 2012), this thesis project next asked whether the molecular clock also influences pigment production (melanogenesis) and/or other aspects of melanocyte biology in the human HF *in situ*. As HF pigment production is tightly coupled to anagen (Slominski et al. 1994), this had to be distinguished from any changes that could be attributed to the anagen prolonging effect of molecular clock silencing.

By knocking down core clock genes *BMAL1* or *PER1* and examining the effect of this manipulation exclusively on anagen VI HFs, the project showed that melanin content, melanocyte dendricity, cell number and melanosome number all are increased by clock gene silencing in a hair cycle-independent manner. Furthermore, transcript levels and activity of the rate-limiting enzyme of melanogenesis, tyrosinase, was similarly increased suggesting the rate of melanin production was also increased. Together this demonstrates for the first time that the peripheral molecular clock influences multiple levels of human melanocyte biology *in situ* (Lengyel et al. 2013b) and therefore implicates the peripheral clock as a new regulator of human pigmentation.

As the molecular clock was shown to exert complex, multi-level influences on both melanocytes and melanogenesis, the potential mechanism of action was next investigated. MITF is the master regulator of melanocytes and causes the transcription of tyrosinase, *TYRP1* and *TYRP2* (Cheli et al. 2010). As all enzymes listed were increased by clock gene silencing, changes in *MITF* gene and protein expression as well as in MITF protein phosphorylation were investigated in clock-silenced HFs. Although MITF expression did not significantly change, the immunoreactivity of activated (phosphorylated) MITF was increased in *PER1* knock-down HFs. This suggests that an increase in MITF activation is at least in part responsible for the increase in melanin content observed in clock-

silenced human HFs, and provides the first evidence in the literature that MITF phosphorylation is clock-regulated, namely by PER1.

To conclude the investigation into the molecular clock's role in human melanogenesis and to address whether this newly uncovered regulation is HF-specific, the impact of clock silencing on epidermal melanocytes *in situ* and on isolated epidermal melanocytes was investigated. Together these experiments demonstrate that the clock regulation of human pigmentation is not HF-specific and also applies to human epidermal melanocytes *in situ* and *in vitro*.

Moreover, the fact that core clock silencing induces an increase in melanin production, tyrosinase expression and activity as well as *TYRP1* and *TYRP2* transcription in isolated, cultured human epidermal melanocytes, demonstrates that these effects are independent of clock inputs arising from other cells (such as epidermal keratinocytes). Furthermore, these experiments imply that it is the intrinsic molecular clock of melanocytes that regulates melanin production. In contrast, the increase in melanocyte number and dendricity seen after clock silencing in human epidermis and HFs may well be caused by cross-talk between melanocytes and their epithelial environment, as these effects are not seen in isolated melanocytes. Thus, this thesis project has revealed that human pigmentation and melanocyte biology are differentially regulated by the peripheral clock on multiple levels of complexity, including the regulation of MITF activity independently from central clock inputs.

Finally, this thesis concluded by asking whether the well-known, clinically important hair cycle- and hair pigmentation- modulatory steroid hormone, thyroxine (T4), also influences clock gene and protein activity in the human HF. Interestingly, both *BMAL1* and *PER1* protein and gene expression are significantly reduced by T4 after 24 hours. Time course analyses evaluated, next, whether T4 eliminates intrafollicular circadian rhythmicity of clock gene expression. This showed that circadian rhythmicity is maintained even under the influence of T4 stimulation, albeit at lower amplitudes. The discovery that T4 dampens intrafollicular clock gene expression over 48 h may explain at least in part why and how T4 prolongs anagen and stimulates melanogenesis in human scalp HFs (van

Beek et al. 2008). In contrast to this, long-term stimulation with T4 (over 6 days) significantly up-regulates clock gene expression.

Furthermore, as T4 has been shown to influence K15-positive human HF epithelial stem/progenitor cells (Tiede et al. 2010), it was important finding that K15-positive stem cells express clock proteins *in situ*, and that the expression of PER1 is increased by T4 in human HFs. This shows that T4 does influence the molecular clock activity of the human HF, including peripheral clock activity in the progenitor cell population of the human HF. Together this highlights the complex role of the peripheral clock in human HF biology.

In summary this thesis has answered the three major open questions relating the chronobiology and the human HF posed: 1) that the human HF does have functional peripheral clock activity which can regulate the human hair cycle; 2) that the peripheral clock also regulates human pigmentation; and 3) that the intrafollicular clock, including that of human HF epithelial stem/progenitor cells is hormonally regulated, namely by the steroid hormone, T4.

In contrast to the previously published literature (Lin et al. 2009; Plikus et al. 2013), this thesis has focused on the human HF, and the model system chosen (i.e. isolated human hair follicles in organ culture) has clearly excluded any influence from the central clock. Using this organ-culture system any possible off-target effects of a global clock gene knock-out, species differences between mice and humans and SCN inputs were excluded (Kondratov et al. 2006; Lin et al. 2009; Akashi et al. 2010; Geyfman and Andersen 2010; Kondratova and Kondratov 2012).

However, one major methodological limitation of human HF organ culture is that conclusions can only be drawn on a functional role of the intrafollicular clock system in regulating the anagen-catagen transition of the human hair cycle. In mice, telogen has been demonstrated to have high levels of clock activity, where BMAL1-kock-out was shown to delay anagen onset when compared to wild-type littermates (Lin et al. 2009). Since the telogen stage is increasingly being recognized as having regulatory importance in the human HF, it is important to check, next, whether the telogen-anagen transition of the human hair cycle is influenced by peripheral clock activity. This could be

achieved by transplanting human scalp skin onto immunocompromised mice (Gilhar 1987, 2013), a model which can encapsulate all human hair cycle stages *in vivo*. Recently developed techniques for gene silencing in intact human skin (For example, gene gun, liposomes, electroporation etc.) (Aleku et al. 2008; Huang et al. 2011; Jensen et al. 2014) could then be applied to the human skin transplants to assess how this impacts on human HF cycling *in vivo.*

Regarding the regulation of melanocyte biology and human pigmentation by the peripheral clock, mechanistically, the current data suggest that the increase in MITF activation by *PER1* silencing plays a key role in mediating the observed hyperpigmentation. However, a direct molecular link between MITF activation and clock silencing remains to be demonstrated. Due to the crucial role of MITF in many aspects of melanocyte biology (Tachibana 2000), blocking MITF activity (e.g. with neutralizing antibodies or by MITF knock-down) will inevitably inhibit pigmentation on multiple levels (Du et al. 2003; Vachtenheim and Borovanský 2010; Praetorius et al. 2013).

One potential mechanism by which the clock influences pigmentation may be via the mitogen-activated-kinase (MAPK) pathway, specifically ERK 1/2 activation which subsequently activates MITF (Du et al. 2003; Su et al. 2013; Jin et al. 2014). This is supported by the fact that ERK1/2 is regulated by the circadian clock (in *Neurospora*) (Bennett et al. 2013). It may be possible to further explore direct molecular interactions of MITF with specific clock proteins by running pull-down assays (Hsieh et al. 2003), e.g. in protein extracts from melanocytes before and after clock silencing.

Alternatively, the increase in pigmentation by clock knock-down could be mediated indirectly by a change in cAMP levels, acting as a secondary messenger. In human pigmentation, cAMP is indeed important for MITF activation and MAPK activation (Buscà and Ballotti 2000; Rodríguez and Setaluri 2014). Moreover an increase in melanogenesis can be induced pharmacologically by increasing intracellular cAMP levels, thus mimicking the pigmentation-stimulatory effects of MSH or ACTH administration (Englaro et al. 1995). Interestingly, clock down-regulation, e.g. in mice with a knock-out of CRY1 and 2, leads to an up-regulation of cAMP (Zhang et al. 2010). This strongly supports cAMP as a potential mechanism of action. Therefore, it should be studied, next, whether cAMP

levels are altered in clock-silenced HFs. This could be assessed using an enzyme-linked immunosorbent assay (ELISA) on cultured, clock-silenced HFs (Lambers et al. 2014).

It can therefore be hypothesised that *PER1* and *BMAL1* knock-down may increase melanogenesis by up-regulation of cAMP and/or activation of the MAPK pathway, specifically by ERK1/2 activation (Robertson et al. 2010). Both of which induce MITF phosphorylation and thus activation whilst simultaneously targeting it for degradation (Buscà and Ballotti 2000; Kim et al. 2014). Thus, it is particularly promising to further study these pathways in future research. A key place to start would be to stain clock-silenced HFs for ERK1/2 in order to determine whether activation of this protein is increased. If this is the case, this would suggest that ERK1/2 activation increases MITF phosphorylation. Ultimately this would imply that the molecular clock primarily influences human HF pigmentation by influencing the MAPK pathway. As modulators of this pathway exist, specifically inhibitors and regulators for ERK1/2 such as *PD98059* (Selleck Chemicals LLC, Houston TX, USA) and ERK inhibitor II (FR180204) (Santa Cruz) (Ohori et al. 2007; Arana-Argáez et al. 2010), they could be used to experimentally reduce ERK1/2 activity in clock-silenced HFs. If no changes in pigmentation are observed it could be conclusively demonstrated that MAPK pathway up-regulation by molecular clock silencing leads to an increase in pigmentation.

Evidently, one key translational aspect of this research is that the results designate the peripheral core clock, specifically PER1 and BMAL1 activity, as promising novel targets for therapeutic hair growth and pigmentation modulation, namely by targeted modulation of the HF molecular clock.
The current work suggests that inhibiting PER1 and/or BMAL activity in scalp HFs should promote hair growth and may thus become a useful therapeutic strategy in various forms of alopecia and effluvium, ranging from androgenetic alopecia to endocrine disorder-associate telogen effluvium to chemotherapy-induce alopecia, either as an alternative to existing hair loss management strategies (Paus et al. 2013; Valente Duarte de Sousa and Tosti 2013) or as an adjuvant therapy. Even though this has not been specifically tested in the current thesis project, up-regulating intrafollicular clock activity should, in theory, serve to inhibit unwanted hair growth (hirsutism, hypertrichosis (Blume-Peytavi 2013).

Several different molecules have already been described and explored *in vitro* that can reduce clock activity (Chen et al. 2012b), which may be studied as initial lead substances in order to obtain proof-of-principle that pharmacological clock activity inhibition does stimulate human hair growth, at least in HF organ culture. For example, GSK4112 and SR9011 can reduce clock activity in mice (Gibbs et al. 2011; Solt et al. 2012), while CEM1 or CEM2 can up-regulate it in mice (Chen et al. 2012a).

The main clinical challenge here will be a) to administer such candidate clock inhibitors topically to minimise the risk of adverse systemic effects, and b) to selectively target the human HF. Such HF selectively may be achievable by utilising recent advances in the development of HF-targeting topical vehicles, such as HF-targeting liposomes or nanoparticles (Chourasia and Jain 2009; Knorr et al. 2009; Patzelt et al. 2011; Chen et al. 2012b; Gu et al. 2012).

As the MAPK pathway is integral to many different biological systems including proliferation, differentiation and cell survival and since its overexpression is linked to cancer development (Grossi et al. 2014; Korc 2014), targeting this pathway may not be ideal for therapeutic manipulation of hair growth and pigmentation disorders. Obviously, a therapeutic strategy that might increase the risk of developing skin cancer by chronically stimulating the MAPK pathway would be undesirable. For example, the vast majority of melanomas shows signs of over-activation of the RAS/RAF/MEK/ERK pathway (Wang and Qi 2013). Thus, one would not wish to increase a patient inherent risk to develop this highly malignant and potentially deadly tumour by prolonged MEK/ERK stimulation. This provides further support for the use of targeted small modifiers of clock expression (Chourasia and Jain 2009; Chen and Chuong 2012; Janich et al. 2014). Yet, this does not completely exclude the potential risks of chronically stimulating the MAPK pathway. Therefore, it would appear ideal to develop topically applicable small molecule clock activity inhibitors that do not over-stimulate MAPK signalling.

An alternative to clock modifiers may be the usage of T4. As this study was able to demonstrate that T4 transiently down-regulates clock activity and as T4 is already one of the clinically most frequently prescribed hormones in medical therapy (Mitchell et al. 2009), T4 may be an ideal

candidate for topical modulation of clock activity. However, the current study revealed that clock activity was actually up-regulated by T4 in longer-term HF organ cultures. Therefore, it is conceivable that chronic T4 application may actually induce catagen (and thus promote hair loss) upon long-term application. Indeed, hyperthyroid patients with excessive T4 and T3 serum levels can show marked diffuse hair loss (effluvium). As a consequence of these considerations it may be advisable to administer topical T4 only as a short pulse therapy, so as to inhibit clock activity only briefly.

Supporting this, mathematical modelling of the HF cycle has suggested that pulsatile treatment would have a much more beneficial role in prolonging anagen in the HF (Al-Nuaimi et al. 2012). Such a pulsatile T4 treatment regimen would coincide with the constitutive circadian fluctuations in the T4 serum level (Dardente 2012; Dardente et al. 2014), further encouraging a pulsatile/circadian T4 treatment schedule to promote hair growth and pigmentation. Circadian based administration of drugs is also in line with recent studies that have demonstrated improved side-effects from circadian regulated chemotherapy (Giebultowicz 2004; Innominato et al. 2014).

This could be tested repeating the methodology used in chapter 6. By culturing HFs with T4 for 12 hours a day, for 6 days and observing the effects on clock gene/protein levels. This could be assessed by immunofluorescence and qRT-PCR. Doing so may support the use of pulsatile treatment as opposed to continuous which could potentially scale up to a clinical level (Ortiz-Tudela et al. 2014; Vásquez-Ruiz et al. 2014). One critical question in this regard is whether topical T4 administration is most effective i.e. when the serum T4 level is maximal or when it is minimal.

Besides T4, other hormones known to modulate clock activity, such as glucocorticosteroids (Balsalobre 2000; Cheon et al. 2013) and melatonin (Dardente 2012), could also be studied in human HF organ culture in order to assess their influence on clock gene expression. It may be speculated that these hormones could impact on intrafollicular clock gene activity in a similar manner to T4 treatment and may be one way by which the SCN synchronises peripheral clock activity in the human HF. Following this, as with T4 treatment, circadian based stimulation on organ-cultured human HFs could be implemented. Being more physiologically relevant; these cultures

may actually show different human HF response compared to previously published neuroendocrine HF organ culture work which use non-circadian administration schedules of hormones (Pérez 2011; Choi et al. 2013).

