A Circadian Clock in HaCaT Keratinocytes

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To anticipate daily environmental changes, most organisms developed endogenous timing systems, the so-called circadian (~24 hours) clocks. Circadian clocks exist in most peripheral tissues and govern a huge variety of cellular, metabolic, and physiological processes. Recent studies have suggested daytime-dependent variations in epidermal functions such as barrier recovery and pH homeostasis. However, a local circadian clock in epidermal keratinocytes has not been reported yet, and as such the molecular link between the circadian system and epidermal physiology remains elusive. In this study we describe a functional cell autonomous circadian clock in human adult low calcium temperature (HaCaT) keratinocytes. Using live-cell bioluminescence imaging and mRNA expression time series, we show robust circadian transcription of canonical clock genes in synchronized HaCaT keratinocytes. Genetic and pharmacological perturbation experiments as well as the phase relations between clock gene rhythms confirm that the molecular makeup of the HaCaT keratinocyte clock is very similar to that of other peripheral clocks. Furthermore, temperature was identified to be a potent time cue (*Zeitgeber*) for the epidermal oscillator. Temperature cycles entrain HaCaT keratinocytes, leading to the identification of rhythmic expression of several genes involved in epidermal physiology such as cholesterol homeostasis and differentiation. Thus, we present HaCaT keratinocytes as an excellent model to study the regulation of keratinocyte physiology by the circadian clock in a simple yet robust *in vitro* system.

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INTRODUCTION

Most organisms are subjected to daily variations in environmental cues such as light–dark cycles, temperature, and food availability. This periodicity has led to the evolution of highly conserved molecular circadian (~24 hours) clocks governing many metabolic and physiological processes. In mammals, circadian rhythms are orchestrated by a master pacemaker, the suprachiasmatic nucleus (SCN), located in the hypothalamus (Reppert and Weaver, 2002). On the molecular level, circadian rhythms are cell autonomous and are generated by interlocked transcriptional/translational feedback loops. The circadian transcription factors CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein-1) activate as a heterodimer the transcription of *Period1–3 (Per1–3)* and *Cryptochromes 1 and 2* (*Cry1 and Cry2)* genes as well as that of the orphan nuclear receptor *Rev-Erbα* via binding to E-box elements in their promoters. In turn, PER and CRY proteins lead to repression of CLOCK/BMAL1, thereby inhibiting their own transcription (Schibler, 2006). In an additional feedback loop, REV-ERBα rhythmically represses *Bmal1* transcription, adding to the robustness of the circadian circuitry (Liu *et al.*, 2008). Post-translational modifications of clock proteins, such as PER protein phosphorylation by CKIε/δ and CK2, are crucial for generating the correct circadian period (Meng *et al.*, 2008; Maier *et al.*, 2009).

In mammals, the circadian system is hierarchically organized: the SCN as the master pacemaker in the brain receives environmental timing information by inputs coming from the eyes and thus is necessary for synchronization of the organism with the outside world. Most peripheral tissues (and even immortalized cell lines) also contain cell-autonomous circadian oscillators with a similar molecular makeup to the SCN (Balsalobre *et al.*, 2000). Peripheral oscillators are synchronized by the SCN via systemic time cues (*Zeitgebers*). Little is known about the mechanisms of this communication: hormonal signals, sympathetic enervation, and/or indirect cues such as body temperature, feeding time, and activity rhythms have been discussed (Dibner *et al.*, 2010). Peripheral clocks are crucial for circadian regulation of local physiology (Lamia *et al.*, 2008). They control daily rhythms in physiology and metabolism predominantly by circadian regulation of transcription factors (Fonjallaz *et al.*, 1996) or rate-limiting enzymes (Le Martelot *et al.*, 2009; Nakahata *et al.*, 2009). The transcriptional regulation of these clock output genes is

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Abbreviations: BMAL1, brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein-1; CLOCK, circadian locomotor output cycles kaput; Cry, Cryptochrome*; dex, dexamethasone; HaCaT, human adult low calcium temperature;* Per1–3, Period1–3*; SCN, suprachiasmatic nucleus*

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highly pervasive (i.e., ~5–20% of the transcriptome of a given tissue is rhythmic, e.g., see Keller *et al.*, 2009) and also tissue specific (i.e., the overlap of rhythmic transcripts between tissues is surprisingly small; Storch *et al.*, 2002).

As the skin forms the barrier between body and environment, it is not surprising that it is profoundly subjected to time-of-day-dependent changes in environmental conditions, such as exposure to UV irradiation and temperature. Despite this, very little is known about a potential circadian regulation of metabolic and physiological processes in the skin: time-of-day-dependent variations in human skin functions such as barrier recovery (Yosipovitch *et al.*, 2004), transepidermal water loss (Yosipovitch *et al.*, 1998), sebum secretion, skin temperature, and skin pH (Le Fur *et al.*, 2001) have been reported. Moreover, circadian expression of core clock genes has been shown in murine skin (Tanioka *et al.*, 2009) as well as in human skin fibroblasts (Brown *et al.*, 2005). However, the molecular mechanisms of how the circadian clock controls skin function remain elusive. It is even unclear to date whether, for example, epidermal keratinocytes possess a functional cell-autonomous circadian clock at all and, if yes, how this clock might regulate epidermal physiology.

