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Short Article

Human Epidermal Stem Cell Function Is Regulated by Circadian Oscillations

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SUMMARY

Human skin copes with harmful environmental factors that are circadian in nature, yet how circadian rhythms modulate the function of human epidermal stem cells is mostly unknown. Here we show that in human epidermal stem cells and their differentiated counterparts, core clock genes peak in a successive and phased manner, establishing distinct temporal intervals during the 24 hr day period. Each of these successive clock waves is associated with a peak in the expression of subsets of transcripts that temporally segregate the predisposition of epidermal stem cells to respond to cues that regulate their proliferation or differentiation, such as TGFβ and calcium. Accordingly, circadian arrhythmia profoundly affects stem cell function in culture and in vivo. We hypothesize that this intricate mechanism ensures homeostasis by providing epidermal stem cells with environmentally relevant temporal functional cues during the course of the day and that its perturbation may contribute to aging and carcinogenesis.

INTRODUCTION

Stem cells and their differentiated progeny must cope with daily variations in a number of factors that can affect their function. The nature of these elements will vary from tissue to tissue, but knowing how and when to react to them is essential to the overall fitness of tissues. For instance, while the liver must metabolize potentially harmful substances ingested during each round of feeding, the skin copes with UV radiation and pathogens in a circadian manner. Our tissues have evolved means to adapt to these changes, and most cells in our body possess an inherent and specific self-sustained clock that allows them to anticipate their behavior according to these daily fluctuations (Dibner et al., 2010). This anticipatory clock mechanism therefore confers a functional advantage to the tissue, and decreasing its robustness reduces the overall fitness of the organism (Dibner et al., 2010). In this sense, whole-body circadian arrhythmia in mice causes premature aging and reduces their life span (Bunger et al., 2000).

Although recent studies have shown that the behavior of some types of stem cells is circadian, we are still far from understanding how circadian rhythmicity contributes to their function. For instance, hematopoietic stem cells (HSCs) egress from their niche and enter into circulation in a circadian manner (Lucas et al., 2008; Méndez-Ferrer et al., 2008). Yet whether the circadian clock affects the proliferation and differentiation of HSCs is not known. The keratinocyte compartment of the skin also behaves according to a circadian pattern, and whole-body arrhythmic *Bmal1* knockout mice show a delay in the growth phase of hair follicles (Lin et al., 2009; Geyfman and Andersen, 2010). Circadian oscillations in the expression of signaling pathways, including Wnt and TGFβ, predispose different populations of hair follicle stem cells to remain dormant or to respond to activating cues (Janich et al., 2011). Perturbation of this mechanism causes a progressive change in the number of dormant hair follicle stem cells as well as age-related hair follicle cycling defects (Janich et al., 2011). However, epidermal-specific arrhythmic mice do not show changes in the first wave of postnatal hair growth, suggesting that changes in the oscillation of systemic or niche-derived cues are likely responsible for the delay in hair follicle growth observed in complete *Bmal1* knockout mice (Lin et al., 2009; Janich et al., 2011; Geyfman et al., 2012).

Murine interfollicular epidermis is also under circadian control. For instance, proliferation of mouse basal epidermal cells peaks at night, whereas accumulation of ROS is antiphasic to it (Geyfman et al., 2012). Interestingly, mouse epidermis is more susceptible to UVB radiation during the night when its cells are more proliferative, and mice significantly develop more squamous tumors when exposed to UVB in the night than during the day (Geyfman et al., 2012; Gaddameedhi et al., 2011). By separating proliferation from the response to UVB and ROS metabolism, circadian rhythms likely protect keratinocytes