Although the current work has generated convincing evidence that the peripheral clock has a functionally important role in modulating the hair cycle, the underlying mechanisms of action remain unknown. With the molecular clock reportedly regulating at least 10% of the genome (Bellet and Sassone-Corsi 2010) it is clear that there are many potential mechanisms that must be taken into consideration and much additional research is required to fully take into account the impact of circadian variations in gene expression on human HF and skin biology. DNA microarray analysis on both *BMAL1* and *PER1* knock-down HFs, compared to a scrambled oligo control followed by qRT-PCR for validation, is an obvious start to obtain first mechanistic candidates of how the molecular clock influences HF biology. This will help to focus future research by highlighting key genes and pathways that are altered in the human HF when clock genes expression is reduced. Simultaneously such a microarray-based, non-hypothesis-driven search strategy for underlying mechanisms of action may also highlight key genes and pathways involved in the clock-regulated pigmentary phenomena identified by this thesis project.

Credible candidate genes and targeted pathways that may underlie the observed anagen prolongation by *PER1* or *BMLA1* silencing include cell-cycle modulation (Lowrey and Takahashi 2004; Miller et al. 2007; Geyfman and Andersen 2010; Plikus et al. 2013). Supporting this, BMAL1 protein expression was found in the dividing MKs during both anagen and catagen. Furthermore, recognised hair cycle-regulatory genes, *c-Myc* (Bull et al. 2001, 2005) and *cyclin D1* (Mitsui et al. 2001; Ohtani et al. 2007) are reduced by PER1 knock-down. Moreover, T4 experiments have demonstrated that not only does *cyclin D1* show strong circadian rhythmicity over 48 hours, but that it is maintained by T4 treatment. That T4 is able to prolong the rhythmic expression of *cyclin D1* suggests that the anagen-prolonging effect of T4 may be mediated by maintaining robust, extended circadian expression of *cyclin D1*. Since a reduction in *Bmal1* increases proliferation and cyclin D1 expression in both murine cells and tumours and decrease apoptosis (Zeng et al. 2010), suggests one potential mechanism for action in which the molecular clock may influence the human hair cycle.

In summary, the current work has shown that the peripheral clock is intrinsic to human HF biology and forms an integral component of the elusive human “hair cycle clock”. Moreover, it has uncovered the presence of a novel ‘pigmentation clock’ that regulates not only HF, but also epidermal pigmentation in human skin. This operates within primary human melanocytes in a cell-autonomous manner, independent of local influences from the skin environment or inputs of the central clock. In a broader sense, this thesis project has therefore further opened the door to translationally relevant chronobiological research in the human system, namely to better understanding the role of the *peripheral* core clock in regulating human tissue physiology *in situ* and under clinically relevant conditions. It is hoped that the concrete concepts emanating from this thesis project will translate into advances in clinical therapy in future.

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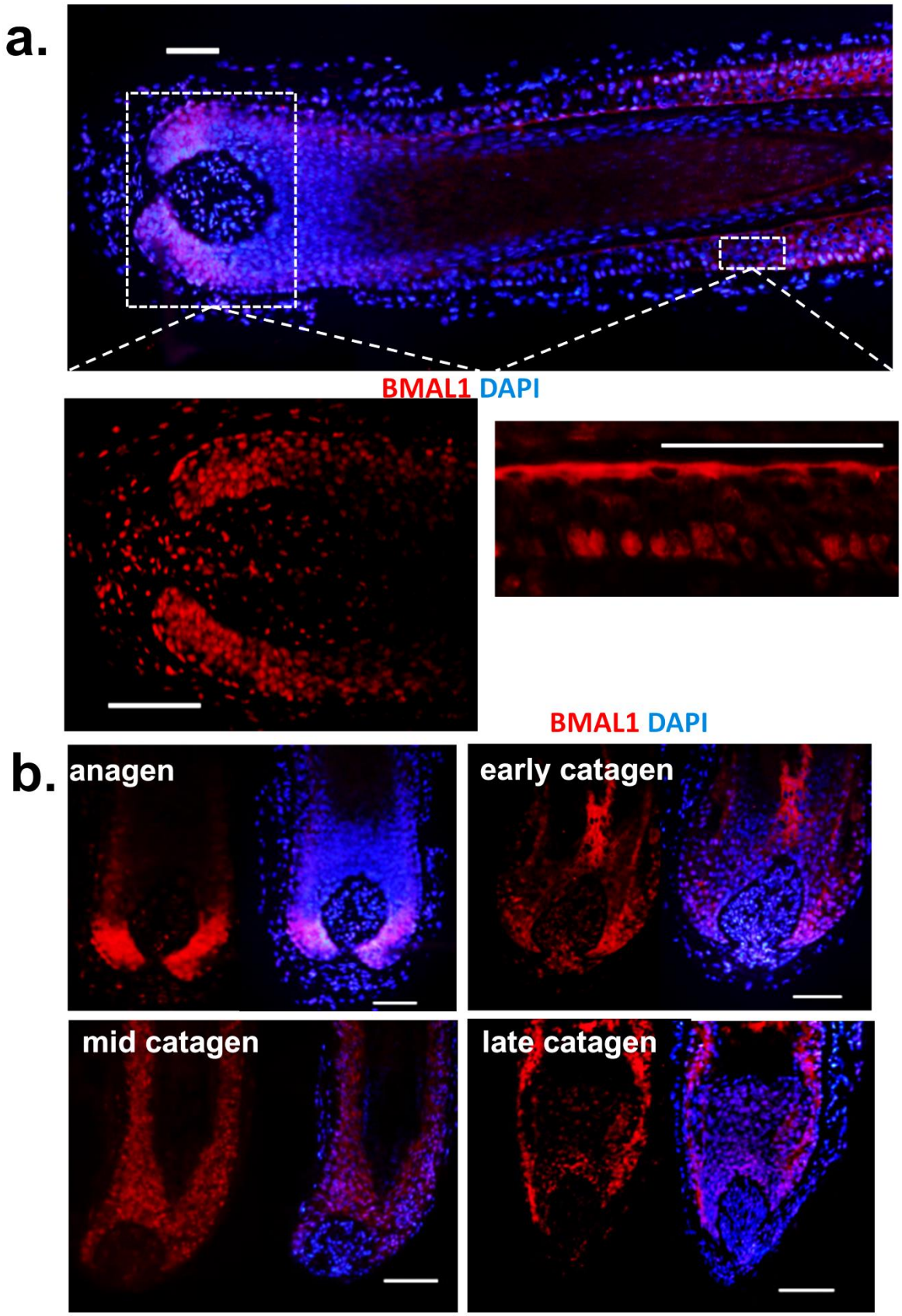
Appendices

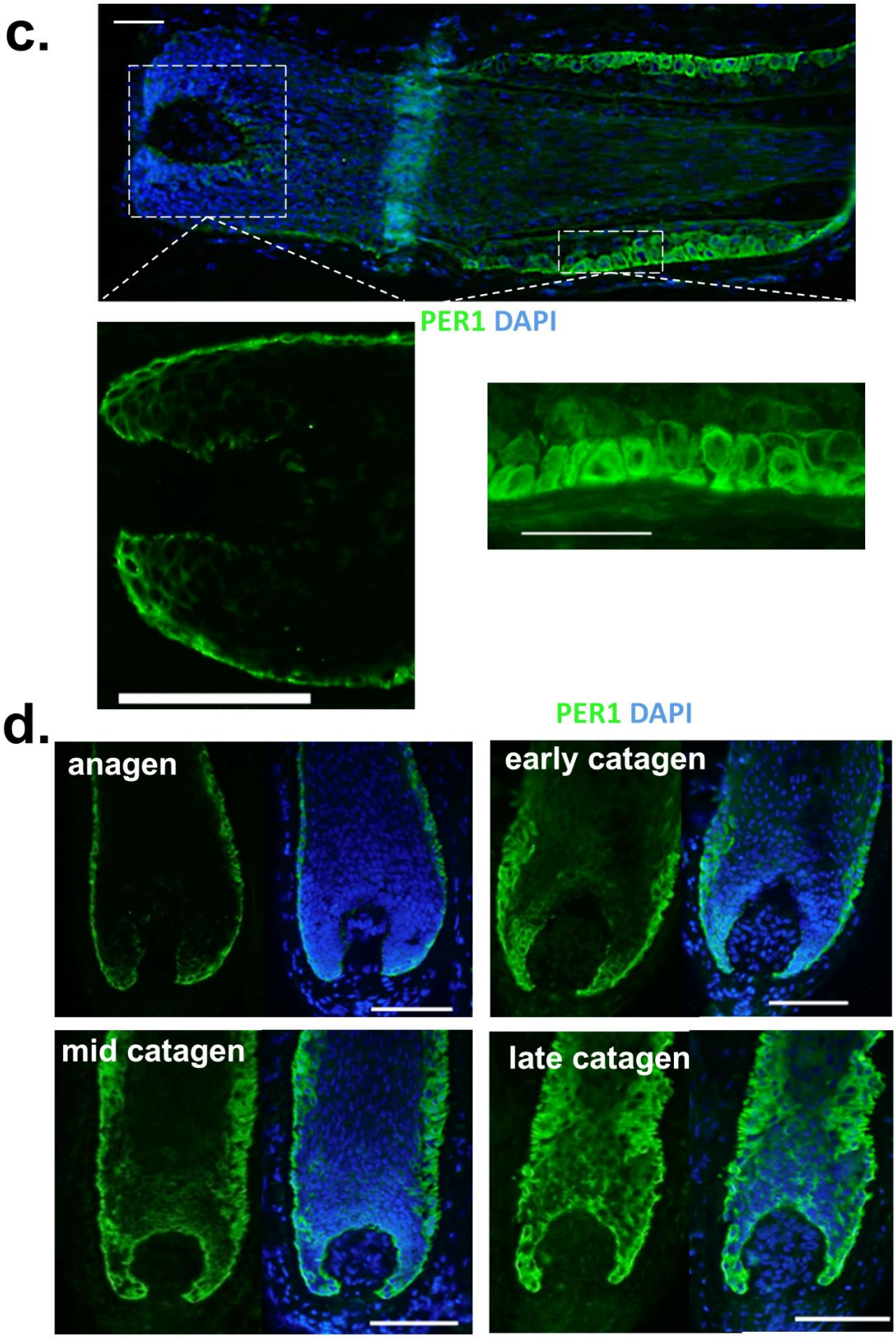
Appendix 1: Chapter 3 high-resolution images.

Due to the alternative format of this thesis images have been assembled into panel figures. The following appendices contains higher resolution images of all immunofluorescent and immunohistochemical stains used in this chapter.