The use of immortalized cell lines has greatly advanced our understanding of the molecular mechanisms in circadian biology. However, no model system has yet been described for the study of circadian rhythms in epidermal cells. In this study we provide evidence for a functional circadian clock in the epidermal keratinocyte cell line, human adult low calcium temperature (HaCaT). This spontaneously *in vitro* transformed cell line is derived from histological normal adult body skin and closely reflects keratinocyte cell behavior (Boukamp *et al.*, 1988; Altenburger and Kissel, 1999). We show that the HaCaT clock is cell autonomous and drives rhythmic expression of core clock genes with phase relations similar to known clocks. When this clock is perturbed by genetic or pharmacological means, the consequences for clock dynamics are as predicted from the canonical clock circuitry. Furthermore, we report temperature as a potent *Zeitgeber* for the circadian clock in HaCaT keratinocytes. Temperature cycles enhance circadian gene expression and allow identification of clock output genes relevant for skin physiology. Consequently, the model system we present here provides a simple yet robust tool for studies of epidermal physiology controlled by the circadian clock.

RESULTS

HaCaT keratinocytes possess a functional circadian clock

To test whether canonical clock genes such as *Per1* and *Clock* are expressed in HaCaT cells as well as primary keratinocytes, we performed immunocytochemistry experiments using antibodies against endogenous PER1 and CLOCK. In both cell types, PER1 and CLOCK are expressed as reported by others (Zanello *et al.*, 2000) with CLOCK being localized predominantly in the nucleus and PER1 in both cytoplasm and nucleus (Figure 1a and b). This subcellular localization is consistent with the known roles of these proteins within the circadian oscillator—CLOCK being a transcription factor and PER1

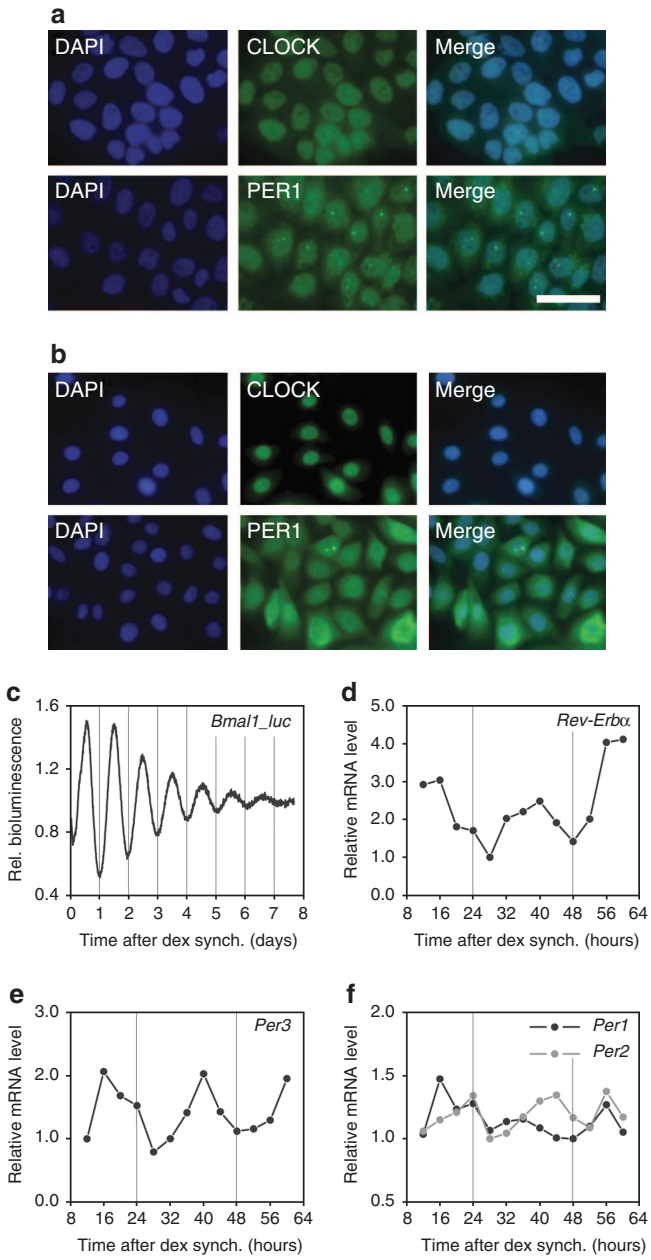

Figure 1. A functional circadian clockwork in HaCaT keratinocytes. (**a**, **b**) HaCaT keratinocytes (**a**) and primary neonatal keratinocytes (**b**) were fixed using paraformaldehyde and CLOCK, and PER proteins were labeled using mAbs. Co-staining of the nucleus was performed using DAPI (4′-6-diamidin-2′-phenylindol-dihydrochlorid). Bar = 50 μm. (**c**) HaCaT keratinocytes were lentivirally transduced with a reporter construct, where luciferase expression is driven by a 0.9-kb fragment of the circadian *Bmal1* promoter. Circadian rhythms of single reporter cell clones were synchronized using dexamethasone (dex), and bioluminescence was recorded for eight consecutive days. Shown is a de-trended time series of a representative single reporter cell clone. (**d–f**) Circadian rhythms of HaCaT keratinocytes were synchronized using dex. Cells were harvested in regular 4-hour intervals and mRNA levels of depicted genes were determined using quantitative reverse transcription (RT)-PCR. Expression levels were normalized to non-oscillating ribosomal 18S RNA levels, de-trended using a linear regression (see Materials and Methods), and given relative to minimum expression. Statistical analysis for circadian oscillation was performed using CircWave software: *Per3*: $P = 0.01$, *Rev-Erbα*: $P = 0.02$, *Per1*: $P = 0.83$, and *Per2*: $P = 0.33$.

being part of the negative feedback complex that shuttles between cytoplasm and nucleus.