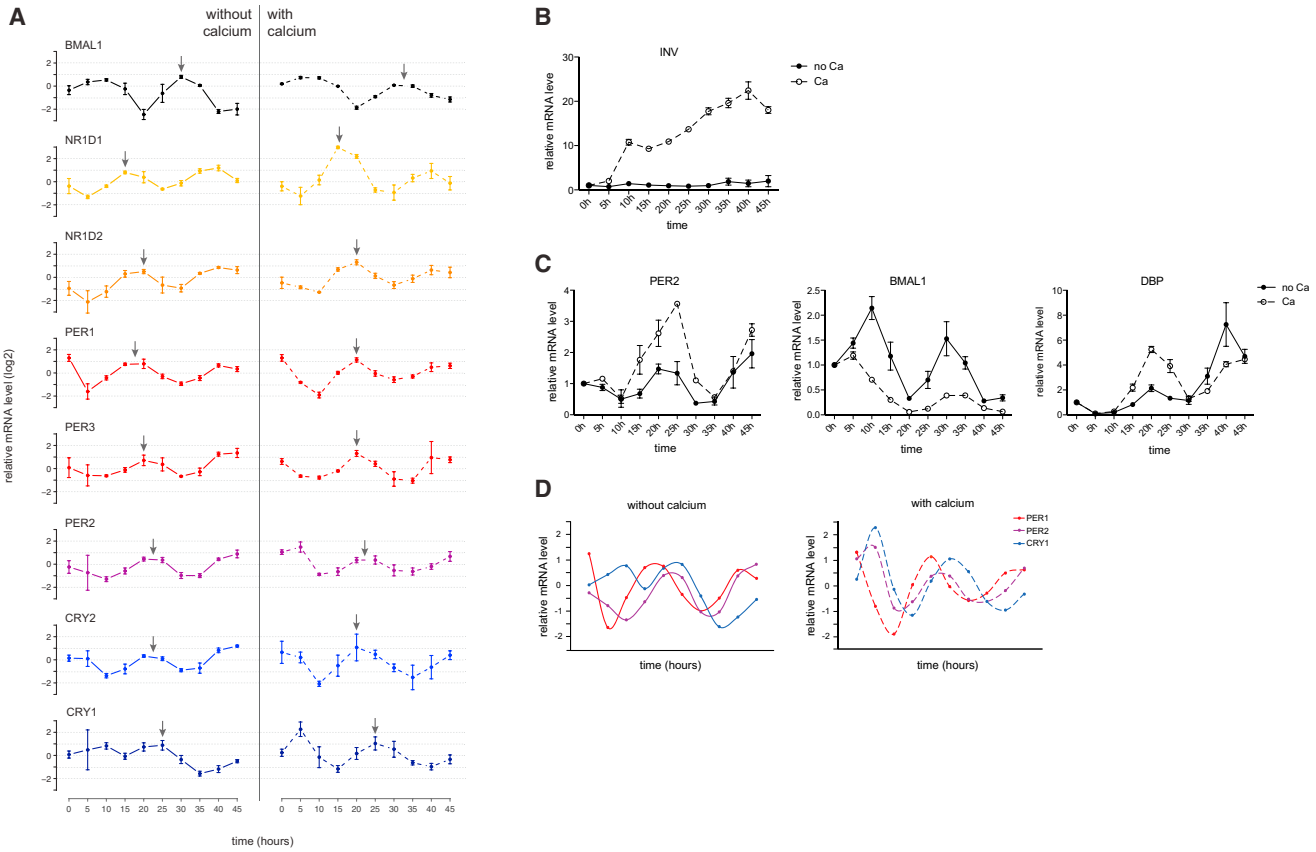


Figure 1. Core Clock Genes Peak in a Successive and Phased Manner in Human Epidermal SCs

(A) Relative mRNA levels of Involucrin (INV) in undifferentiated and calcium-differentiated PHKs normalized to time point 0 hr.

(B) PHKs were synchronized and gene expression wasmeasured at indicated times. The right and left panels show expression of clock genes in cells with and without prior calcium treatment, respectively, at 0 hr.

(C) Relative mRNA levels of PER2, BMAL1, and DBP in undifferentiated and calcium-differentiated primary human keratinocytes normalized to time point 0 hr.

(D) Superimposition of PER1, PER2, and CRY1 expression profiles. Although the amplitude of expression is different, the phase shifts between the genes remains highly consistent whether or not differentiation is induced through calcium treatment.

n = 3 in all panels; results are shown as mean ± SEM. See also Table S1.

from the potentially harmful situation of proliferating when exposed to UVB or when ROS levels are highest. Loss of this mechanism might be one of the causes underlying the premature aging observed in epidermal-specific circadian arrhythmic mice (Janich et al., 2011).

DNA synthesis and mitosis show circadian fluctuations in human epidermis (Schell et al., 1981a, 1981b, 1983; Brown, 1991). However, it is unclear whether (and if so, how) human epidermal stem cells know which is the best time for performing a specific function during the 24 hr period of the day. We have addressed this question by combining timed mRNA expression analysis of primary human keratinocytes with functional data. Our results show that, intriguingly, the expression of specific core clock genes is phase delayed within 24 hr and that this establishes successive windows of time in which epidermal stem cells are more predisposed to perform certain functions than others. These include proliferation, differentiation, and response to UV, all of which are paramount to ensure that epidermal stem cells fulfill their basic function to maintain homeostasis.

RESULTS

Core Clock Genes Peak in a Successive Manner along a 24 hr Period

We obtained the stepwise global changes in gene expression of primary human keratinocytes (PHKs), either in their stem cell state or induced to differentiate with calcium, every 5 hr during 2 consecutive days (Table S1). To synchronize the circadian rhythms, cells were given a short serum pulse for 2 hr prior to the time course of RNA collection. Expression of the core clock genes *Bmal1*, *period1–3* (*PER1–3*), *Nr1d1/2*, and *cryptochrome 1/2* (*CRY1/2*) confirmed that the serum pulse properly synchronized the circadian rhythm of stem cell and differentiating keratinocytes (Figures 1A and 1B). As expected, PHKs stimulated with calcium progressively differentiated, as shown by the increased expression of differentiation markers such as *involucrin* (*IVL*), *filaggrin* (*FLG*), and *Late Cornified Envelope* (*LCE*) genes, whereas undifferentiated PHKs did not spontaneously differentiate during the time course (Figure 1A, Table S1 available online). Calcium-induced differentiation did not alter the oscillation

period of core clock genes but increased the amplitude of oscillation of *PER1–3* and *DBP*, while decreasing that of *Bmal1* compared to undifferentiated cells (Figure 1C and Table S1).

PER1–3 and *CRY1/2* are direct transcriptional targets of BMAL1 and CLOCK. However, intriguingly, when plotted together, their peaks showed phase delays with respect to each other. For instance, the expression of *PER1* and *PER3* peaked approximately 20 hr after the synchronization pulse, whereas *PER2* and *CRY2* peaked 5 hr after *PER1* and *PER3* (Figure 1B). *CRY1* peaked 5 hr later than *PER2*/*CRY2*, and *NR1D1* and *NR1D2* also showed a phase delay with respect to each other, with peaks of expression between 15 hr and 20 hr postsynchronization, respectively (Figure 1B). As expected, *Bmal1* was predominantly antiphasic with its core clock targets (Figure 1B). When we plotted all these clock core genes together, an interesting pattern of concatenated oscillations emerged, whereby *Bmal1* is the first to peak at around 5–10 hr postsynchronization, followed by *Nr1d1* and *Nr1d2* (15–20 hr), *PER1*/*PER3* (20 hr), and subsequently by *PER2*/*CRY2* (20–25 hr) and CRY1 (25 hr) (Figure 1D; for simplicity only one core clock gene is shown for each peak). This pattern of expression was unaffected during differentiation, further indicating that differentiation only affects the amplitude and not the period of oscillation of the core clock machinery (Figures 1B and 1D).