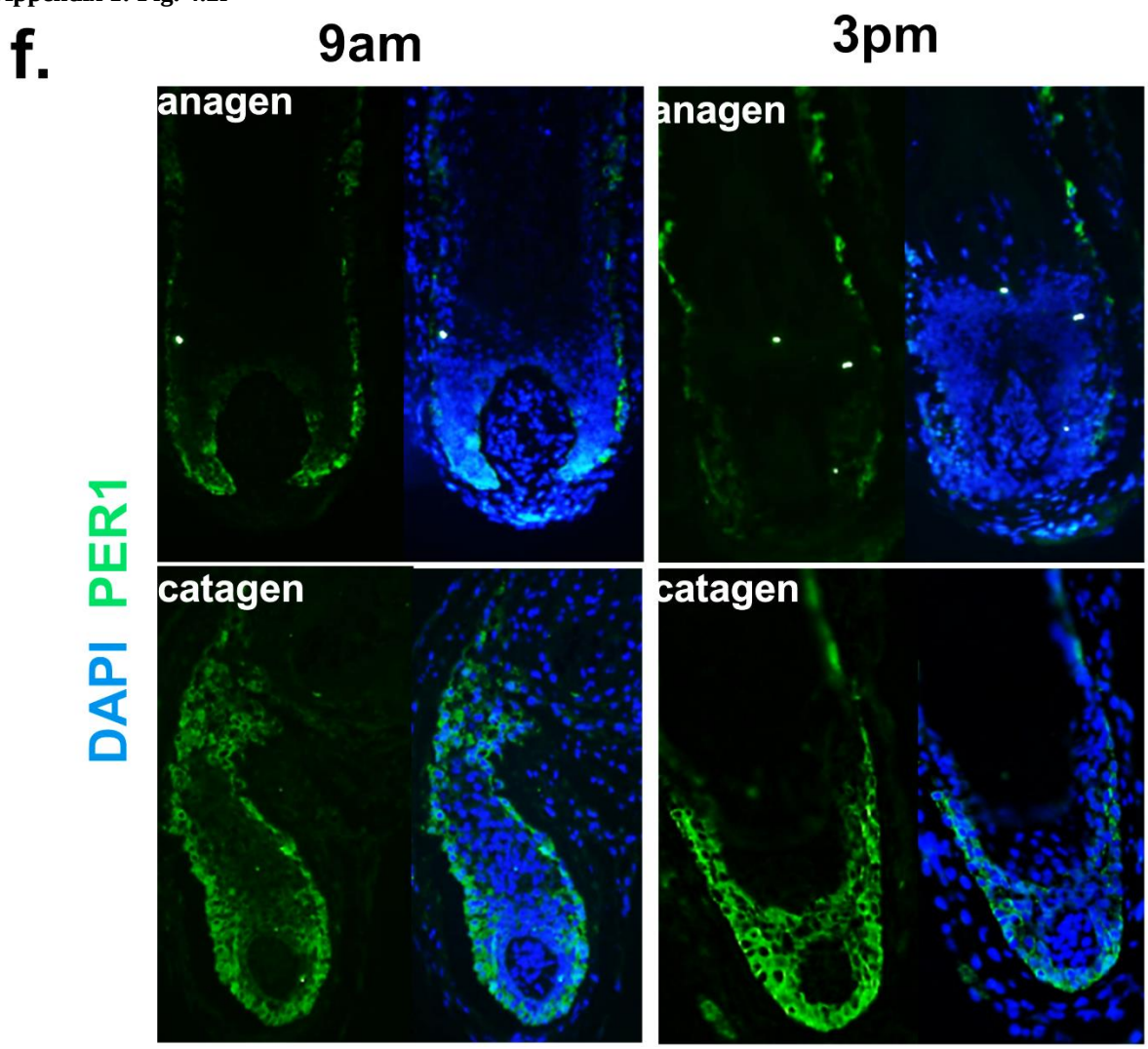
Appendix 1: Fig. 4.2a & b



Appendix 1: Fig. 4.2c & d



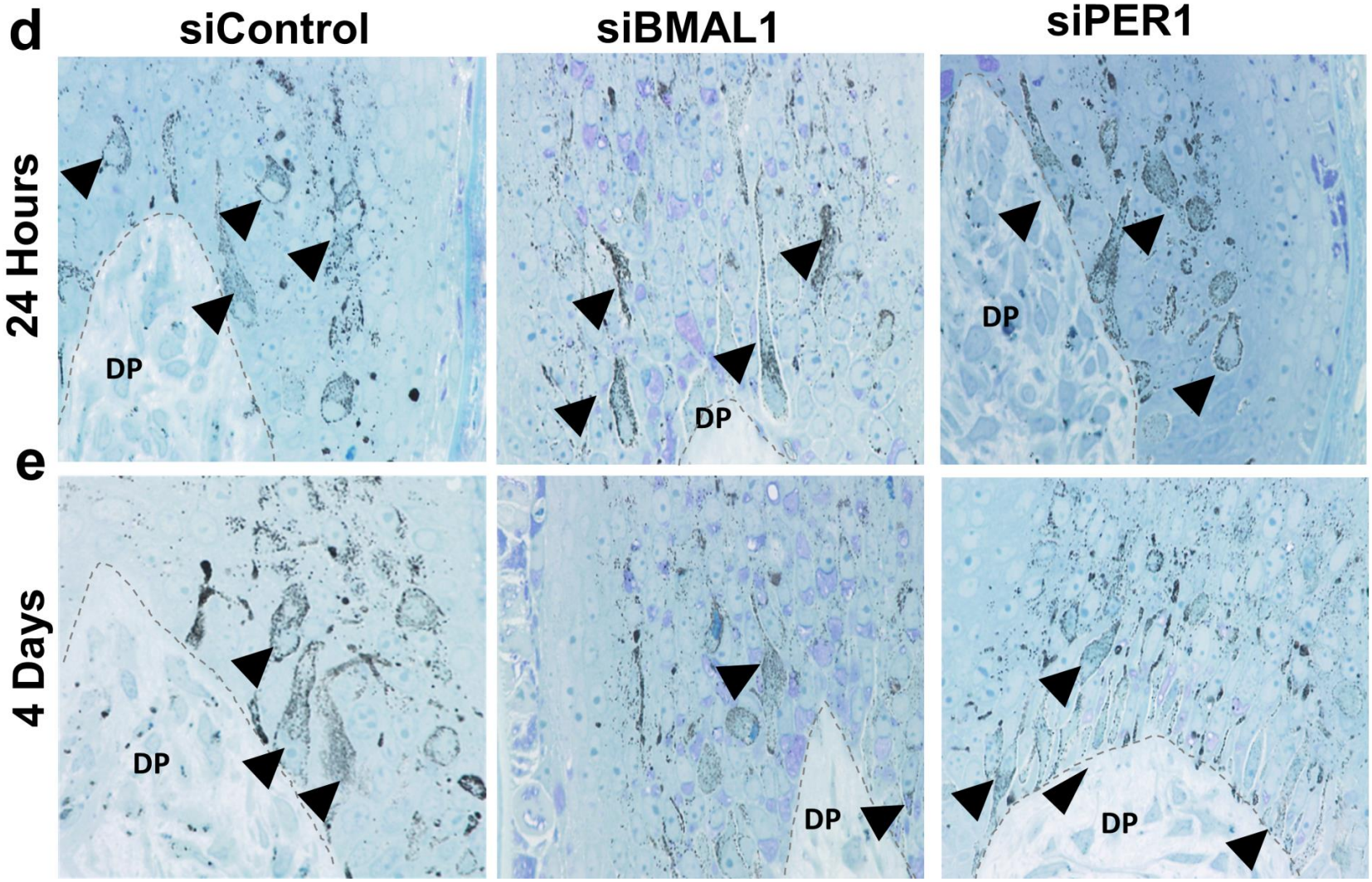
Appendix 1: Fig. 4.2f



Appendix 2: Chapter 4 high-resolution images

Due to the alternative format of this thesis images have been assembled into panel figures. The following appendices contains higher resolution images of all immunofluorescent and immunohistochemical stains used in this chapter.

Appendix 2: Fig. 5.1d & e



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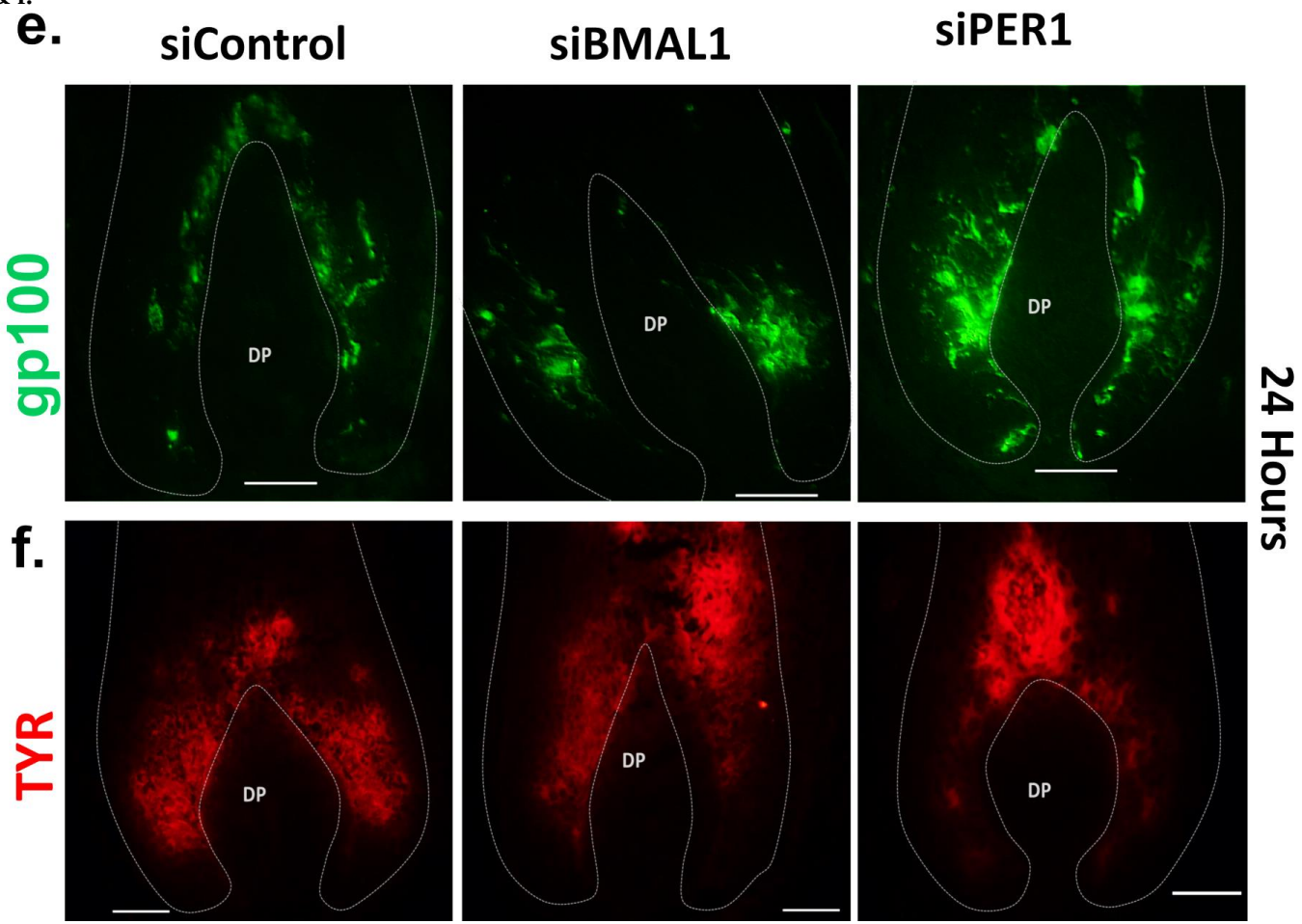
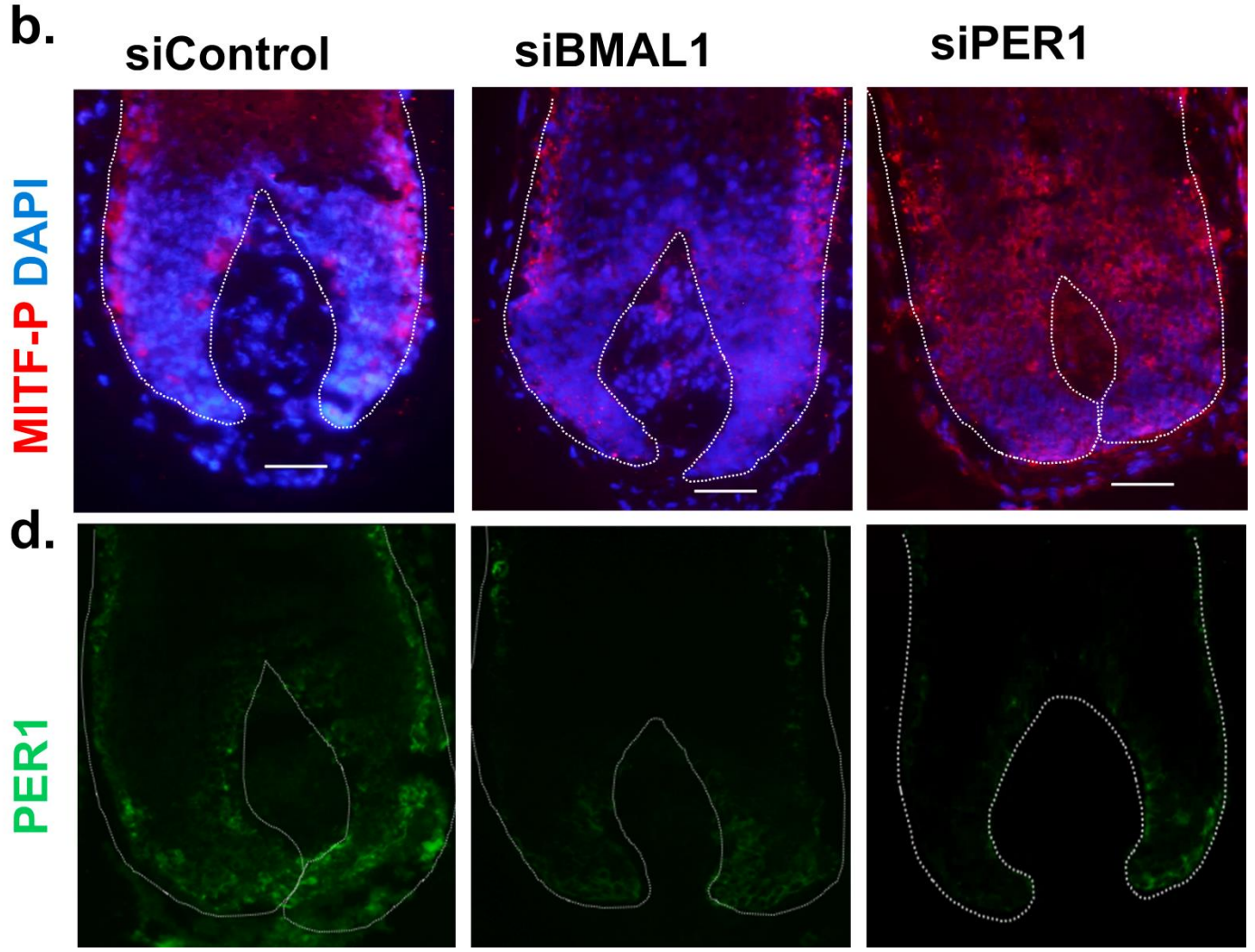
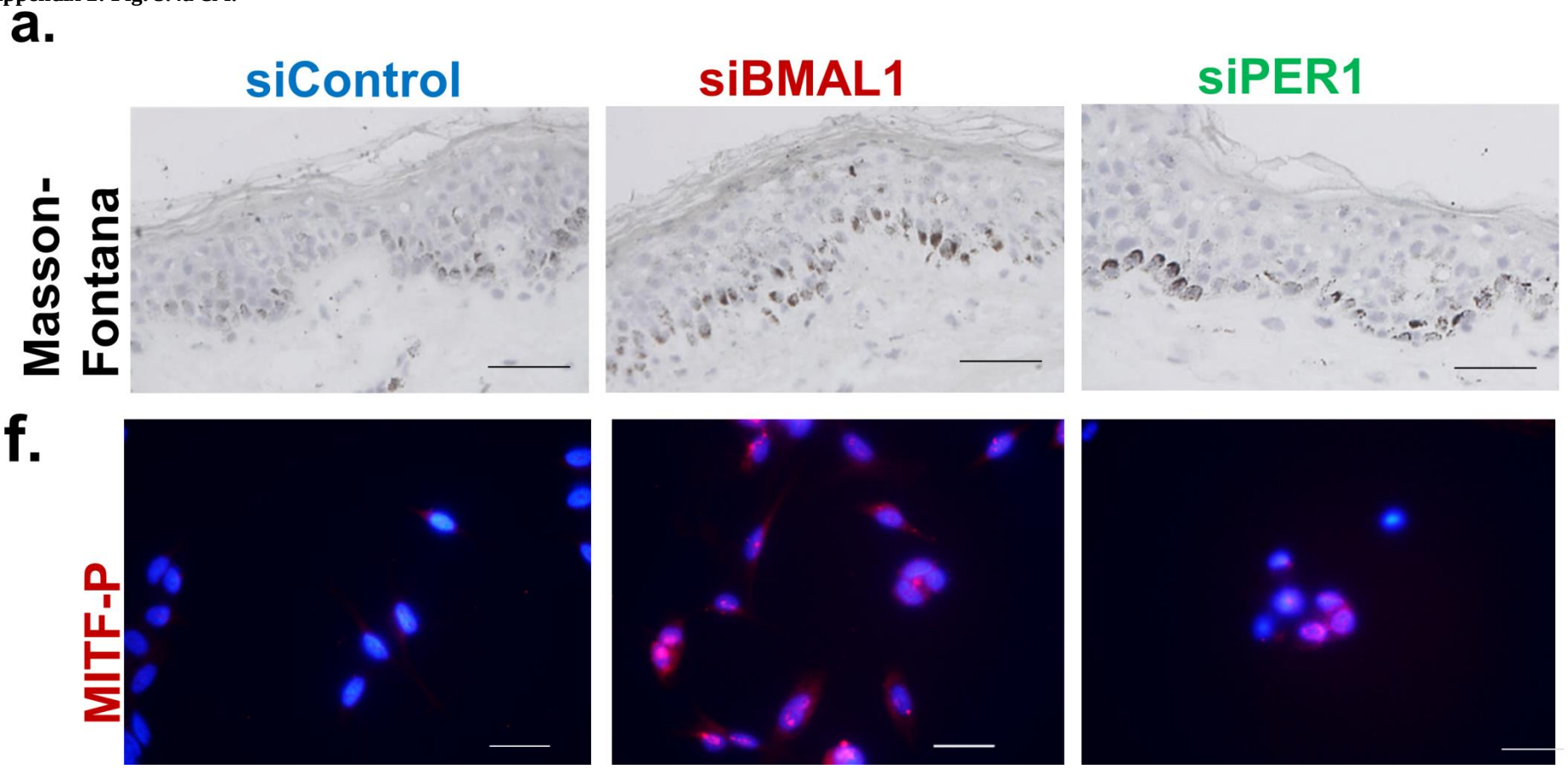