If keratinocytes possess a functional circadian oscillator, it should drive rhythmic transcription of clock genes as well as clock output genes. To test this, we made use of a luciferase-based reporter system, where a 0.9 kb promoter fragment of the rhythmic clock gene *Bmal1* is driving the expression of luciferase. HaCaT keratinocytes were lentivirally transduced with this reporter construct (resulting in stable integration), and single reporter cell clones were derived. Circadian rhythms were synchronized with dexamethasone (dex) and rhythmic luciferase activity of live cells was monitored for several days. Persistent circadian oscillation of *Bmal1* promoter activity was observed for 17 individual keratinocyte reporter cell clones with periods of 23.3 ± 0.1 hours (SEM, $n = 17$; Figure 1c)—a period typical for mammalian cells. The damping of the oscillation is very likely because of a gradual desynchronization of the individual keratinocytes in the population known for non-SCN mammalian cells and tissues in culture (Nagoshi *et al.*, 2005). Together, the circadian rhythms in HaCaT keratinocytes are very similar to those detected in primary fibroblasts as well as in many cells known to have a functional circadian clock.

To test whether other canonical clock genes also show rhythmic transcriptional profiles, we harvested total RNA from dex-synchronized HaCaT keratinocytes in regular 4-hour intervals and performed quantitative reverse transcription-PCR experiments. We detected robust circadian rhythms of *Rev-Erbα* as well as of *Per3* mRNA levels (Figure 1d and e). These transcripts have been reported to exhibit high-amplitude circadian rhythms in the human cell line U-2 OS, whereas others, such as *Per1,2* or *Cry1,2*, usually display lower amplitude oscillations (Hughes *et al.*, 2009)—a finding that we also confirmed for HaCaT keratinocytes (Figure 1f and data not shown).

If the circadian clock in HaCaT keratinocytes is similar to those in other peripheral tissues and cells, clock dynamics should be altered in a comparable manner when the clock is perturbed. To test this, we used pharmacologic as well as genetic means to target different crucial parts of the molecular clock. Upon interference with PER protein phosphorylation—a critical step for a normal circadian period (Vanselow *et al.*, 2006)—we observed alterations of circadian dynamics that have been described for other model systems (Maier *et al.*, 2009): when we pharmacologically inhibited CKIε/δ or CK2 activity, the circadian period significantly and dose-dependently lengthened (Figure 2a, b, and d). Also, when we downregulated *Fbxl3*, which is required for CRY protein degradation, with RNA interference, we observed a significant and substantial lengthening of the circadian period (Figure 2c and d), as it has been reported for hypomorphic mutations of *Fbxl3* in mice (Busino *et al.*, 2007; Godinho *et al.*, 2007; Siepka *et al.*, 2007). Furthermore, short hairpin RNA (shRNA)-mediated silencing of *Bmal1* resulted in a complete abrogation of rhythmicity in HaCaT cells (Figure 2e), which has also been reported in other mammalian systems (Bunger *et al.*, 2000).

Together, our data—rhythmic clock gene transcription and abundance, correct clock protein localization, as well as predicted alterations in circadian dynamics upon genetic and pharmacological perturbations—clearly demonstrate that HaCaT keratinocytes possess a robust circadian clockwork with properties indistinguishable from other known peripheral clocks.

Temperature is a potent *Zeitgeber* for HaCaT keratinocytes

Circadian regulation of body temperature has been proposed to synchronize peripheral oscillators *in vivo*, and temperature cycles have been shown to drive circadian gene expression in human fibroblasts (Brown *et al.*, 2002). Daytime-dependent changes in human skin temperature have been previously reported with temperature variations of up to 1–2 °K (Yosipovitch *et al.*, 1998). Therefore, we hypothesized that temperature cycles might contribute to the synchronization (entrainment) and/or enhancement of epidermal circadian oscillators including keratinocyte clocks.

To test whether the circadian clock in HaCaT keratinocytes can be entrained to 24-hour temperature cycles, we subjected our reporter HaCaT cells to a 12-hour 37 °C/12-hour 33 °C temperature cycle (with 2-hour transitions) and continuously monitored *Bmal1*-driven bioluminescence. As predicted for true entrainment, *Bmal1-luc* reporter activity adopted a stable phase relation to the temperature cycle and an increased amplitude during entrainment compared with HaCaT cells treated with dex alone (Figure 3a). When temperature-entrained cells were released into constant 37 °C conditions, oscillations persisted with a phase predicted from the previous cycles, indicating true entrainment rather than mere temperature-driven effects (so-called ''masking''). The delay in phase compared with the oscillations of dex-only-treated cells (see day 5 in Figure 3a) further showed that HaCaT cells subjected to the temperature cycle adopted a 24-hour rhythm during entrainment, whereas cells in constant conditions oscillated with their intrinsic non-24-hour period, which eventually led to the observed phase angle difference upon release in constant temperature.

In order to unambiguously exclude that temperature cycles merely drive bioluminescence rhythms rather than entrain them, we again subjected HaCaT keratinocytes to 24-hour temperature cycles. This time, HaCaT reporter cells were synchronized 8.5-hour apart by dex treatment, resulting in a stable phase difference of 8.5 hours in constant temperature (Figure 3b). However, when parallel cultures with the same phase difference were entrained to 24-hour temperature cycles, typical so-called ''transient cycles'' can be observed for one of the cultures, whereas the other culture almost immediately adopted a stable phase relation to the temperature cycle. During ''transients'' (i.e., the gradual synchronization to the *Zeitgeber* cycle) two daily peaks of *Bmal1* promoter activity can be seen: one decreasing peak during the warm phase corresponding to the phase before entrainment, and a second increasing peak during the cold phase representing the new, entrained phase. Thus, depending on the phase before the temperature cycle, HaCaT keratinocytes entrained to 24-hour temperature cycles within