The Successive Peaks of Clock Genes Temporally Segregate Different Epidermal Stem Cell Biological Functions

Our analysis indicated that the core clock machinery displays successive oscillations that subdivide the day into at least five temporal intervals (i.e., those established by the peaks of *NR1D1*/*NR1D2*, *PER1/3*, *PER2*/*CRY2*, *CRY1*, and *Bmal1*), which we refer to as peaks A–E. We next sought to determine whether each peak defined different functional categories in both stem cell and differentiating keratinocytes. To do so, we developed a polynomial curve fitting analysis to identify cohorts of genes whose expression displayed a pattern of peaks and troughs similar to each of these clock subsets (Supplemental Information; Table S2; Figure S1). In undifferentiated PHKs, the expression of 2,013 genes correlated with the pattern of oscillation of core clock genes, distributed more or less equally at each peak (A, 374 genes; B, 454 genes; C, 695 genes; D, 575 genes; E, 415 genes; Table S2). Differentiating keratinocytes showed 2,498 genes with a similar distribution to core clock genes (A, 327 genes; B, 566 genes; C, 899 genes; D, 494 genes; E, 832 genes; Table S2) (Figure 2A).

We then performed gene ontology (GO) analyses on the list of genes for each peak to obtain the biological functions represented by each (Table S3 and Table S4) (Huang et al., 2009). Several studies have shown that the mRNAs of *PER1* and *PER2* peak early during the morning in human epidermis, which we used as a reference point to extrapolate the approximate time of the day corresponding to each peak (Akashi et al., 2010; Spörl et al., 2012; Watanabe et al., 2012). The most statistically significant biological categories represented in peak A included protein localization and regulation of transcription, cytoskeleton, cAMP metabolism, and collagen metabolic process, among others (Figure 2B and Table S3). Interestingly, these biological categories included genes previously shown to be involved in keratinocyte differentiation, such as *Notch3*, *KLF9* (a prodifferentiation transcription factor recently shown to be circadian in human keratinocytes; Spörl et al., 2012), *p57*, and *SPRR1A/B* (Watt et al., 2008). Peak B in turn predominantly consisted of genes involved in calcium homeostasis, cholesterol metabolism, RNA modification, amino acid metabolism, response to vitamin D, and ribosome biogenesis, which has been recently shown to be circadian in murine liver (Jouffe et al., 2013) (Figure 2B). Several of these categories, such as calcium homeostasis, cholesterol metabolism, and response to vitamin D, are directly related to keratinocyte differentiation (Bikle et al., 2001) (Table S3). This list also included *NSUN2/Misu*, a downstream target of Myc that poises epidermal stem cells for differentiation, and *CAV1*, which regulates the formation of the lamellar granules required for keratinocyte cornification (Blanco et al., 2011; Sando et al., 2003). Although peak C also comprised genes pertaining to calcium signaling and ribosome biogenesis, it now included genes involved in response to glucocorticoids, glucose and lipid metabolism, and (albeit with a lower statistical significance) cell cycle progression (Figure 2B). Hence, our results suggest that peaks A–C primarily correspond to a peak in expression of pathways involved in predisposing human epidermal stem cells for the onset of differentiation and the metabolic changes associated with it.

Interestingly, peak D (corresponding approximately to 5–10 hr after the peaks of PER1-peak B and PER2-peak C) no longer showed genes related to keratinocyte differentiation, but rather to those involved in organization of the nuclear lumen, DNA-damage response and repair, ribosome biogenesis, mitochondrial morphogenesis, cell cycle and DNA replication, and regulation of the splicing, pigmentation, and ATP metabolism (Figure 2B). These included genes such as *SSRP1*, *EXO1*, *SMC6*, *POLD1*, *RAD54B*, and *APRT*, which are involved in the response and repair of DNA upon UV radiation, and *TP63* and *HELLS*, both of which are required to kick-start epidermal stem cell proliferation (Sertic et al., 2011; Santa Maria et al., 2007; Ogi et al., 2010; Truong et al., 2006; Sen et al., 2010). Lastly, peak E comprised genes regulating endosome membrane, RNA localization, chromatin remodeling, DNA metabolism/stress response, and cell division/mitotic phase (Figure 2B).

A pattern emerges from this data whereby peaks A–C, corresponding to late-night to early-morning hours, are mostly related to pathways predisposing keratinocytes to differentiate, whereas peaks D and E, representing the afternoon and evening hours, corresponded to pathways inducing DNA replication, protection to UV, and, subsequently, cell division. This overall pattern of successive peaks suggests that human epidermal stem cells proceed through functional landmarks that segregate their predisposition to respond to proproliferative and differentiation cues, while providing them with the necessary metabolism and protection against UV radiation. This pattern of functional segregation was also present in differentiating keratinocytes (Table S4), yet each peak was represented by different biological functions than those observed in the undifferentiated state. For instance, peaks D and E no longer included genes involved in DNA replication and mitosis, but contained those related to keratin filament organization and *Runx1*, which are required for human keratinocyte differentiation (Masse et al., 2012) (Figure S2). In addition, the category of defense response was only