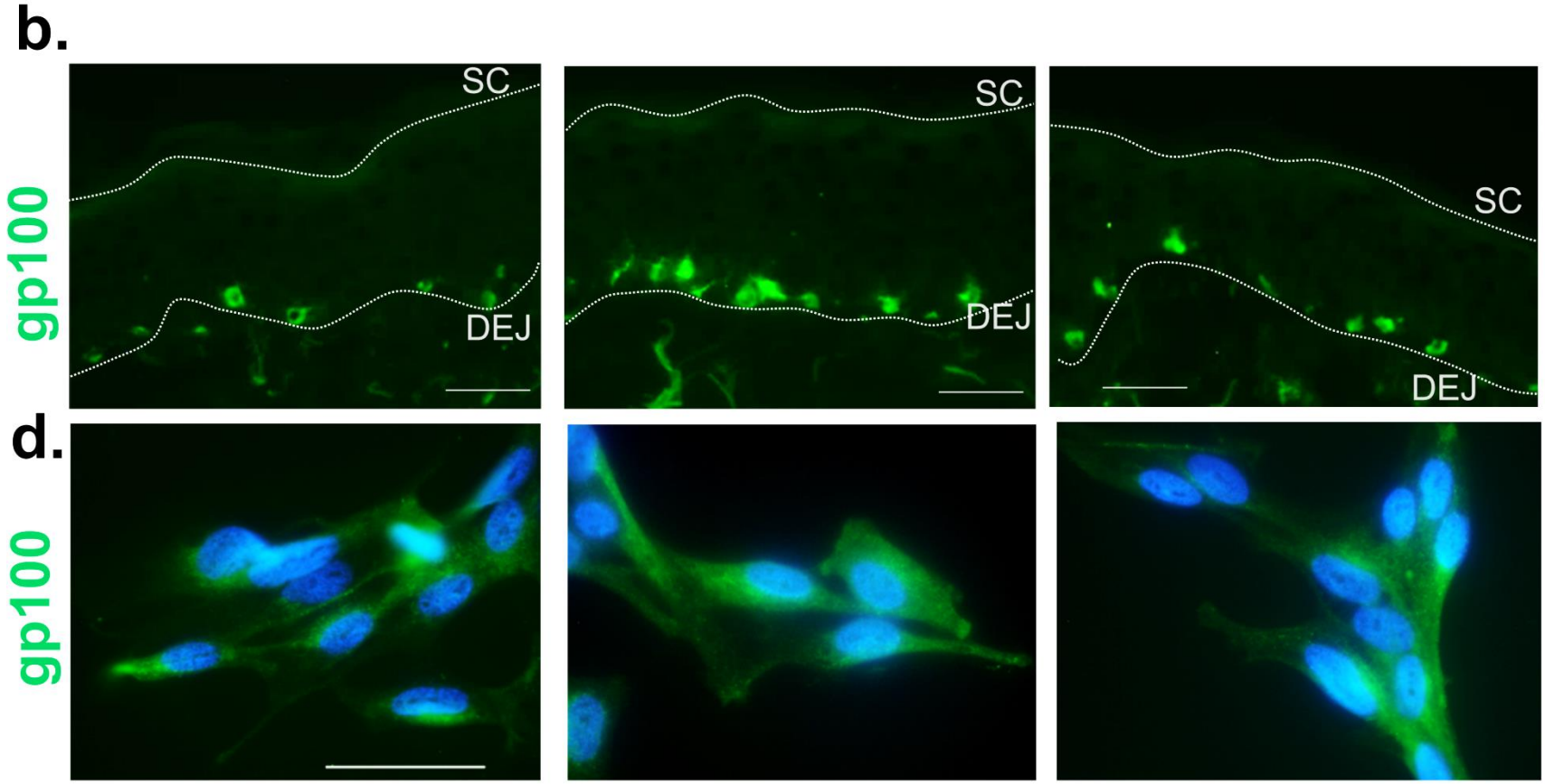
Appendix 2: Fig. 5.3b & d.



Appendix 2: Fig. 5.4a & f.



Appendix 2: Fig. 5.4b & d.



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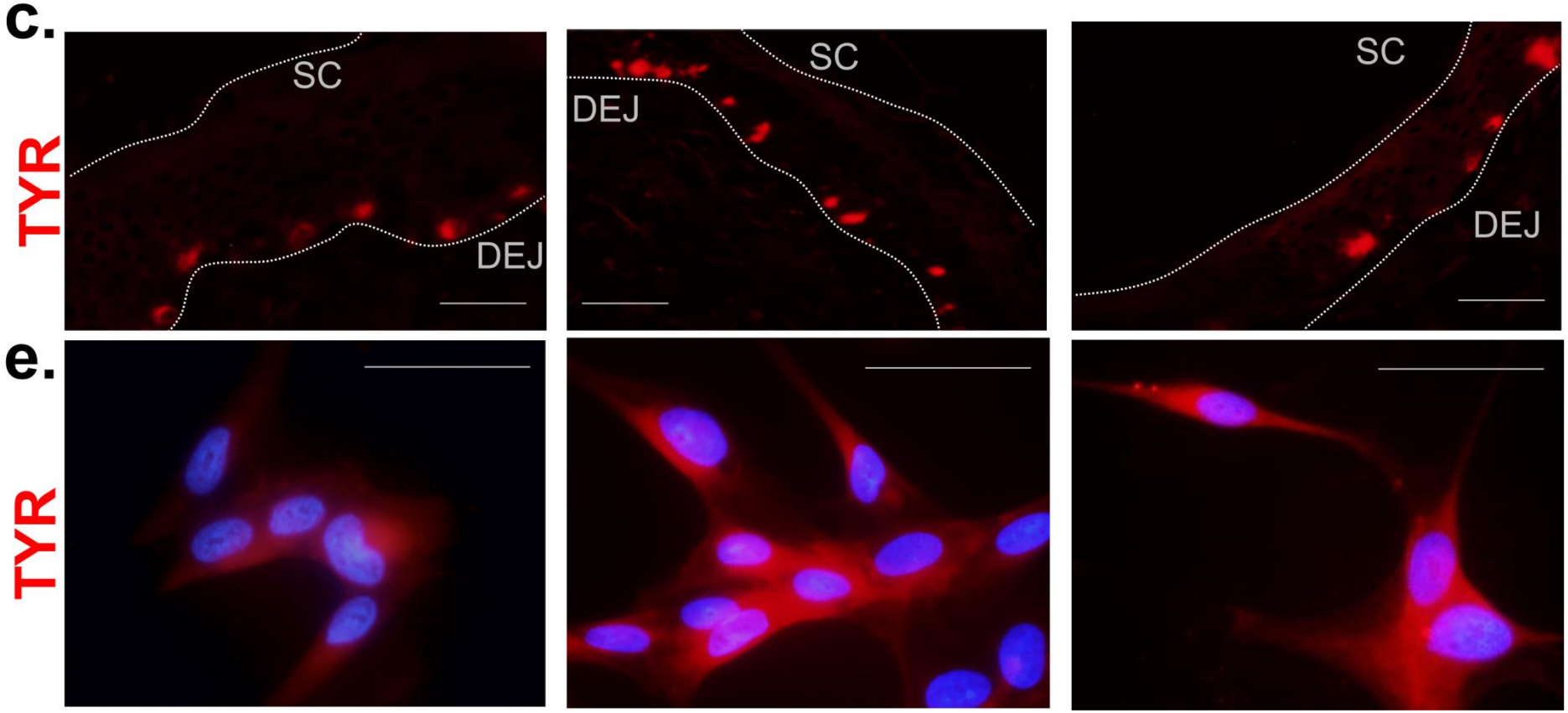
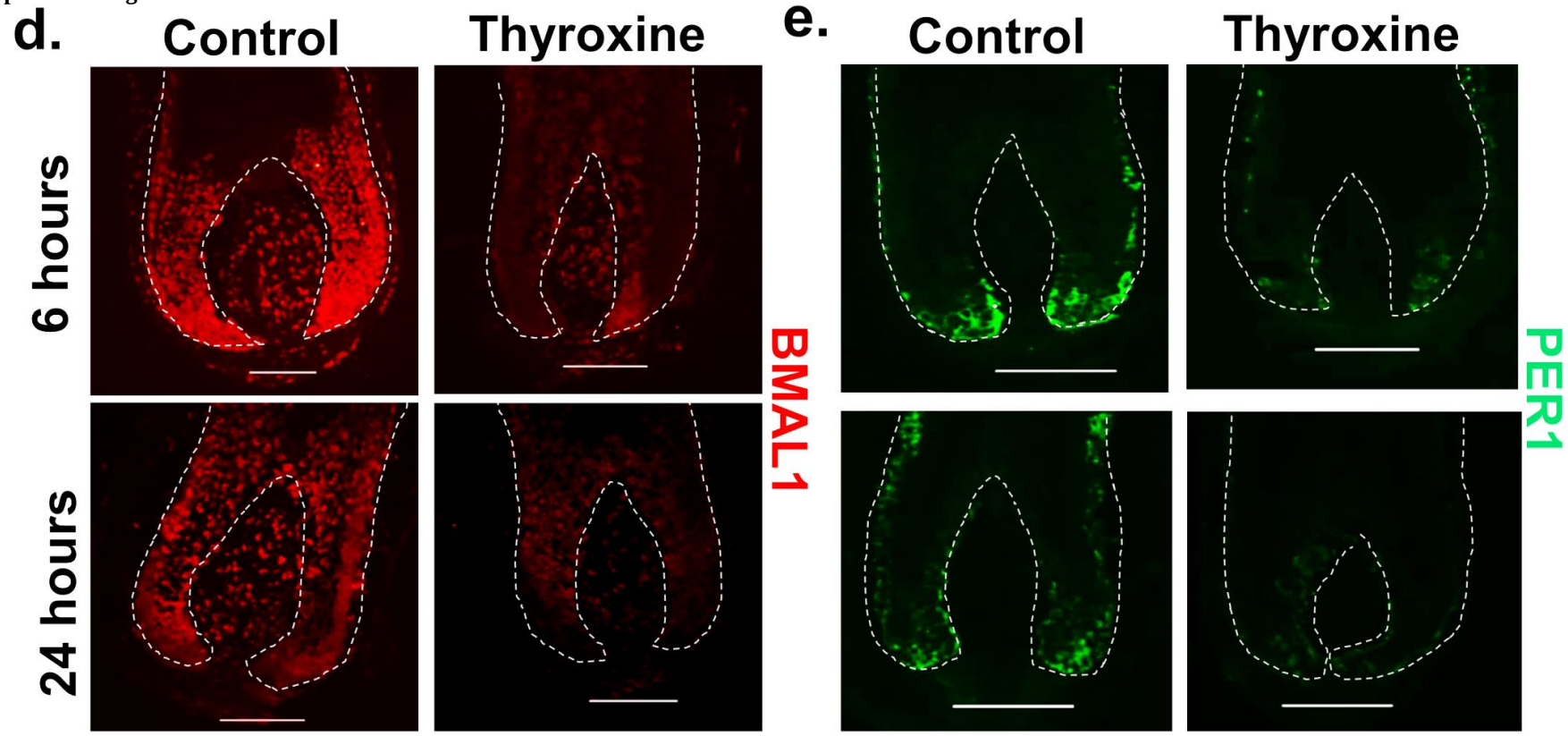


Appendix 3: Chapter 4 high-resolution images

Due to the alternative format of this thesis images have been assembled into panel figures. The following appendices contains higher resolution images of all immunofluorescent and immunohistochemical stains used in this chapter.

Appendix 3: Fig 6.1d & e.