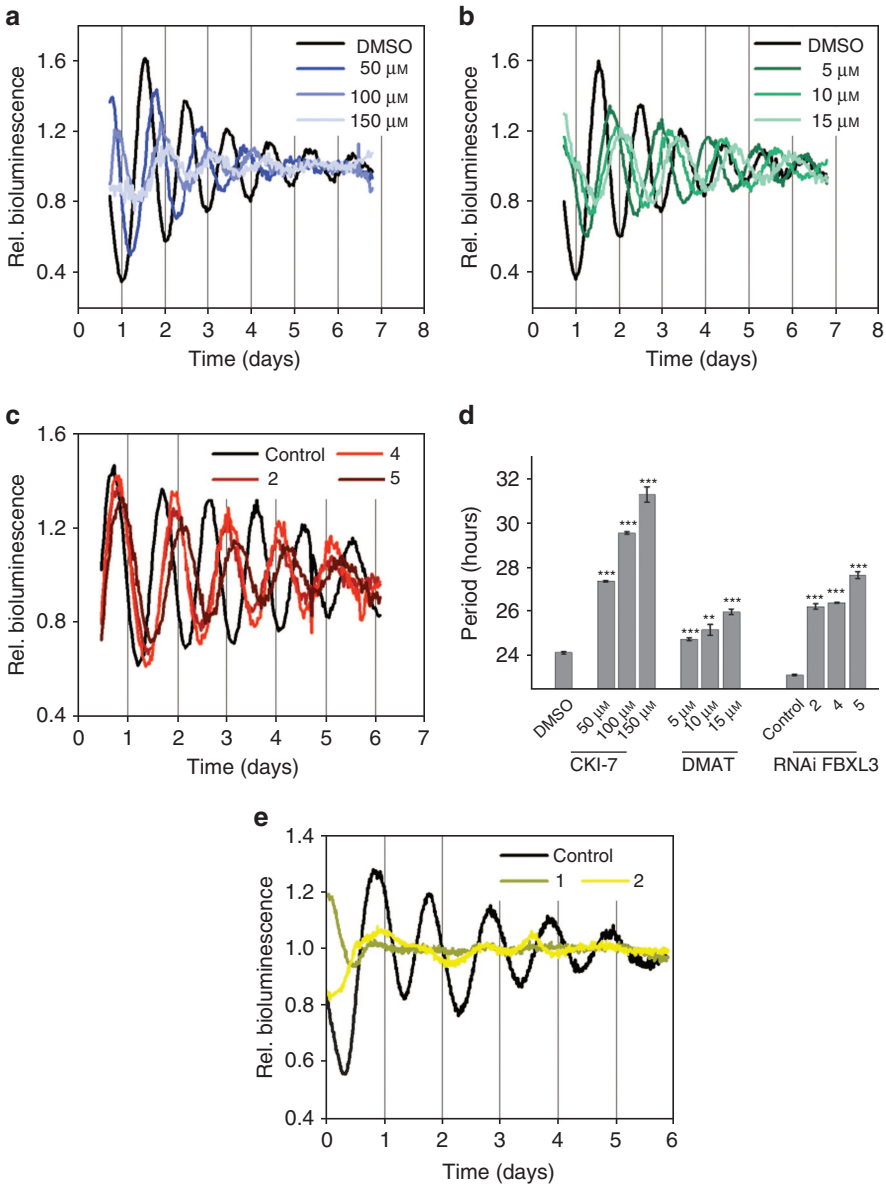

Figure 2. The circadian dynamics in HaCaT keratinocytes can be modulated by genetic and pharmacological means. (**a**, **b**) HaCaT reporter cells were cultured in the presence of the specific (**a**) CKI or (**b**) CK2 inhibitors CKI-7 or DMAT (2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole). Circadian rhythms were synchronized with dexamethasone and bioluminescence was recorded for eight consecutive days. Shown are average de-trended time series ($n=6$). (**c**, **e**) HaCaT reporter cells were lentivirally transduced with short hairpin RNA (shRNA) constructs targeting (**c**) *Fbxl3,* (**e**) *Bmal1*, or a non-silencing control. RNA interference (RNAi)-mediated knockdown—measured using quantitative real-time PCR—of *Fbxl3* resulted in residual *Fbxl3* mRNA levels of 32, 34, and 16% for the anti-*Fbxl3* constructs 2, 4, and 5 (compared with control), respectively, and <10% for *Bmal1* constructs 1 and 2. Circadian rhythms were synchronized with dexamethasone and bioluminescence was recorded for seven consecutive days. Shown are average de-trended time series ($n=3$–4). (**d**) Alterations of circadian periods upon pharmacological or genetic perturbation are given in comparison to the period of HaCaT reporter cells treated with solvent (DMSO) or a non-silencing shRNA control. Given is the mean ± SEM (**$P<0.01$, ***$P<0.001$; paired *t*-test).

a few days (Figure 3c). Note that entrainment of one of the cultures is not yet complete after 5 days, because upon release into constant conditions a small phase difference between the two cultures remained (Figure 3c).

In summary, temperature cycles can entrain circadian clocks in keratinocytes to a particular specific phase, irrespective of the previous phase. These results strongly suggest that temperature is a *Zeitgeber* for the circadian clock in HaCaT keratinocytes.