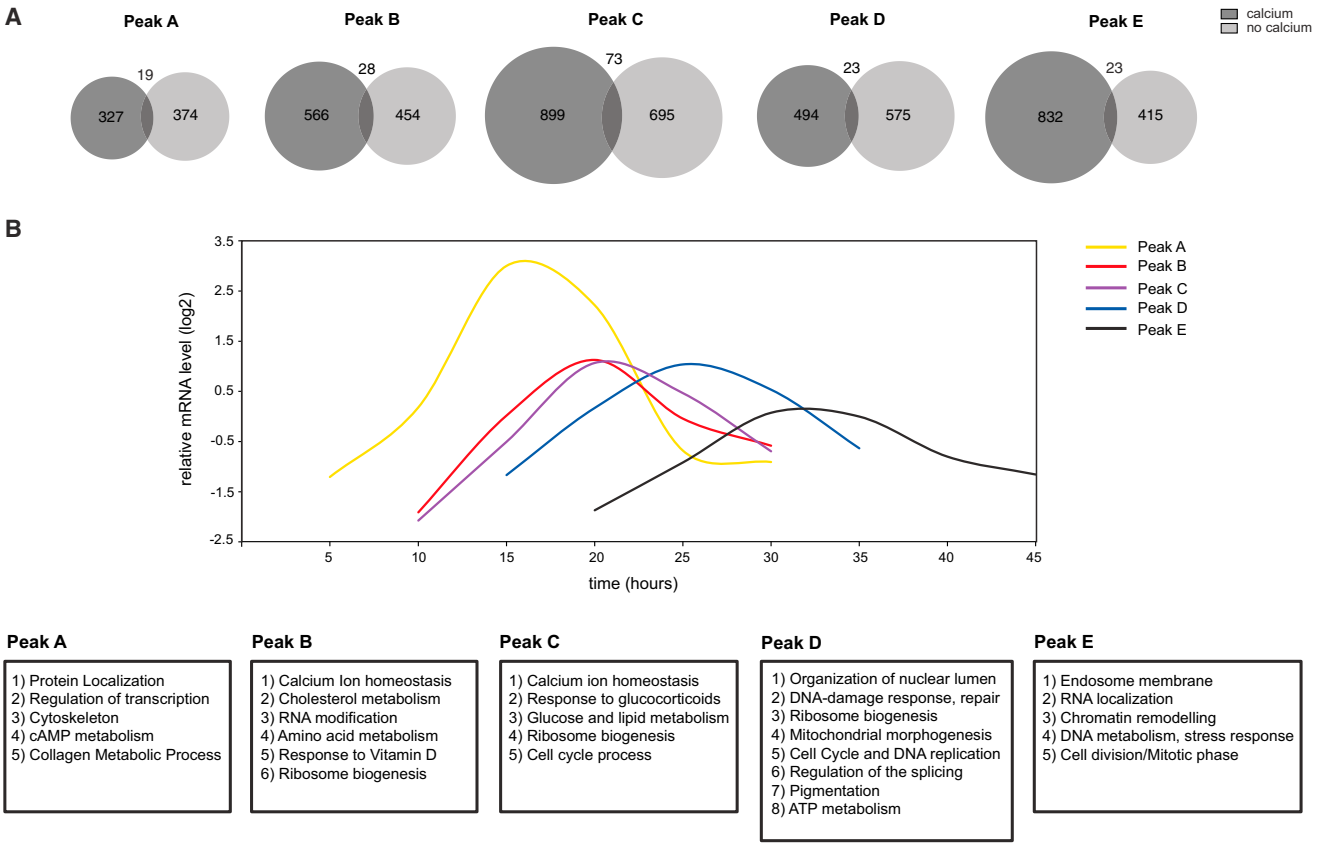


Figure 2. Successive Core Clock Gene Peaks Establish Distinct Functional Intervals during the 24 hr Period

(A) Gene lists were defined in accordance with their compatibility to peaks A–E ($p < 0.05$) for the two experimental conditions (with calcium, dark gray; without calcium, light gray). The Venn diagrams show the overlap between those lists.

(B) The five curves represent each of the peaks A–E in the undifferentiated cells along with their topmost enriched GO categories ($p < 0.05$).

See also Figures S1 and S2 and Table S2, Table S3, and Table S4.