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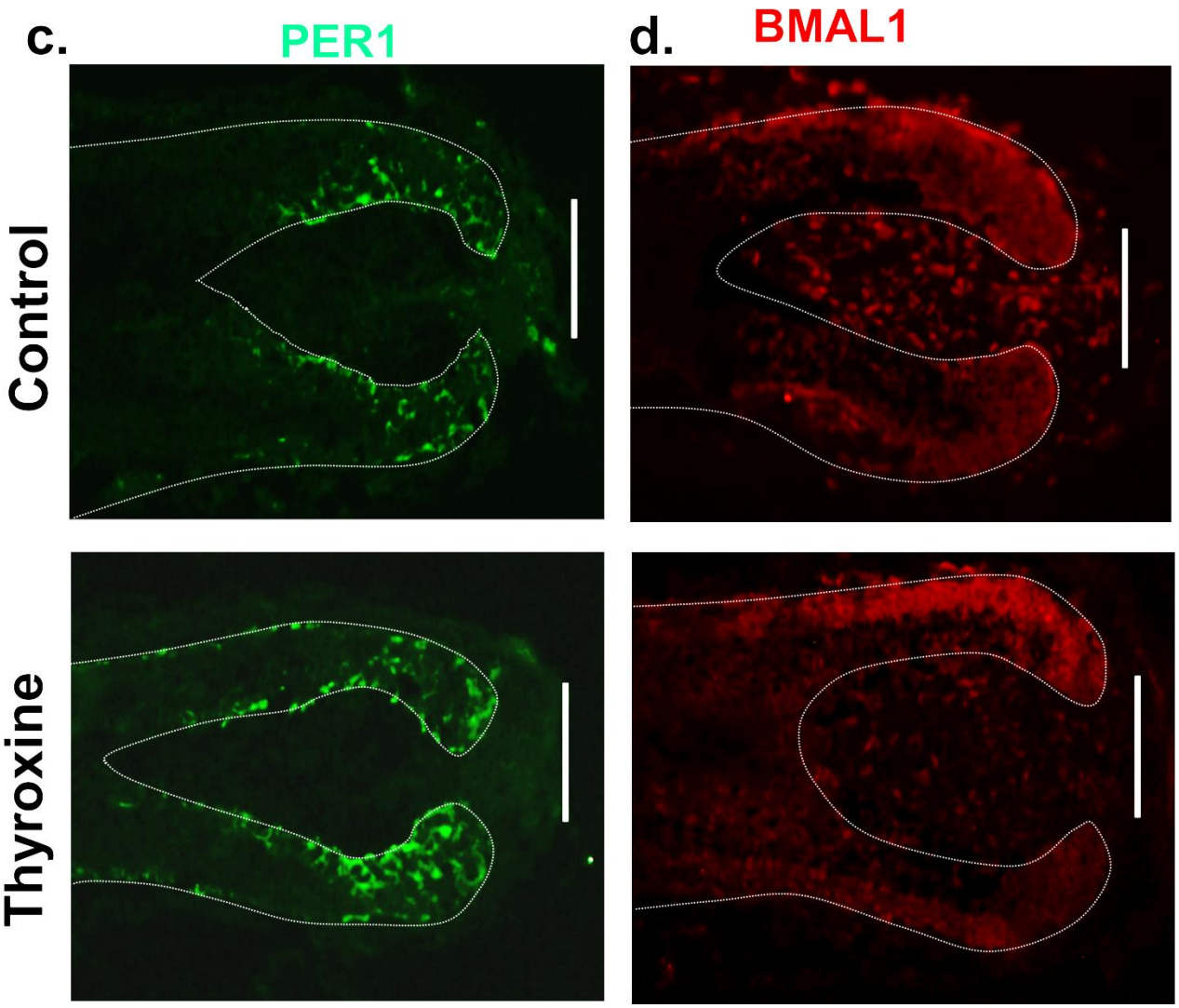
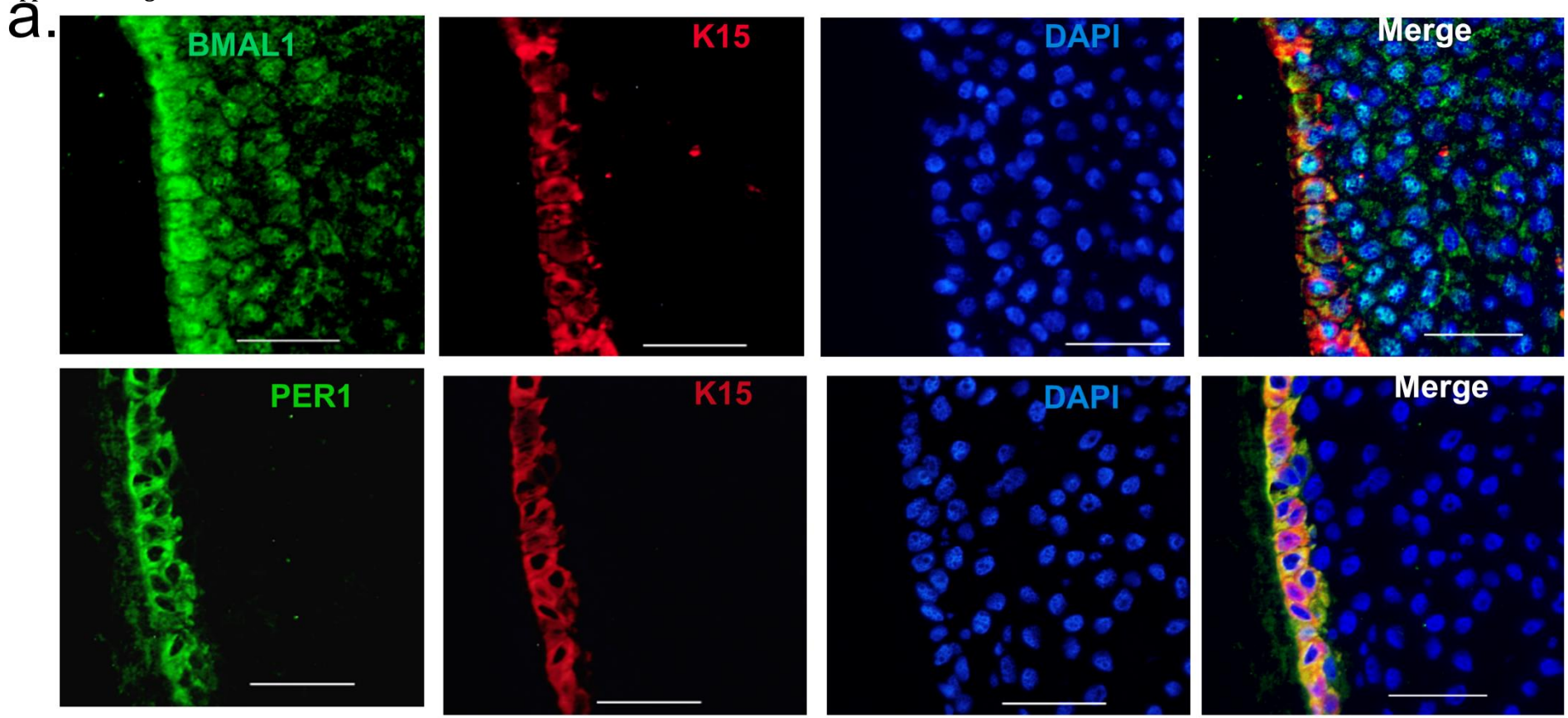
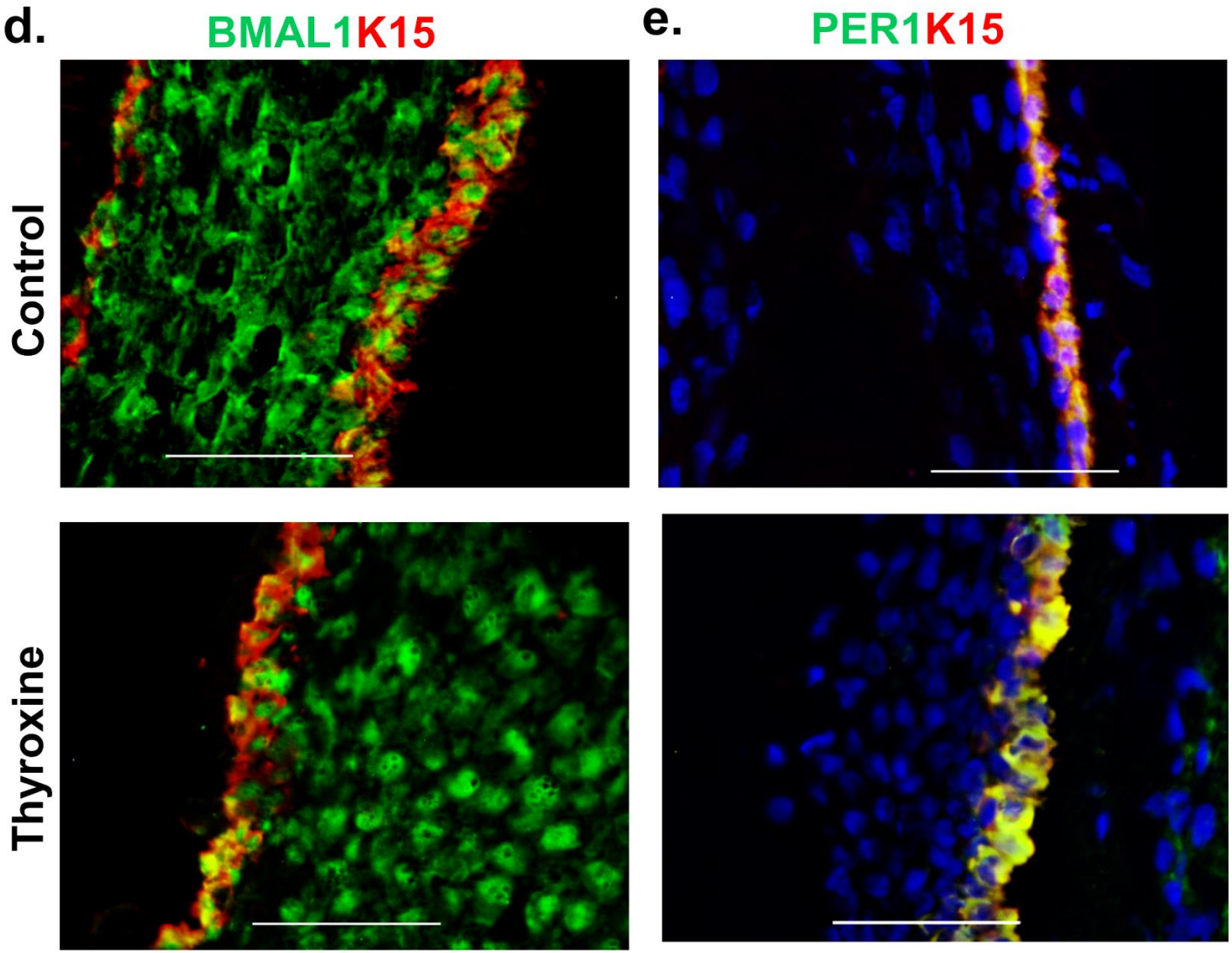


Appendix 3: Fig. 6.4a



Appendix 3: Fig. 6.4d &e.



Appendix 4: Chronobiology in the skin.

The following article is currently being prepared for submission to *Bio Reviews* once the melanocyte article is published and additional input from collaborators (Maksim Plikus and Benedetto Grimaldi), who specialise in chronobiological research, is received. This article is based on the introduction and future perspectives of this thesis.

PRELIMINARY DRAFT

1st target journal: **Biol Rev**

➔ senior author contacts *Biol Rev* editor with pre-submission inquiry in Oct 2014

Chronobiology and the hair follicle

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Overview and scope of review

Here, we explore why the hair follicle, one of the defining features of mammals, offers a unique, translationally relevant model system for studying how molecular mechanisms that govern circadian and perennial biorhythms impact on peripheral tissue physiology. After defining the field of chronobiology and its general importance for tissue physiology and pathology, we briefly synthesize key concepts of clock regulation. On this basis, we explain the theoretical background into why clock genes and the pathways they target are an obvious candidate system for regulating the cyclic growth and regression activity of the hair follicle (HF), and why this new frontier in hair research has important clinical implications. After reviewing recent evidence that HFs show circadian variations of peripheral clock gene activity, it is delineated that the peripheral clock regulates not only murine (HF) cycling, but also human hair growth and, strikingly, also pigmentation, at least in human epidermal and HF melanocytes. This review closes by defining key open questions in the field and by sketching concrete translational perspectives on how HF chronobiology research may lead to new therapeutic approaches in the treatment of skin and hair diseases.

What is chronobiology?

Chronobiology is the study of the biological timing systems that control living processes including metabolism, DNA replication and hormone production (Gérard and Goldbeter 2012; Baron and Reid 2014; Dardente et al. 2014; Orozco-Solis and Sassone-Corsi 2014). The purpose of chronobiology is to dissect how environmental time cues produce daily rhythms and long term seasonal rhythms that mediate and adapt biological processes (Giebultowicz 2004). It is now understood that most cells have an intrinsic timing system that is essential for biological organisation (Karatsoreos et al. 2011; Engelberth et al. 2014; Orozco-Solis and Sassone-Corsi 2014). It is when normal biological timing, e.g. during nightshift work, psycho-emotional stress and diet, that normal tissue homeostasis is disrupted triggering or aggravating diseases, including metabolic syndrome, Alzheimer's disease, hypertension, diabetes, and cancer (Kondratov and Antoch 2007; Baron and Reid 2014; Kalsbeek et al. 2014; Mazzoccoli et al. 2014; Robinson and Reddy 2014; Sheikh-Ali and Maharaj 2014). It is

clear that both a better understanding of biological time-keeping and a more easily accessible model that has intrinsic clock activity is required.

How the molecular clock impacts on tissue physiology and disease

It is increasingly appreciated that disruption to the molecular clock and loss of synchronisation from external cues can lead to tissue malfunction and clinical pathologies. Such pathologies include hypertension, with *bmal1* knock-out mice having low blood pressure. It is currently thought that the molecular clock balances the level of vascular reactive oxygen species (ROS) via nicotinamide adenine dinucleotide phosphate-oxidases (NADPH-oxidases) such as Nox1,2,4,5 leading to vascular tissue maintenance including vasodilation and vasoconstriction and endothelial remodelling (Kunieda et al. 2008; Takac et al. 2012). Further examples were demonstrated in clock knock-out mice which show an increased number of age-related co-morbidities such as ROS accumulation, decreased life span and loss of bone mass (Kondratov et al. 2006; Geyfman and Andersen 2010; Kondratova and Kondratov 2012).

In humans, a similar increase in the prevalence of hypertension has been observed in epidemiological studies of night-shift workers (Ohlander et al. 2014). Other pathologies include metabolic disease, which is also more prevalent in shift workers (Sheikh-Ali and Maharaj 2014), and age-related pathologies. Furthermore, there is a growing link between Alzheimer's disease and clock dysfunction as patients with this disease often show decreased amplitude of clock gene expression. This may potentially lead to an increase in amyloid-beta deposit in the brain, exacerbating the condition (Bedrosian and Nelson 2012; Buratti et al. 2014).

With an increasing understanding of how clock, specifically the molecular clock, dysfunction can exacerbate disease, a greater understanding of how the molecular clock controls normal tissue homeostasis and function increase our understanding of how dysfunction leads to disease states and may be used to uncover novel therapeutic targets. Indeed more recently chronobiology based therapies have been successful in treating illness. Studies have shown that administering circadian-

based chemotherapy to cancer sufferers reduced clinical fatigue, prevented a loss in body weight and showed other general improvements when compared to patients with fixed-time therapy (Innominato et al. 2014; Ortiz-Tudela et al. 2014). Moreover, prenatal babies treated in incubators with a light/ dark therapy showed a significant gain in body mass than those in traditional incubators (Vásquez-Ruiz et al. 2014).

It is clear external influences are important for healthy tissue function. However, many of these studies are association based and focus on the role of external cues such as light and dark cycles and therefore fail to take into account the molecular clock which produces these daily rhythms (Stokkan et al. 2001; Roelfsema and Pijl 2012; Vásquez-Ruiz et al. 2014).

What is the molecular clock?