Temperature entrainment enhances circadian expression of clock genes as well as clock output genes

Temperature cycles can entrain keratinocytes clocks, which are predicted to have effects on the amplitude of circadian rhythms. Amplitude expansion of synchronized oscillators is a well-known resonance phenomenon described in many theoretical and experimental studies (Aschoff and Pohl, 1978; Roenneberg, 1995; Kurosawa and Goldbeter, 2006). In addition, temperature cycles might also activate cold- and/or

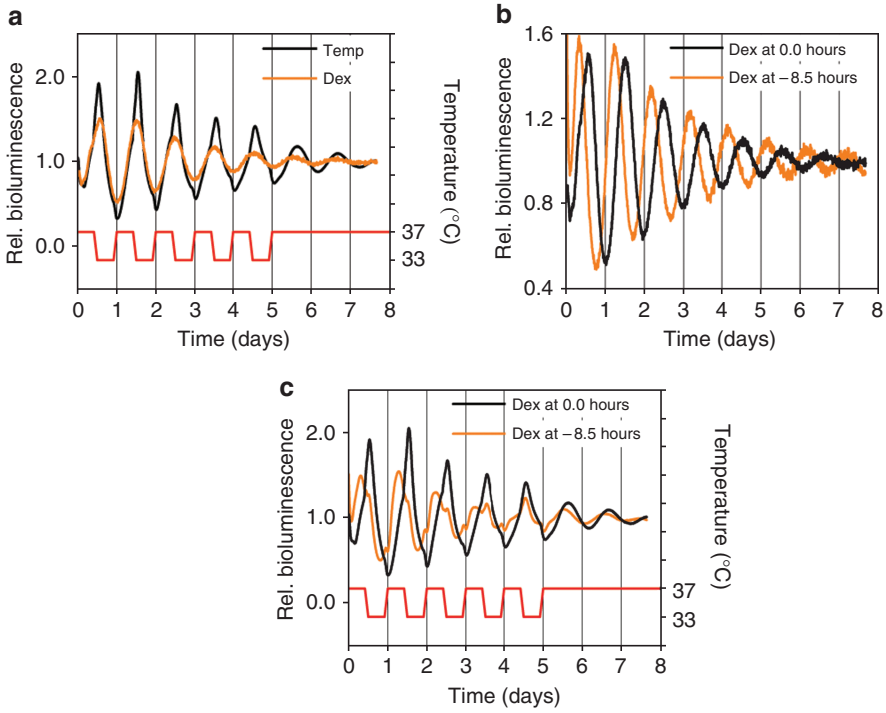

Figure 3. Temperature cycles entrain the HaCaT keratinocyte clock. Circadian rhythms of HaCaT reporter cells were synchronized using dexamethasone (Dex). (**a**, **c**) Subsequently, cells were either subjected to 24-hour temperature cycles with essentially a 12-hour warm phase (37 °C) and a 12-hour cold phase (33 °C) or (**b**) cultured under constant temperature (37 °C), while bioluminescence was recorded for eight consecutive days. In **b**, cells were synchronized 8.5 hours apart. Trend-eliminated bioluminescence time series are shown in black and orange, and the temperature regime is indicated in red.

heat-induced proteins that could act on clock gene expression, leading to increased circadian amplitudes at the single-cell level (Kornmann *et al.*, 2007). To test whether the amplitude of clock-controlled gene expression is increased upon temperature entrainment, we subjected parallel cultures of dex-synchronized HaCaT keratinocytes to 24-hour temperature cycles for 2 days before releasing them into constant conditions. Note that the phase of the temperature cycle was selected to ensure essentially immediate entrainment (see Figure 3c). During the second day of temperature entrainment and the first day in constant conditions, total RNA was prepared from cells harvested in regular 4-hour intervals. Subsequently, we performed quantitative reverse transcription-PCR experiments and detected robust significant and high-amplitude circadian rhythms in mRNA abundance of *Per1, Per2, Per3, Cry1* and *2*, *Rev-Erbα*, and *Bmal1* (Figure 4a). As predicted for entrained oscillators, the amplitudes of these rhythms were substantially larger compared with those in non-entrained keratinocytes (see Figure 1). In addition, these high-amplitude rhythms even persisted upon release in constant temperature, again showing that they were not merely temperature driven. Importantly, the phase relations of these clock gene mRNA oscillations are as expected from the molecular makeup of the circadian circuitry: CLOCK/BMAL1-driven genes, such as *Per* and *Cry* genes, have their peak expression much earlier than *Bmal1*, which is negatively controlled by REV-ERBα.

In every peripheral tissue investigated so far, a huge number (up to 10%) of all transcripts show circadian abundance rhythms. In immortalized cell lines (e.g., NIH3T3 fibroblasts) synchronized with dex, however, only a small subset of known circadian transcripts could be detected to be rhythmically expressed (Hughes *et al.*, 2009). This is probably at least in part due to a less efficient synchronization of individual cellular clocks, and have so far limited the value of cell lines as models for circadian output analysis. To test whether our temperature entrainment paradigm can overcome these limitations—as it seems likely given the amplitude increase of clock gene oscillations (see Figure 3a)—we analyzed the potential rhythmicity of selected putative clock output genes. The transcription factor *Dbp* has been reported to be a direct target of the circadian clock (Wuarin and Schibler, 1990) and, indeed, we found significant circadian expression of *Dbp* in HaCaT keratinocytes (Figure 4b). Moreover, circadian transcription of *Insig2a*, a gene involved in cholesterol homeostasis (Yabe *et al.*, 2002), and *Actin* were observed. These genes have been described to be regulated in a circadian fashion in mouse tissue *in vivo* (Hughes *et al.*, 2009; Le Martelot *et al.*, 2009), but, to our knowledge, not in an *in vitro* cell culture model. Although *Dbp* and *Insig2a* rhythms have a similar phase compared with other CLOCK/BMAL1-driven genes (see Figure 2), the phase of *Actin* mRNA rhythms is more similar to that of *Bmal1*, implying an alternate transcriptional control perhaps by REV-ERBα or other mechanisms (e.g., pre-mRNA/mRNA stability).