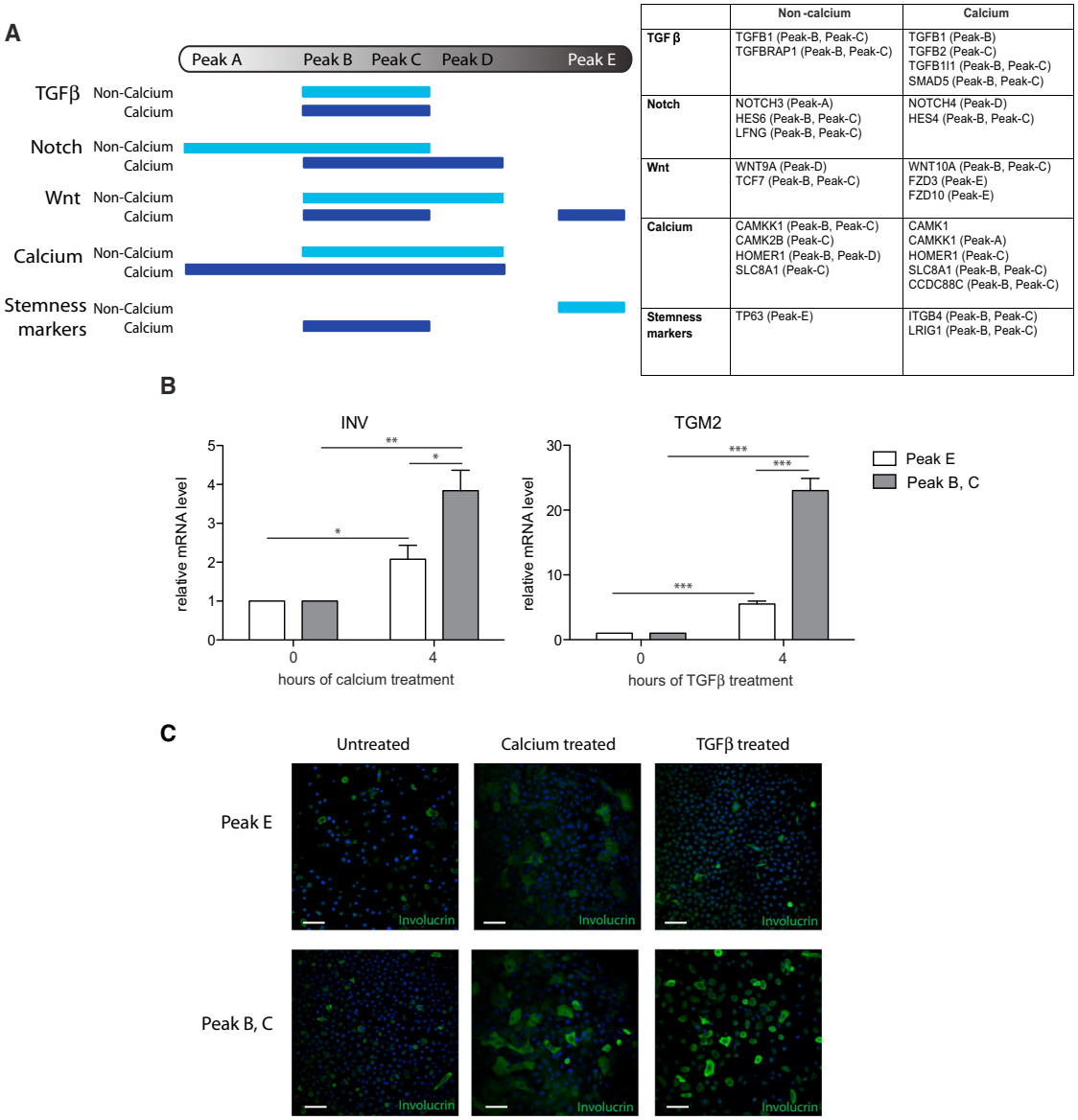
represented in the GO analysis of differentiating keratinocytes, consistent with their role in establishing the protective defense barrier. Interestingly, the fact that UV radiation was also present in both data sets suggests that epidermal keratinocytes, whether undifferentiated or differentiated, remain vigilant from this harmful radiation in a circadian manner.

Epidermal Stem Cells Respond to Differentiation Cues in a Time-of-Day-Dependent Manner

Several pathways involved in epidermal homeostasis were represented in our analysis, including TGFβ, Notch, BMP, Wnt, and calcium signaling (Figure 3A and Table S2). The expression of genes important for epidermal stem cell quiescence, proliferation, and differentiation, such as *LRIG1*, *TP63*, and *ITGB4*, also peaked at a specific time of the day (Figure 3A). Some members of the calcium, TGFβ, and Notch pathways also showed time-of-the-day differences in their expression in murine basal epidermal keratinocytes, indicating that their daytime-dependent expression is conserved between mice and human (Figure S3A).

We next sought to test whether the timed changes in the expression of these genes translated into functional differences regarding the predisposition of PHKs to respond to these cues. We concentrated on two pathways, calcium and TGFβ, as they constitute two major signals driving PHK differentiation. Our results predict that undifferentiated PHKs would be more predisposed to respond to both cues during peaks B–C, corresponding to the late night-morning hours (Figures 1D and 2B). To test this, we synchronized the circadian rhythm of undifferentiated PHKs and then stimulated them with TGFβ or calcium at 12 or 24 hr post-synchronization, corresponding to the summits of peaks E and B–C, respectively. We then collected RNA 4 hr after stimulation to evaluate the response of the cells to each stimulus by measuring the expression levels of *IVL* and *transglutaminase 2*, known calcium and TGFβ target genes (Honma et al., 2006; Ranganathan et al., 2007). Interestingly, PHKs responded more efficiently to both cues 24 hr postsynchronization than at 12 hr, coinciding with the behavior predicted from our peak analysis (Figure 3B). This enhanced response was also evident by the increased expression of IVL within the colonies and by the enlarged cell size associated with differentiation (Figure 3C). Calcium- and TGFβ-induced differentiation of PHKs progressively increased between the trough and the peak, further demonstrating that they depend on an oscillatory mechanism (Figure S3B).

We next verified whether increasing and sustaining the expression of *PER1* or *PER2* would affect the predisposition of PHKs to differentiate. For this, we overexpressed *PER1* or



	Non -calcium	Calcium
TGF β	TGFB1 (Peak-B, Peak-C) TGFBRAP1 (Peak-B, Peak-C)	TGFB1 (Peak-B) TGFB2 (Peak-C) TGFB1I1 (Peak-B, Peak-C) SMAD5 (Peak-B, Peak-C)
Notch	NOTCH3 (Peak-A) HES6 (Peak-B, Peak-C) LFNG (Peak-B, Peak-C)	NOTCH4 (Peak-D) HES4 (Peak-B, Peak-C)
Wnt	WNT9A (Peak-D) TCF7 (Peak-B, Peak-C)	WNT10A (Peak-B, Peak-C) FZD3 (Peak-E) FZD10 (Peak-E)
Calcium	CAMKK1 (Peak-B, Peak-C) CAMK2B (Peak-C) HOMER1 (Peak-B, Peak-D) SLC8A1 (Peak-C)	CAMK1 CAMKK1 (Peak-A) HOMER1 (Peak-C) SLC8A1 (Peak-B, Peak-C) CCDC88C (Peak-B, Peak-C)
Stemness markers	TP63 (Peak-E)	ITGB4 (Peak-B, Peak-C) LRIG1 (Peak-B, Peak-C)

Figure 3. The Predisposition of Human Epidermal SCs to Respond to Calcium and TGFβ Prodifferentiation Cues Segregates during the 24 hr Period

(A) Diagram showing peak fitting of pathways known to be important for differentiation and pluripotency of epidermal cells. The table lists genes belonging to the abovementioned pathways with detailed information on their peak fitting ($p < 0.05$).