While the term chronobiology refers to biological timing systems, it often refers specifically to the molecular clock, which modulates local tissue physiology. It is this molecular system that provides a promising candidate for choreographing the rhythmic processes in peripheral tissues i.e. the human hair cycle. It is now appreciated that most if not all cell types have a molecular clock, with organs including the liver, heart and kidney having peripheral molecular clock activity that with oscillate irrespective of the central pacemaker and external cues (**Fig. 1**) (Schmutz et al. 2011; Bass 2012; Sato et al. 2013).

The molecular clock is a series of molecular feedback loops that are intrinsically involved in controlling the cell cycle with all the check point proteins being under circadian control. Moreover the circadian system temporally segregates DNA replication occurring during the night to reduce potential damage from UV, whilst metabolic genes are up-regulated during light hours. Furthermore, that the apoptotic genes are also under clock control (Gaddameedhi et al. 2012), including p53 (Hamada et al. 2014), further suggests the potential role for the molecular clock in mediating the switch between the HF growth stage anagen, marked by high-levels of proliferation and the apoptotic driven catagen.

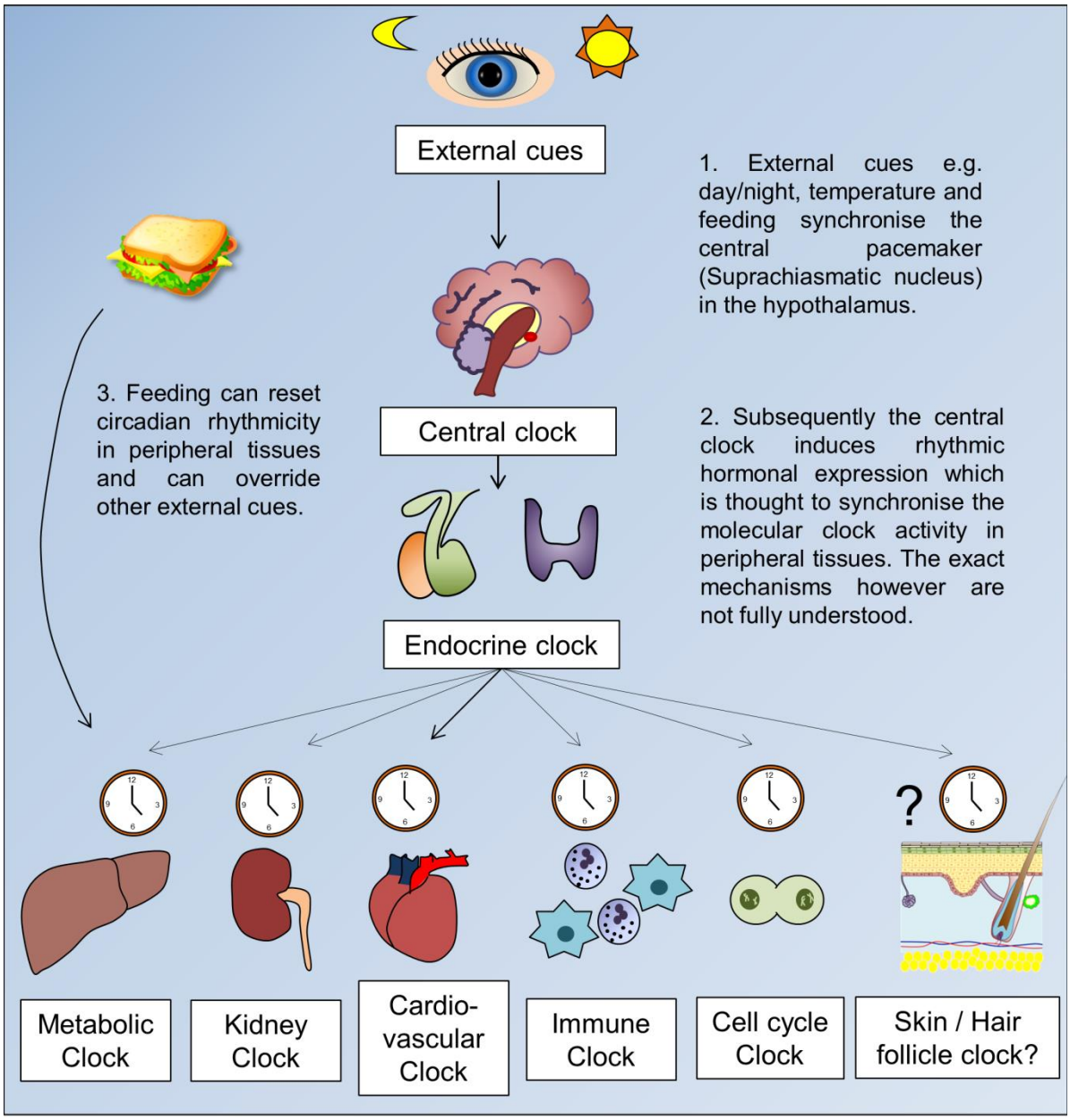

Fig 1: Organisation of the central and peripheral clocks.
(1.) The external cues such as the light and dark cycle are detected by the eye and conveyed to the suprachiasmatic nucleus (SCN) in the hypothalamus. (2.) The hypothalamus induces rhythmic expression of systemic hormones which subsequently synchronise the autonomous peripheral clock activity in peripheral tissues. (3.) Feeding is also one key external cue which can synchronise peripheral clock activity.

The core molecular clock is a series of cellular feedback loops that are synchronised by the master clock, the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN receives external clues or zeitgebers such as light and dark cycles and synchronises the circadian activity of the molecular clock found in peripheral tissues (**Fig. 1**) (Amir and Robinson 2006; Schroeder and Colwell 2013; Robinson and Reddy 2014).

Additional table on target genes of the molecular clock.

The core molecular clock begins when CLOCK and BMAL1 proteins come together, forming a heterodimer complex (Kondratov et al. 2003). This heterodimer translocates to the cell nucleus where it binds to those genes with an EBOX motif in their promoter region (Verastegui et al. 2000; Yoo et al. 2005). Genes with such a motif are collectively termed clock controlled genes (CCGs) and it's thought that up to 10% of the genome may be controlled in this manner (Panda et al. 2002). The binding of the CLOCK:BMAL1 heterodimer leads to the transcription of CCGs (Gekakis 1998; Bunger et al. 2000; Robinson and Reddy 2014). Two examples of CCGs are the period family (PER1,2 3) and the cryptochrome family (CRY1,2) (Darlington 1998; Sangoram et al. 1998; Robinson and Reddy 2014). As PER and CRY accumulate they form a second heterodimer which inhibits the formation of the initial CLOCK:BMAL1 heterodimer thereby inhibiting their own transcription. As these proteins degrade the CLOCK:BMAL1 heterodimer can reform, restarting the cycle (Kondratov et al. 2003; Robinson and Reddy 2014) (**Figure 2**). This process occurs over approximately 24 hours producing a circadian expression pattern of genes/proteins. Additional CCGs are the retinoic acid receptor related orphan receptors RORα and REV-ERBα/β which form accessory loops to the 'core' clock. These additional loops help maintain the 24 hour rhythmicity along with input from the SCN.

Additional input from Benedetto and Maksim: e.g. epigenetics in clock activity control etc., molecular basis of the autonomy of clock activity changes (parallels to hair cycle clock!!) e.t.c.

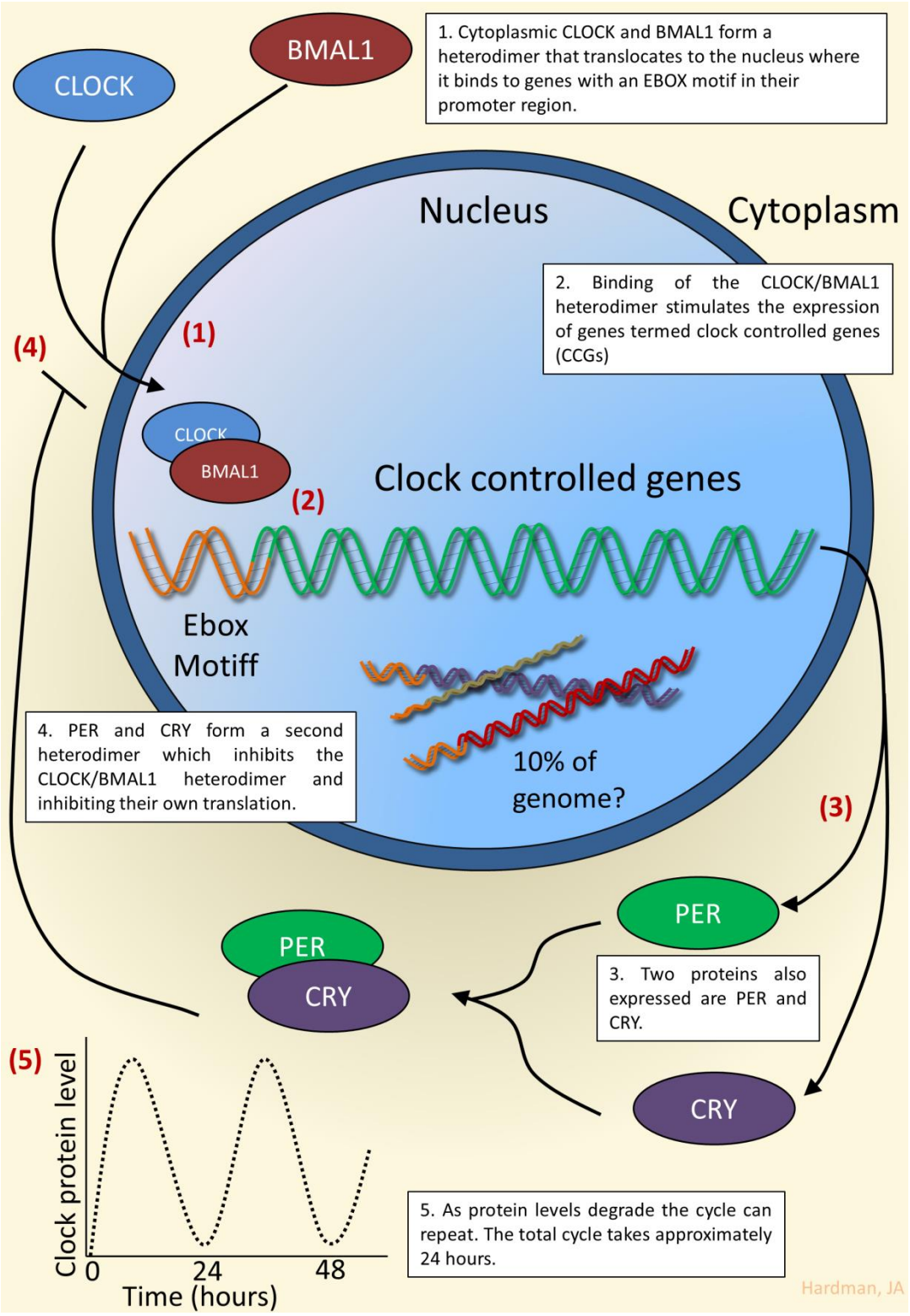

Fig. 2: The core molecular clock system.
The core molecular clock is an oscillating system lasting 24-hours. (1) The transcribed protein CLOCK and BMAL1 form a heterodimer which translocates to the cell nucleus where it binds to genes containing an EBOX motif in its promoter region or clock controlled genes (CCGs). (2) This binding leads to the transcription of CCGs and two families of proteins, the Period (PER) and Cryptochrome (CRY) family. (3) Proteins PER and CRY form a second heterodimer which prevents the formation of the first heterodimer and thereby inhibiting its own transcription. (4) This cycle leads to a 24-hour oscillatory rhythm.

The human hair follicle as an ideal target for translationally relevant chronobiology research

Additional figure showing the HFs anatomy and cycling

The human hair follicle (HF) is a cyclically regenerating clinically relevant mini-organ which traverses three stages, one of growth, apoptosis-driven regression and relative quiescence (Schneider et al. 2009). The HF is structurally, molecularly and temporally complex undergoing this life-long cyclic transformation. While there are many molecular components appreciated to be involved in hair cycle control, the intrinsic timing-mechanism or “hair cycle clock” that drives this long-term oscillatory system is not understood making the HF a key target and model for chronobiology research (Paus and Foitzik 2004; Kloepper et al. 2010).

Experimentally the human HF can be cultured, during which it will encapsulate both hair growth and the anagen-catagen stages of the HF cycle at rates close to *in vivo* (Philpott et al. 1990). By studying the complex system that is the HF we can begin to understand how biological timing systems affect cellular and physiological processes and how different cellular populations interact but also gain a better understanding of how the HF functions and how dysfunction can lead to pathologies including androgenic alopecia (Inui and Itami 2011).

HF cycling is a highly conserved yet the mechanisms as to why it cycles still remain a mystery (Paus et al. 1999b). Proposed theories include seasonal change, limitation of hair shaft length, cleansing the skin surface of toxins, disposing of chemicals from the body in trichocytes and to prevent the accumulation of mutations by shedding cells (Paus and Foitzik 2004; Cotsarelis 2006a). HF cycling has been extensively studied in murine models but there is minimal data available on this cyclic behaviour in the human HFs which, unlike the murine follicle, undergoes asynchronous cycling.

Although there are many appreciated controls involved in modulating the rhythmically process that is the human hair cycle, the timing mechanism controlling the switch between each cycle stage is yet to be fully described (Paus et al. 1999b). Chronobiology, the study of biological timing system is becoming increasingly appreciated in tissue physiology (Giebultowicz 2004). With the molecular clock now established as a novel candidate in controlling many biological processes including metabolism, the cell-cycle, the immune system and the murine hair cycle amongst other things (Lin et al. 2009; Bellet and Sassone-Corsi 2010; Bailey et al. 2014; Ben-Shlomo 2014; Kalsbeek et al. 2014), it may be that this oscillatory molecular system is one of the main regulators of the elusive 'hair cycle clock'.

Chronobiology has long been associated with the study of daily rhythms and the impact on physiology and health when normal rhythms are perturbed. Key examples of this are jetlag and night-shift work which are frequently associated with an increasing prevalence of diseases including obesity, diabetes and cancer (Davis and Mirick 2006; Buxton et al. 2012; Kalsbeek et al. 2014). Since the discovery of the genes and proteins that produce the biological clock the 'molecular biological clock' this system is now understood to be intrinsic to all cells (Partch et al. 2014). Furthermore, they are appreciated to be integral in choreographing complex processes involved in tissue maintenance including balancing reactive oxygen species (Lai et al. 2012).