In liver, elegant genetic experiments by the Schibler laboratory have demonstrated that the rhythmicity of certain

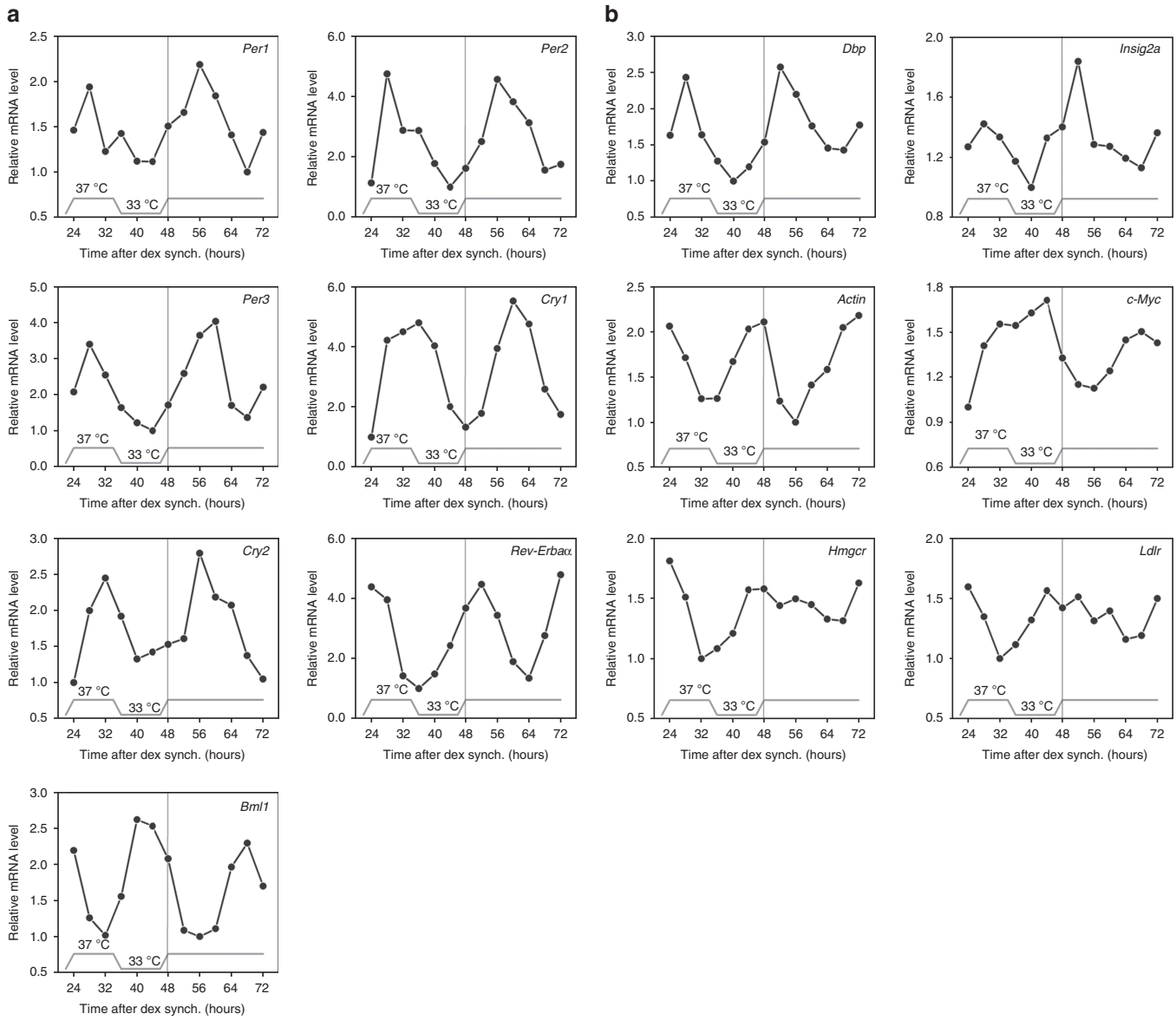

Figure 4. Temperature cycles enhance the amplitude of circadian gene expression in HaCaT keratinocytes. Circadian rhythms of HaCaT keratinocytes were synchronized using dexamethasone (dex) and subjected to temperature cycles (as indicated) for 2 days. Subsequently, cells were released into constant temperature conditions (37 °C). During the second day of temperature entrainment and the first day in constant conditions, cells were harvested at regular 4-hour intervals and mRNA levels of depicted genes were determined using quantitative PCR. Expression levels of clock-genes (**a**) and output-genes (**b**) were normalized to non-oscillating ribosomal 18S RNA, de-trended using a linear fit (see Materials and Methods), and given relative to minimum expression. Statistical analysis for circadian gene expression was performed using CircWave software. Because of the differential effects of temperature on circadian gene expression, the statistics for *c-Myc*, *Aqp3*, *Hmgcr*, and *Ldlr* was performed separately for each day. *Per2*, *Per3*, *Cry1*, *Cry2*, *Bmal1*, *Rev-Erbα*, *Dbp*, *Actin*: $P<0.01$; *Per1*, *Insig2a*: $P<0.05$; *c-Myc*: $P=0.16$ (day 1) and $P<0.01$ (day 2); *Hmgcr*: $P<0.01$ (day 1) and $P=0.27$ (day 2); *Ldlr*: $P<0.01$ (day 1) and $P=0.12$ (day 2).

transcript levels is not (only) controlled by the local liver clock but is driven by systemic factors—such as rhythmic core body temperature (Kornmann *et al.*, 2007). Therefore, it was also conceivable in our keratinocyte model that some transcriptional rhythms are only detected during the temperature cycle but not in constant temperature. Indeed, we found temperature-driven circadian gene expression of the *Ldl receptor* and *Hmgcr*—both key players in cholesterol homeostasis. Interestingly, the oncogene *c-Myc* only showed significant circadian transcription after release into constant conditions (Figure 4c). These observations suggest additional temperature-dependent effects on gene expression of *c-Myc* that might mask circadian transcription.