(B) Undifferentiated primary human keratinocytes were treated 12 hr or 24 hr after serum synchronization with calcium or TGFβ for a period of 4 hr. Graphs show relative mRNA levels of involucrin (INV, n = 4) and transglutaminase-2 (TGM2, n = 3) after 4 hr of treatment.

(C) Same experiment as (B) showing protein levels of IVL by immunofluorescent staining (green fluorescence; blue fluorescence corresponds to DAPI). Results are shown as mean ± SEM normalized to the expression levels of INV and TGM2 at 12 or 24 hr postsynchronization (referred to as 0 hr of treatment).

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t test). See also Figure S3, Table S2, Table S3, and Table S4.

PER2 in undifferentiated PHKs (Figure 4A) and confirmed that their overexpression perturbed their circadian rhythm (Figures S4A and S4B). PHKs overexpressing *PER1* or *PER2* spontaneously differentiated as shown by a significantly reduced clonogenic capacity (Figure 4B) and an increased expression of the differentiation markers IVL and loricrin (Figure 4C). We also performed an in vivo competition assay based on an orthotopic method of transplantation of PHKs in immunodeficient mice. Three different transplants were established: (1) a 1:1 mixture of PHKs tagged either with mCherry or GFP; (2) a 1:1 mixture of control mCherry cells and *PER1*-overexpressing GFP cells; and (3) a 1:1 mixture of control mCherry cells and *PER2*-overexpressing GFP cells (Figure 4D). In each instance, control cells contributed to the basal layer in a stable manner, and columns of tagged basal and suprabasal cells were visible throughout the transplants. On the other hand, PHKs overexpressing PER1 or PER2 only located at the uppermost suprabasal differentiated layers (Figure 4D). Endogenous expression PER2 at the