The peripheral clock and the hair follicle: current evidence and concepts

Although circadian clock gene/protein expression was demonstrated in many of the cell types found in the skin (keratinocytes, melanocytes and fibroblasts) (Zanello et al. 2000; Sandu et al. 2012) it wasn't until Lin *et al.* in 2009 published their study that a link between circadian biology and the HF was established (Lin et al. 2009). In this murine study, distinct clock activity was observed throughout the hair cycle with Dbp, a CCG which acts as a robust marker for clock activity, showing the highest amplitude of expression in the secondary hair germ during telogen. Utilising a targeted knock-down of *bmal1* activity, thereby eliminating clock activity (Bunger et al. 2000), anagen onset was delayed when compared to littermate controls suggesting a role for the molecular clock in modulating hair cycle activity (Lin et al. 2009). It was further demonstrated that the bulb and bulge region of the murine HF had the highest level of clock activity, thereby identifying distinct, localised expression of clock genes/protein activity within the murine HF (Plikus et al. 2013).

The clock activity within the bulge stem cells specifically coincides with the activation status of the stem cells. Utilising a *Per1* promoter attached to a fluorescent reporter has shown that high levels of *Per1* expression coincide with activated stem cells, which have high levels of Wnt and low tgfβ expression, whereas low *Per1* expression represented the dormant stem cells. This demonstrated that the HF bulge stem cells exist in a heterogeneous state, half being primed for activation whilst the other remain dormant (Janich et al. 2011). Furthermore, the circadian clock has been implicated in controlling stem cell division, differentiation and is integral in maintaining stem cell heterogeneity (Brown 2014). These studies together first identified the robust circadian activity of the murine HF which has a distinct role in both the murine hair cycle and also in stem cell activity.

In humans, rhythmic clock gene expression was observed in plucked HFs identifying a novel tool by which to observe an individual's circadian profile however, this investigation failed to take into account the synchronising activity of the SCN, ignoring any potential intrinsically oscillating clock activity that may be present in the human HF and that may contribute to innate HF function (Akashi et al. 2010). However, more recent studies uncovered that the human HF does indeed express

several clock and clock-controlled in a circadian manner, when isolated from the SCN (Al-Nuaimi et al. 2014). This study also demonstrated that clock the clock protein PER1 had a strong hair cycle-dependent expression pattern, increasing as HFs entered catagen. Importantly, it showed that silencing of *PER1* and *BMAL1*, impacts on human HF cycling *in vitro* significantly prolonging anagen in the absence of central clock inputs, thus documenting that the molecular clock has a major a functional role in human HF biology. Given the paramount importance of abnormalities in HF cycling for undesired hair growth or loss (Paus and Cotsarelis 1999; Schneider et al. 2009) this is translationally important as this discovery identifies a new molecular control through which human hair growth might be manipulated in a clinically desired manner. (**Fig. 3**)

Using human HFs as a model system clearly excluded any influence from the central clock allowing the peripheral molecular clock to be studied, whilst avoiding any possible off-target effects of a global clock gene knock-out, species differences between mice and humans that may make it unclear whether murine hair research concepts apply to the human HF, and SCN inputs were excluded (Kondratov et al. 2006; Lin et al. 2009; Akashi et al. 2010; Geyfman and Andersen 2010; Kondratova and Kondratov 2012). That recent microarray analyses of synchronized murine HFs show peripheral clock gene activity in other cycle transformation stages further supports a role for the peripheral clock in influencing the 'hair cycle clock' as such. (Lin et al. 2009; Geyfman and Andersen 2010; Geyfman et al. 2012a; Plikus et al. 2013).

One unavoidable major methodological limitation of human HF organ culture is that conclusions can only be drawn on a functional role of the intrafollicular clock system in regulating the anagen-catagen transformation of the human hair cycle. Yet, in mice, telogen has been demonstrated to have high levels of clock activity, and the main hair cycle-regulatory effect of clock manipulation was that BMAL1-kock-out delayed the entry of telogen HFs into anagen (Lin et al. 2009). Since the telogen stage is increasingly being recognized as being everything but "quiescent", and is of major, often underestimated regulatory importance for hair cycle control, it is mandatory to check, next, whether the telogen-anagen transition of the human hair cycle also underlies regulation by changes in intrafollicular peripheral clock activity. This may be achieved by transplanting human scalp skin

onto immunocompromised mice (Gilhar 1987, 2013) so that clock gene expression and activity can be systematically characterized during a full human hair cycle *in vivo*.

Recently developed techniques for gene silencing in intact human skin (For example, gene gun, liposomes, electroporation etc.) (Aleku et al. 2008; Huang et al. 2011; Jensen et al. 2014) can then be applied to the human skin transplants in order to assess at the level of preclinical research how this impacts on human HF cycling *in vivo.*

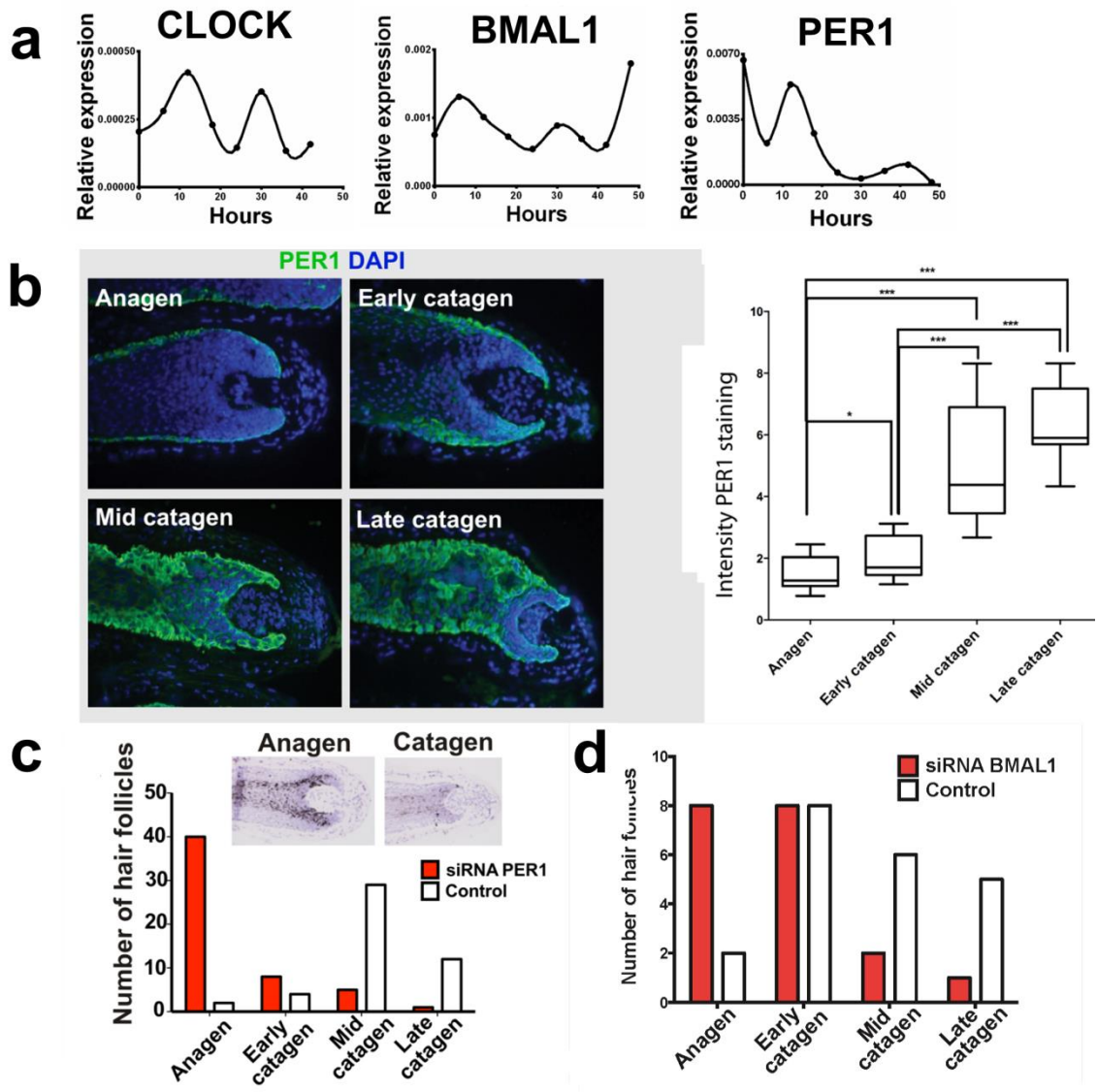

Fig. 3: The human HF has a functional molecular clock.
(a) Time course experiments on the human HF demonstrated that clock gene activity oscillated over 48 hours therefore the human HF has rhythmic clock activity when isolated from the SCN. (b) Protein analysis of PER1 showed that its expression was hair cycle-dependent increasing as HFs enter catagen. (c) Finally after silencing PER1 in cultured human HFs it was found that HFs remained in anagen longer than the scrambled oligo control. (d) This was mirrored when BMAL1 was silenced therefore uncovering a functional role for the molecular clock in the human hair cycle, specifically the anagen-catagen transition. [permission to be requested from the Journal of investigative dermatology for usage of these images].

A new horizon in chronobiology: the peripheral clock as a modulator of human melanogenesis and melanocyte biology

In addition to the hair cycle, the HF undergoes many other complex processes, one of which is the formation of hair pigmentation. Melanocytes produce hair pigment in the process of melanogenesis that converts L-tyrosine to the pigment molecular melanin (Chávez-Béjar et al. 2013), a process that is tightly coupled to the hair cycle and occurs exclusively in anagen (Slominski et al. 1994). With the close coupling of melanogenesis and hair cycle which is known to be influenced by the peripheral clock, first suggested a link between the molecular clock activity and pigmentation (Slominski et al. 2005a; Al-Nuaimi et al. 2014).

Interestingly human melanocytes have been linked with the molecular clock having been demonstrated to express both clock genes and proteins when cultured (Zanello et al. 2000; Sandu et al. 2012). As the process of melanogenesis and therefore if the HF has peripheral molecular clock activity is likely it will control many aspects of melanocyte biology and melanogenesis. Despite many candidates that control these processes being identified (**table 2.2**) what ultimately choreographs these systems remains elusive. This implicates the presence of an autonomously oscillating system, potentially the molecular clock, in controlling the complex on/off switch of melanogenesis.

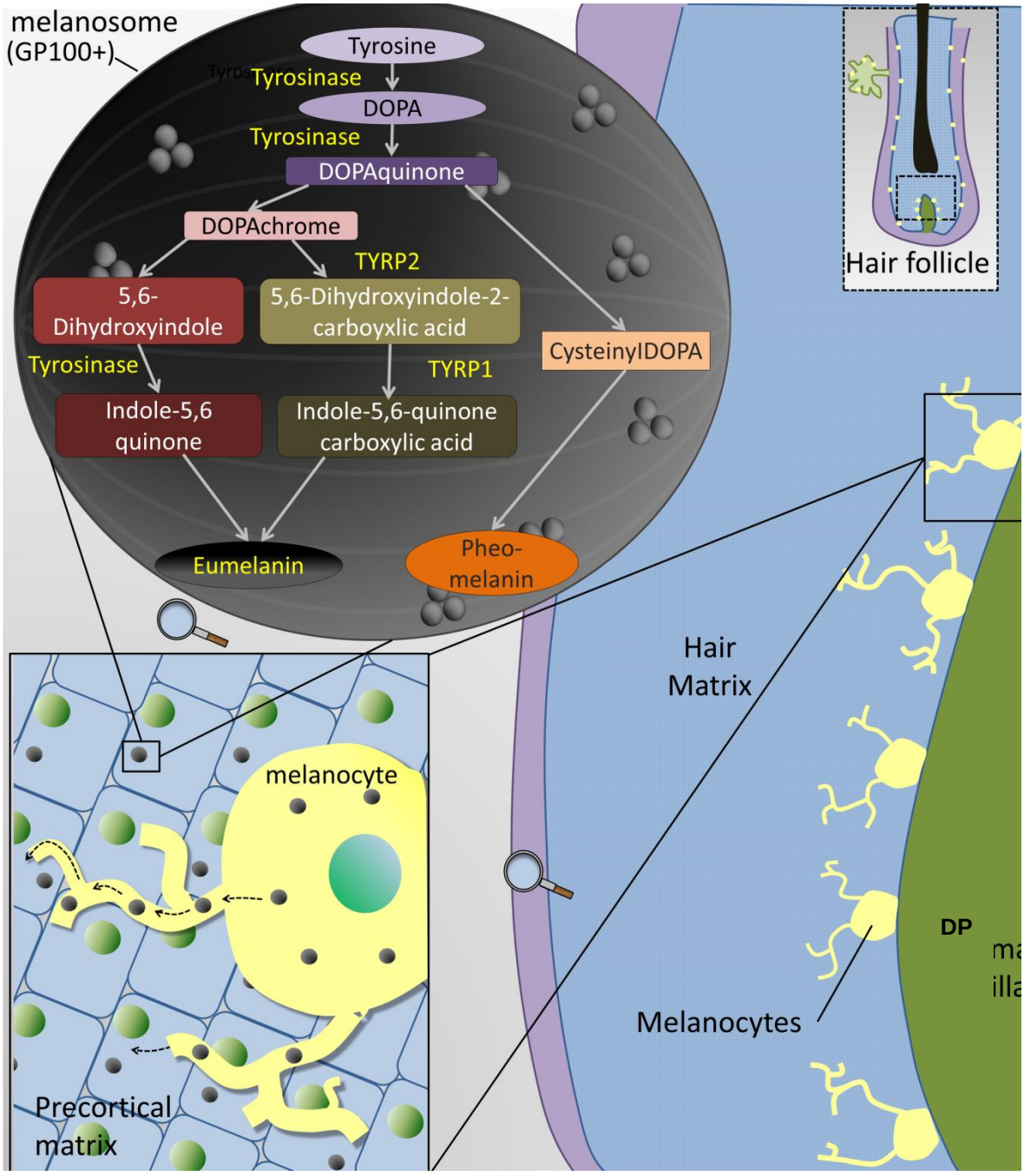

Fig. 4: Hair follicle pigmentation and intrafollicular melanogenesis: Overview
Hair follicle pigmentation is a complex process collectively known as melanogenesis. The pigment molecule melanin is produced by specialised dendritic cells, melanocytes, which locate adjacent to the dermal papilla within the hair matrix. These melanocytes contain organelles known as melanosomes within which the reaction to create melanin occurs. The formation of melanin involves a cascade of various chemical reactions catalysed mainly by a rate limiting enzyme tyrosinase (Tobin 2011). Once the melanosome has accumulated melanin, the organelle itself is transferred to those keratinocytes in the hair matrix destined to differentiate into the hair shaft via the melanocytes' dendrites.