HaCaT cells have long been used as a model system to study keratinocyte cell behavior (Boukamp *et al.*, 1988; Altenburger and Kissel, 1999). However, immortalized cell lines have obvious limitations (e.g., disrupted cell cycle control and limited differentiation capacities) compared with primary cells. Therefore, the feasibility of the HaCaT model to study circadian rhythms in epidermal physiology largely

depends on how closely it reflects the circadian oscillator in primary keratinocytes. To elucidate whether our temperature paradigm also allows entrainment of the circadian clock in primary epidermal cells, we subjected neonatal foreskin keratinocytes to temperature cycles (similar to HaCaT keratinocytes) and analyzed expression levels of canonical clock genes that showed high-amplitude oscillations in HaCaT keratinocytes (i.e., *Per2, Per3,* and *Rev-Erbα*). All three clock genes also showed robust high-amplitude oscillations in primary keratinocytes with similar phase relations to HaCaT cells (i.e., *Per* genes peaking later than *Rev-Erbα*, Figure 5a–c, compare Figure 4a). Furthermore (in a proof of concept experiment), circadian control of Insig2a gene expression could be validated in primary keratinocytes (Figure 5d). These observations show clearly that (1) primary epidermal keratinocytes also possess a cell-autonomous circadian clockwork, (2) this clock behaves quite similar to the HaCaT clock, and (3) clock output genes identified in the HaCaT model can be (at least in some cases) validated in primary keratinocytes.

Together, we show that temperature entrainment expands the amplitudes of not only circadian clock genes, but also clock output genes in HaCaT keratinocytes. This new paradigm allows for genome-wide expression analysis of clock outputs using cell culture models, facilitating the identification of physiological processes that are controlled by the circadian system.

DISCUSSION

HaCaT keratinocytes as a new model system to study circadian control of epidermal physiology

This study sets out to establish a viable model system for studying circadian rhythms in epidermal keratinocytes. Our data clearly show robust circadian rhythms in HaCaT keratinocytes that are comparable to circadian rhythms found in other peripheral tissues *in vivo* and *in vitro*. In addition, genetic and pharmacologic perturbation experiments demonstrate that these rhythms are generated by an endogenous clock that is indistinguishable from those described in other human and murine model systems. The use of immortalized cell lines (such as murine NIH3T3 fibroblasts or human U-2 OS cells) has greatly advanced our understanding of molecular mechanisms governing circadian rhythmicity. The HaCaT model reported here complements the set of existing systems and represents a new cell line to study circadian rhythms on a cellular level. Importantly, HaCaT cells are widely used to study various aspects of keratinocyte/epidermal physiology ranging from apoptosis to epidermal differentiation (Micallef *et al.*, 2009; Wells *et al.*, 2009; Calay *et al.*, 2010). Therefore, the findings of this study open the door to study the molecular link between the circadian clock and epidermal physiology.

We used our HaCaT keratinocyte model to investigate the effects of temperature cycles on the epidermal oscillator, as physiological temperature rhythms in the skin have long been known (Yosipovitch *et al.*, 1998). Interestingly, temperature acts as potent *Zeitgeber* of the HaCaT clock, synchronizing clock gene expression as well as expanding the amplitudes of clock-controlled genes. Our results are in accordance with studies in rat-1 fibroblasts showing circadian gene expression of the clock genes *Dbp*, *Bmal1*, *Per2*, and *Cry1* after temperature entrainment (Brown *et al.*, 2002). However, an increase in amplitudes of circadian gene expression by temperature entrainment compared with dex synchronization has not been reported before. This amplitude expansion may be because of a more efficient synchronization of individual cellular oscillators. In addition, it is conceivable that the temperature cycle-induced increase in rhythm amplitudes is mediated by the diurnal expression of heat- and cold-induced proteins (see Kornmann *et al.*, 2007). Although (at least in liver) heat-shock proteins are reported to be expressed at times when body temperature is high, CIRP, a cold-induced RNA-binding protein, is maximal at times when body temperature is low. Thus, it appears that body temperature cycles can be translated into antiphasic expression of heat- and cold-induced proteins. Therefore, it is also possible that in HaCaT keratinocytes, a rhythmic expression of temperature-sensitive clock components such as *Per2* (Kornmann *et al.*, 2007) is enhanced in amplitude by such a mechanism. Indeed, we found significant *Per2* oscillation only after temperature entrainment, suggesting that *Per2* may convey temperature information to the clock also in keratinocytes.

Importantly, our temperature entrainment paradigm greatly facilitates the identification of clock output genes in cultured cell lines. Using temperature as additional *Zeitgeber* to dex, we found more transcripts cycling in HaCaT keratinocytes in the small subset of genes tested than Hughes *et al.* (2009) reported in a genome-wide expression profiling in NIH3T3 fibroblasts after dex treatment. Therefore, we propose that the limitations of *in vitro* models for studying circadian rhythms can at least be partly overcome by applying temperature cycles. This experimental setup should allow genome-wide expression profiling in HaCaT cells, which likely reveals interesting aspects of circadian physiology in the epidermis.

To qualify the HaCaT model as a viable tool to study circadian modulation of keratinocyte physiology, it was vital to compare the circadian clockwork in HaCaT and primary keratinocytes. To this end we applied our temperature entrainment paradigm to primary neonatal keratinocytes. Interestingly, we found a similar molecular clock in primary keratinocytes compared with HaCaT cells underlining the validity of the HaCaT model system.