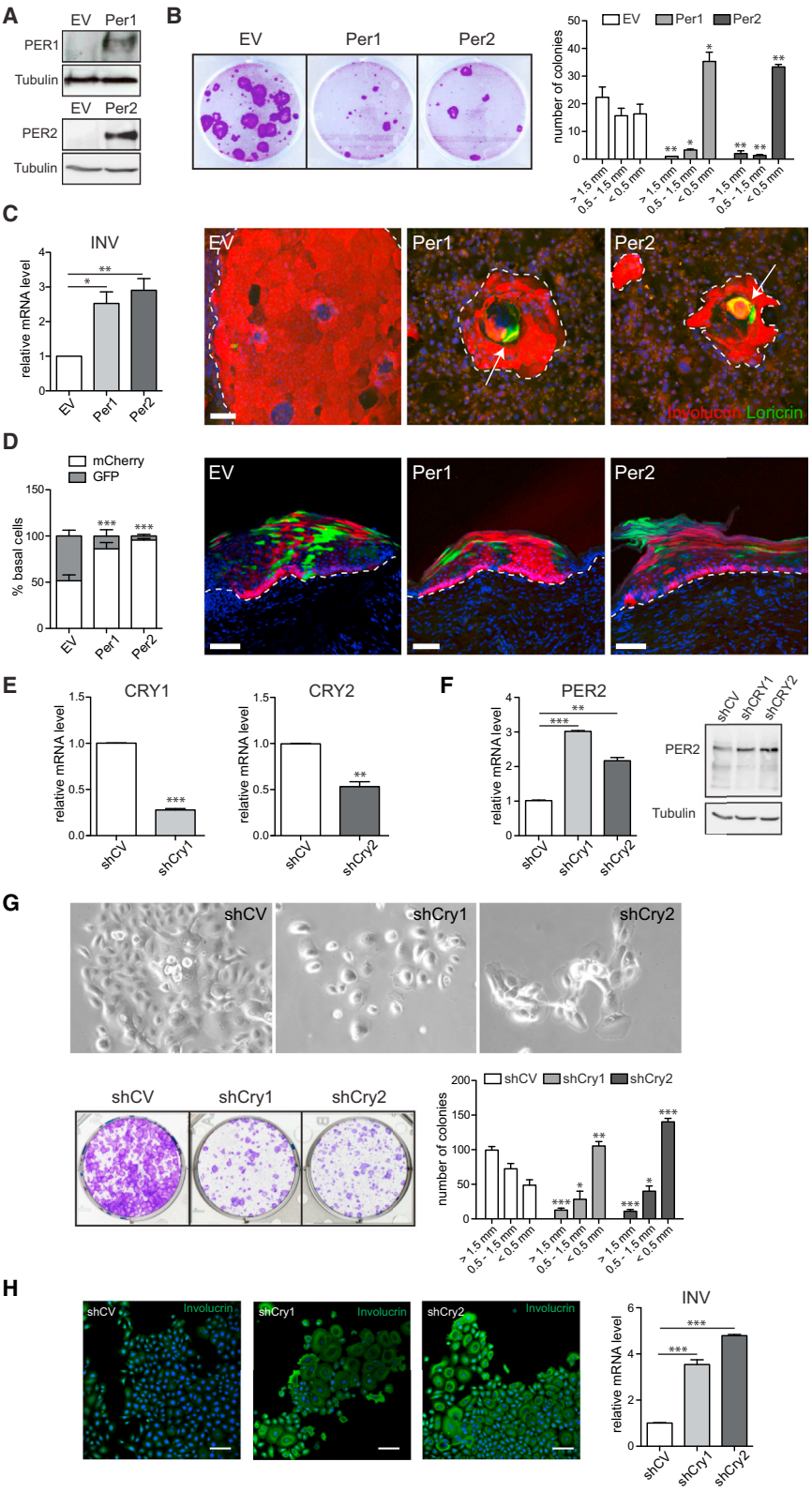


Figure 4. Circadian Arrhythmia Induces Spontaneous Differentiation and Loss of Self-Renewal of Human Epidermal SCs

(A) Western blot analysis shows the overexpression of PER1 and PER2 in PER1- or PER2-infected PHKs compared to empty vector (EV) control.

(B) Clonogenic assay of PER1- or PER2-overexpressing PHKs compared to EV-infected cells. Images are representative for n ≥ 4 independent infections. Colony size was quantified according to their diameter and differences in colony number in PER1- or PER2-infected keratinocytes were calculated compared to the control (n = 3).

(C) PER1- or PER2-infected PHKs have increased expression of IVL and loricrin. Left panel: graph shows relative mRNA levels of INV in PER1- or PER2-infected keratinocytes compared to the control (mean ± SEM, n = 3). Right panel: immunofluorescence reveals high expression of IVL (red) and loricrin (green, arrow) in PER1- or PER2-infected keratinocytes compared to the control. Scale bar: 100 μm.

(D) Competitive skin reconstitution assay of EV or PER1- or PER2-infected PHKs (green) mixed with equal numbers of mCherry-infected cells (red) shows the exclusion of PER1- or PER2-infected keratinocytes from the basal cell layer (dashed line) and their localization to the outermost differentiated cell layers (n = 3 mice per infection). Left panel: graph shows percentage of mCherry and GFP-positive cells in the basal layer of EV or PER1- or PER2-overexpressing transplants. Right panel: direct fluorescence of mCherry or GFP. Scale bar: 75 μm.

(E) mRNA levels of endogenous CRY1 and CRY2 in PHKs stably expressing shRNAs specific to each protein (n = 3 independent infections).

(F) mRNA and protein levels of endogenous PER2 in CRY1 and CRY2 knockdown cells (n = 3).

(G) PHKs with reduced levels of CRY1 and CRY2 show a differentiated morphology and reduced clonogenic potential (n = 3 independent infections).

(H) Elevated levels of IVL (protein and transcript) in PHKs with reduced levels of CRY1 or CRY2 (n = 3 independent infections).

In all panels, *p < 0.05, **p < 0.01, and ***p < 0.001 (Student's t test), and data are represented as mean ± SEM. See also Figure S4.

mRNA and protein level increases upon knockdown of CRY1 and CRY2 (Figures 4E and 4F; Dibner et al., 2010). Consequently, PHKs with lower levels of CRY1 and CRY2 spontaneously differentiated, determined by their cellular morphology, decreased clonogenic potential, and higher expression of IVL than control cells (Figures 4G and 4H).

DISCUSSION

Our results indicate that the oscillation of the core clock transcriptional machinery consists of successive waves along the 24 hr of the day. Intriguingly, these phase shifts of 4–5 hr define a continuum of functional landmarks that segregate vital functions of keratinocytes.

They predispose undifferentiated keratinocytes to respond to differentiation cues between the late-night and morning transition. Subsequently, during the hours of exposure to light, two different and consecutive cohorts of genes predominantly involved in protection from UV and in inducing proliferation reach their zenith. Thus, human keratinocytes anticipate their protection against UV at the time when they are more prone to duplicate their DNA and more susceptible to radiation-induced DNA damage. During the evening-night transition, keratinocytes then become predisposed to undergo mitosis, but also start to express genes involved in the onset of differentiation, thereby establishing the beginning of a new cycle.

Calcium-induced differentiation of keratinocytes maintained the period of the oscillations of core clock genes, although the amplitude of the negative limb of the clock was increased. The molecular and functional reasons underlying this change in amplitude are currently unknown, but previous works have shown that activation of calcium signaling directly increases the expression of *PER1* and *PER2* in brain cells (Nomura et al., 2003, 2006; Akiyama et al., 2001). Functionally, since the peak of *PER1* and *PER2* correlates with the predisposition of PHKs to differentiate, we hypothesize that the increase in their amplitude of oscillation confers a more deterministic propensity toward differentiation (i.e., once a cell has committed to differentiate, it is advantageous that it does so in an irreversible manner). Conversely, a lower oscillating amplitude might endow stem cells with the necessary plasticity to adapt and respond to the changing conditions of the tissue. It is important to underscore that very few biological categories defined by each intradiurnal peak overlap between the undifferentiated and differentiated states, indicating that even within the same lineage (i.e., epidermal keratinocytes) the circadian transcriptome is highly cell specific. However, the biological functions shared in both cellular states relate to basic cellular functions, such as the protection against UV, metabolism, and ribosome biogenesis. On the other hand, only differentiated keratinocytes show time-dependent changes in the expression of genes involved in the formation of an impermeable barrier and defense against pathogens, both of which are essential in the epidermis. Intriguingly, plant cells also show a circadian immune protection against pathogens that display circadian rhythms in their infectivity (Wang et al., 2011).