The molecular clock is appreciated to be involved in the modulation of cellular metabolism and replication in response to UV light (Geyfman et al. 2012b) and can also relay light cues (Magalhães Moraes et al. 2014). Moreover, both *BMAL1* (Elshazley et al. 2012; Geyfman et al. 2012b; 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. In addition to this, the strength of cellular responses to DNA damage is under circadian control (Oklejewicz et al. 2008; Sancar et al. 2010). That melanocytes also respond to DNA-damaging UVR stimulation by increasing dendricity, hormone production and pigmentation, as well as acting as "sensory" cells within the epidermis implies that there may be a link between these two systems.

Furthermore, 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 ROS accumulation and reduction of life-span, fat, muscle and bone mass (Kondratov et al. 2006; Geyfman and Andersen 2010; Kondratova and Kondratov 2012), that a loss in hair pigmentation, or hair greying, is one of the first signs of aging in the HF further supports this. Indeed ROS accumulation in HF melanocytes is believed to be involved in age-related greying (Arck et al. 2006; Wood et al. 2009). As both and clock genes and melanogenesis are linked with ROS homeostasis, further supports a link between melanocyte biology and chronobiology (Geyfman et al. 2012b; Lai et al. 2012; Lee et al. 2013; Avitabile et al. 2014). 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).

Recently, the molecular clock was shown to have a functional, multi-level influence on both melanocytes and melanogenesis in a hair-cycle independent manner. When clock genes (BMAL1 or PER1) were silenced in human HF and skin melanin content, and melanosome number was increased. Gp100 immunoreactivity, melanocyte number and dendricity, tyrosinase activity and expression, of TYR, TYRP1 and 2 similarly increased. Melanocyte cell culture supported this finding

that tyrosinase activity and TYRP1 and 2 were increased when clock genes were silenced. In contrast to *in situ* work however, gp100 and melanocyte dendricity did not increase. These experiments were the first to demonstrate that human pigmentation is carefully modulated by an autonomous molecular clock activity in the melanocytes of both human HFs and human skin. Other aspects of melanocyte biology such as the increased dendricity observed appeared to be due to complex cross-talk between the melanocytes and the surrounding epithelial tissues (**Fig. 5**).

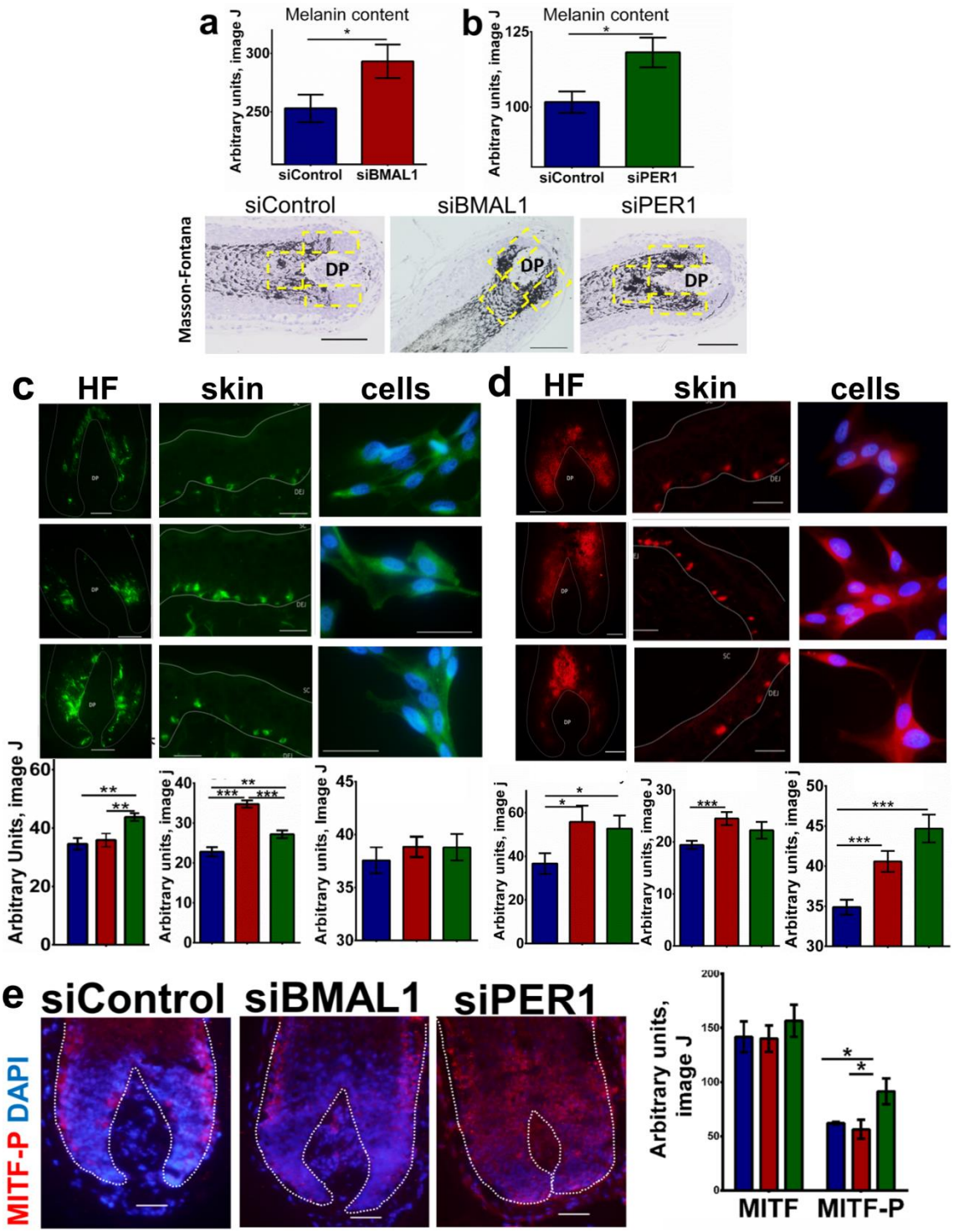

Fig. 5: The molecular clock influences human pigmentation.
Clock genes in human HFs, skin biopsies and isolated melanocytes were silenced. Subsequently the influence on various melanocyte and melanogenesis read-out parameters was quantitatively assessed. (a,b) a significant increase was observed in intrafollicular melanin content when either *BMAL1* or *PER1* was silenced in human HFs. (c) Gp100 expression was increased in both human HFs and human skin, but showed no significant differences in isolated melanocytes. (d) Tyrosinase activity also increased in human HFs, skin and also in isolated melanocytes. (e) Mechanistically, the activation of MITF, the master regulator of pigmentation was significantly increased when *PER1* suggesting this is how the clock influences human pigmentation.

Regarding the regulation of melanocyte biology and human pigmentation by the peripheral clock, mechanistically, the current data suggest that the increase in MITF activation by *PER1* silencing plays a key role in mediating the observed hyperpigmentation. However, a *direct* molecular link between MITF activation and clock silencing remains to be demonstrated. Unfortunately, due to the crucial role of MITF in many aspects of melanocyte biology (Tachibana 2000), dissecting such a direct link is technically very difficult, since blocking MITF activity (e.g. with neutralizing antibodies or by MITF knock-down) will inevitably inhibit pigmentation on multiple levels (Du et al. 2003; Vachtenheim and Borovanský 2010; Praetorius et al. 2013). One potential mechanism by which the clock influences pigmentation may the mitogen-activated-kinase (MAPK) pathway, specifically ERK 1/2 activation which subsequently activates MITF (Du et al. 2003; Su et al. 2013; Jin et al. 2014). This is supported by the fact that ERK1/2 is regulated by the circadian clock (in *Neurospora*) (Bennett et al. 2013). It may be possible to further explore direct molecular interactions of MITF with specific clock proteins by running pull-down assays (Hsieh et al. 2003), e.g. in protein extracts from melanocytes before and after clock silencing.

Alternatively, the increase in pigmentation by clock knock-down could be mediated indirectly by a change in cAMP levels, acting as a secondary messenger. In human pigmentation, cAMP is indeed important for MITF activation and MAPK activation (Buscà and Ballotti 2000; Rodríguez and Setaluri 2014). Moreover an increase in melanogenesis can be induced pharmacologically by increasing intracellular cAMP levels, thus mimicking the pigmentation-stimulatory effects of MSH or ACTH administration (Englaro et al. 1995). Interestingly, clock down-regulation, e.g. in mice with a knock-out of CRY1 and 2, leads to an up-regulation of cAMP (Zhang et al. 2010). This strongly supports cAMP as a potential mechanism of action. Therefore, it should be studied, next, whether cAMP levels are altered in clock-silenced HFs. This could be assessed using an enzyme-linked immunosorbent assay (ELISA) on cultured, clock-silenced HFs (Lambers et al. 2014).

Therefore it can be hypothesised that *PER1* and *BMAL1* knock-down may increase melanogenesis by up-regulation of cAMP and/or activation of the MAPK pathway, specifically by ERK1/2 activation

(Robertson et al. 2010). Both of which induce MITF phosphorylation and thus activation whilst simultaneously targeting it for degradation (Buscà and Ballotti 2000; Kim et al. 2014). Thus, it is particularly promising to further study these pathways in future research. A key place to start would be to stain clock-silenced HFs for ERK1/2 in order to determine whether activation of this protein is increased. If this is indeed the case, this would suggest that ERK1/2 activation increases MITF phosphorylation. This would ultimately imply that the molecular clock primarily influences human HF pigmentation by influencing the MAPK pathway. As modulators of this pathway exist, specifically inhibitors and regulators for ERK1/2 such as *PD98059* (Selleck Chemicals LLC, Houston TX, USA) and ERK inhibitor II (FR180204) (Santa Cruz) (Ohori et al. 2007; Arana-Argáez et al. 2010), they could be used to experimentally reduce ERK1/2 activity in clock-silenced HFs. If no changes in pigmentation are observed it could thus be conclusively demonstrated that the MAPK pathway up-regulation by molecular clock silencing leads to an increase in pigmentation.

A hair follicle perspective on the (neuro)-endocrine controls of chronobiology

TSH, T4 and T3, all members of the hypothalamic pituitary axis (HPT), demonstrate circadian rhythmicity and have been shown to be expressed in the HF. The HPT axis through which the thyroid hormones thyroxine (T4) and the more active triiodothyronine (T3) are produced, like circadian rhythms begin in the hypothalamus. In this pathway TRH produced by the hypothalamus travels to the anterior pituitary gland where it stimulates the production of thyroid stimulating hormone (TSH). The production of TSH inhibits further production of TRH and stimulates the production of thyrotropin in the thyroid gland. TRH, while directly stimulating the production of thyrotropin also regulates other downstream hormones including prolactin, growth hormone and insulin that also have roles in modulating the HF and hair cycle

It is now understood that human skin and the human HF express many of these hormones and the corresponding receptors and therefore there may be a peripheral HPT axis. TSH receptors for

example have been found in cultured dermal and HF fibroblasts, HF and skin derived keratinocytes (Slominski et al. 2002) which was further confirmed in human skin which expressed TSH and TSH receptor protein and mRNA. Further supporting the presence of a peripheral HPT-axis is TRH and TRH–receptors which are expressed in human HFs, where they stimulate pigmentation, prolong anagen and lead to MK proliferation therefore increasing HF elongation *in-vitro* (Gáspár et al. 2010). Finally, key enzymes involved in converting the terminal product thyroxine (T4) to the more active triiodothyronine (T3) D2 and D3 can be found in the human hair follicle suggests that HFs are actively able to convert T4 to T3 separate from the thyroid gland (van Beek et al. 2008). Together, that the human HF and human skin expresses all components of the HPT-axis strongly suggests that there is indeed a local HPT-axis in human skin and its appendages.

Interestingly, many of the hormones produced systemically are under tight circadian control. Products of this process thyroxine (T4) and triiodothyronine (T3) (**Fig 2.8**) are of particular interest as they influence both peripheral clock activity and have a role in negative feedback, inhibiting upstream products of the axis. T3 and T4, like TRH, have been shown to have a considerable role in the HF (van Beek et al. 2008) where they inhibit apoptosis and prolong anagen durations and have been shown to modulate keratin expression within the HF, namely K15 (van Beek et al. 2008). Moreover, there is a clear link with the circadian clock and thyroid hormones. Animal studies have found that systemic treatment with thyroid hormones can lengthen circadian periodicity in hamsters (Beasley and Nelson 1982). In contrast thyroidectomy can both reduced circadian periodicity but also dampen clock gene expression, specifically it blunts PER2 oscillations (Beasley and Nelson 1982; McEachron et al. 1993; Amir and Robinson 2006). In addition, T4 is essential for seasonal rhythms and mating season timing in mammals and also has a diurnal expression patterns (Dardente 2012; Dardente et al. 2014) where it has been implicated in regulating metabolism (Dardente et al. 2014), a process regulated by the molecular clock (Sahar and Sassone-Corsi 2009; Bass 2012; Buxton et al. 2012). However, direct evidence that T4 modulates the peripheral clock in human tissues *in situ* is still missing. While current studies highlight a potential link between thyroid hormones and the clock system, how thyroid hormones affect peripheral clock activity and whether it is through the molecular clock system that thyroid hormones influence the human HF requires investigation.

Key open questions in hair follicle chronobiology and how to address them

Additional input from Maksim Plikus

Conclusions and perspectives

In summary, this review has shown that the peripheral clock is intrinsic to human HF biology and forms an integral component of the long-elusive human “hair cycle clock”. Moreover, it has described the presence of a novel ‘pigmentation clock’ that regulates not only HF, but also epidermal pigmentation in human skin. This operates within primary human melanocytes in a cell-autonomous manner, independent of local influences from the skin environment or inputs of the central clock. In a broader sense, this thesis project has therefore further opened the door to *translationally relevant* chronobiological research in the human system, namely to better understanding the role of the *peripheral* core clock in regulating human tissue physiology *in situ* and under clinically relevant conditions. It is hoped that the concrete concepts emanating from this thesis project will translate into advances in clinical therapy along the lines discussed above.