Our understanding of how these successive peaks of expression are established and maintained is still poor. A recent rigorous study describing how the basic transcriptional clock machinery works in vivo in the murine liver indicates that the core clock repressors Per1, Per2, Cry1, and Cry2 bind to their respective genomic sites at different times of the day, indicating that the transcriptional regulation of the clock is far more complex than previously anticipated (Koike et al., 2012). The transcription-independent and universal circadian clock based on the oxidation state of peroxiredoxins might also modulate these oscillations (Edgar et al., 2012; O'Neill and Reddy, 2011; O'Neill et al., 2011). Using the available circadian transcriptome of other tissues reported by others, we have observed the same pattern of oscillations or core clock genes as in human epidermal stem cells, suggesting that this mechanism might be relevant for modulating the function of adult stem cells and their differentiated counterparts (Storch et al., 2002) (Figures S4C and S4D).

EXPERIMENTAL PROCEDURES

PHK Culture

PHKs were isolated from neonatal foreskin samples and cultured with a feeder layer of fibroblasts (J2-3T3) as described previously (Gandarillas and Watt, 1997). Keratinocytes were infected with retroviral supernatants produced from Phoenix A or lentiviral supernatants produced by 293T cells transfected with the mentioned plasmids. Cells in clonogenic assays were grown for 8–10 days and then fixed in 4% paraformaldehyde and stained with 0.1% crystal violet/0.1% rhodanile blue (Sigma-Aldrich). For calcium-induced differentiation and non-calcium treated control experiments, keratinocytes were grown in Keratinocyte Serum-Free Medium with supplements (KSFM; GIBCO). After reaching 70% confluence KSFM was exchanged for EMEM (Lonza) supplemented with 8% chelated FBS, EGF (10 ng/ml), 1% penicillin/streptomycin, and 0.05 mM $CaCl_2$. After 12 hr time point 0h was collected, and the residual keratinocytes were synchronized for 2 hr with EMEM containing 20% chelated FBS, EGF (10 ng/ml), 1% penicillin/streptomycin, and 0.05 mM $CaCl_2$. After synchronization cells were washed once with PBS and cultured in EMEM, supplemented with 8% chelated FBS, EGF (10 ng/ml), 1% penicillin/streptomycin, and either 0.05 mM or 1.2 mM $CaCl_2$, corresponding to non-calcium and calcium treatment, respectively. PHKs were treated with either 2 ng/ml TGFβ1 (PreproTech) or 1.2 mM $CaCl_2$ with EMEM/20% chelated FBS.

Immunofluorescence and Western Blot

Keratinocytes grown on glass coverslips were fixed in 4% paraformaldehyde, blocked with 0.25% gelatin/PBS, and stained with primary and secondary antibodies diluted in blocking buffer for 30 min at room temperature. Nuclei were counterstained with DAPI (Roche). Primary antibodies were anti-involucrin (1:1,000, ab68, Abcam) and anti-loricrin (1:1,000, RPB-145P, Covance); secondary antibodies were anti-rabbit Alexa Fluor 647 and anti-mouse Alexa Fluor 594 (1:500, Molecular Probes). Pictures were acquired using a Leica TCS SP5 confocal microscope. Protein extracts were analyzed by SDS-PAGE and western blotting for PER1 (1:500, Affinity Bioreagents), PER2 (1:500, Alpha Diagnostic), and Tubulin (1:5,000, clone SAP.4G5, Sigma-Aldrich).

Skin Reconstitution Assay

Infected human keratinocytes were mixed with mouse newborn fibroblasts and transplanted onto the back of Swiss Nude mice (Lichti et al., 2008). Skin grafts were collected 6 weeks posttransplantation and fixed for 2 hr in 4% paraformaldehyde, embedded in OCT, and sectioned at 8 μm thickness. Nuclei were stained with DAPI. Pictures were acquired using a Leica TCS SP5 confocal microscope. Mice were housed in an AAALAC-I approved animal unit under 12 hr light/12 hr dark cycles and SPF conditions, and all procedures were approved by the Ethical Committee for Animal Experimentation of the Government of Catalonia.

FACS

The back skin of adult C57BL6/J mice sacrificed at ZT10 or ZT22 was incubated for 2 hr at 37°C in 0.25% trypsin, and epidermal cells were isolated as described previously (Jensen et al., 2010). Cell suspensions were incubated for 30 min on ice with the following antibodies at the given dilutions: 1:100 for biotin-conjugated anti-CD34 (clone RAM34, BD PharMingen), 1:200 for PE-conjugated anti-α6-integrin (CD49f clone NKI-GoH3, Serotec), and 1:500 for APC-conjugated streptavidin (BD PharMingen). DAPI staining was used to rule out the presence of dead cells. FACS was performed using FACSAriaII and FACSDiva software (BD Biosciences). CD34-negative/α6-integrin^{bright} epidermal cells were collected in E-medium and subjected further to RNA isolation.

Microarrays, Data Acquisition, and Bioinformatics Methods

Total RNA from calcium and non-calcium treated primary human keratinocytes was hybridized to Human Gene Expression 4 × 44K v2 microarrays and SurePrintG3 Human Gene Expression 8 × 60K microarrays, respectively, both from Agilent. Probe signals were extracted using the Feature Extraction software provided by Agilent and normalized using the Bioconductor R-Project limma package. To compare the expression pattern of individual genes to those of known clock components, we devised the method explained in the Supplemental Information.

ACCESSION NUMBERS

Gene expression data can be accessed at GEO using the accession number GSE50631.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.09.004.

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