Putative role(s) of clock-controlled genes in epidermal physiology

The HaCaT model system presented here allows for identification of clock output genes relevant for epidermal physiology. Although only a small (proof of principle) subset of putative clock-controlled genes were tested in this study, the obtained data offer first insights in the circadian regulation of physiological processes crucial for epidermal function. We found robust circadian transcription of *Insig2a*, a key regulator of cholesterol homeostasis. *Insig2a* is part of a ternary protein complex, which senses cholesterol availability in the endoplasmic reticulum membrane. Upon cholesterol

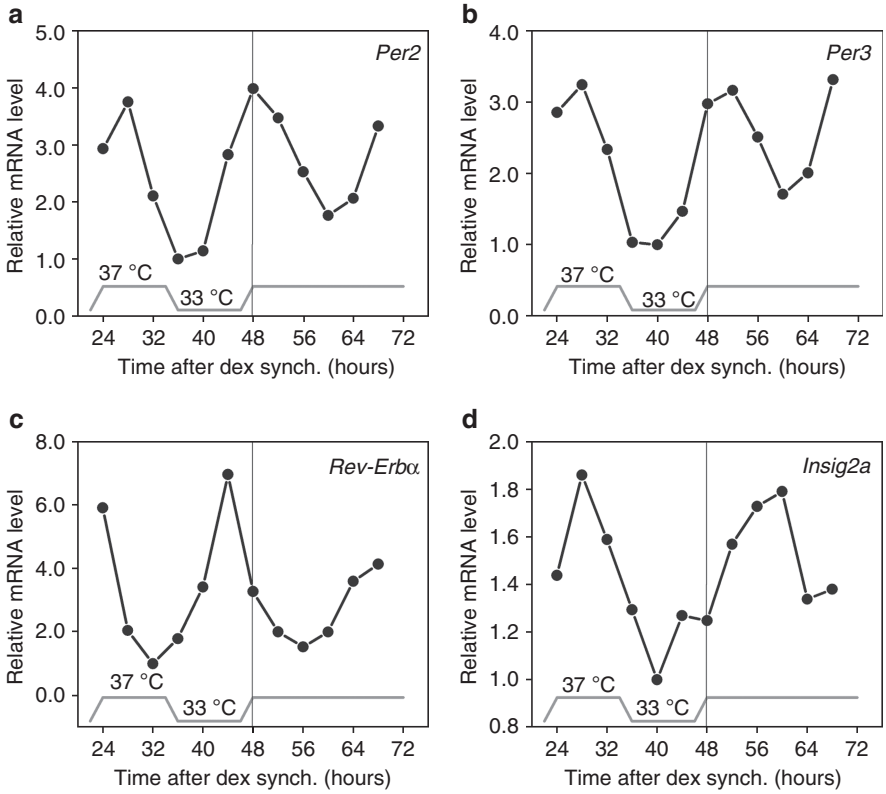

Figure 5. A cell-autonomous clock in primary keratinocytes. Circadian rhythms of confluent primary keratinocytes were synchronized using dexamethasone (dex) and subjected to temperature cycles (as indicated) for 2 days. Subsequently, cells were released into constant temperature conditions (37 °C). During the second day of temperature entrainment and the first day in constant conditions, cells were harvested at regular 4-hour intervals and mRNA levels of depicted genes (**a**–**d**) were determined using quantitative PCR. Expression levels were normalized to non-oscillating ribosomal 18S RNA, de-trended using a linear fit (see Materials and Methods), and given relative to minimum expression. Statistical analysis for circadian gene expression was performed using CircWave software. *Per2, Per3, Rev-Erbα:* $P<0.01$*; Insig2a:* $P<0.05$.

starvation, SREBPs (sterol regulatory element-binding proteins) are released from the complex and subsequently activate genes involved in cholesterol *de novo* synthesis such as *Hmgcr* or cholesterol uptake such as the *Ldl receptor* (Vallett *et al.*, 1996; Radhakrishnan *et al.*, 2007). Indeed, we also observed circadian regulation of *Hmgcr* as well as the *Ldl receptor* gene expression at least during temperature entrainment. We and others reported previously that cholesterol is essential for keratinocyte differentiation/proliferation as well as for cornified envelope formation (Schmidt *et al.*, 1991; Sporl *et al.*, 2009). Moreover, cholesterol (among other lipids) is released from cornifying keratinocytes forming the epidermal lipid barrier. Thus, a circadian regulation of cholesterol metabolism in keratinocytes would imply a strict timing for keratinocyte differentiation and possibly also for skin barrier function. Interestingly, daytime-dependent variations in skin barrier recovery after tape stripping have been reported (Yosipovitch *et al.*, 1998, 2004). In accordance with a putative role of the circadian clock in keratinocyte differentiation/proliferation, circadian regulation of *c-Myc* transcription was observed. The oncogene *c-Myc* has a dual role in epidermal homeostasis by (1) inducing epidermal stem cell proliferation and (2) stimulating terminal differentiation of transit amplifying keratinocytes (Watt *et al.*, 2008).

As we also found robust oscillation in the expression level of the circadian transcription factor *Dbp*, it would be interesting to address the role of *Dbp* in keratinocyte physiology in future studies. Target genes of *Dbp* in other tissues involve the cytochrome *P*450 family (CYPs) (Lavery *et al.*, 1999). A large number of metabolic processes (e.g., steroid/VitD_3 metabolism) in keratinocytes also involve CYPs. Particularly, local VitD_3 metabolism in keratinocytes offers interesting aspects as VitD_3 is a major regulator of keratinocyte differentiation and diurnal variations in serum VitD_3 have already been reported (Rejnmark *et al.*, 2002).

Taken together, we present a robust model system to study circadian rhythms in epidermal keratinocytes, which should facilitate research in the circadian regulation of epidermal physiology. Understanding the circadian clock of the epidermis might ultimately lead to optimized drug delivery and sophisticated treatment of skin disease.

MATERIALS AND METHODS

Cell culture, bioluminescence recording, and data evaluation

Normal human epidermal neonatal foreskin keratinocytes were purchased from Lonza (Rockland, ME), seeded in 75 cm^2 plastic flasks at 1.3×10^4 cells per ml, and cultured in complete keratinocyte growth medium 2 lacking hydrocortisone and

supplemented with 1 mM $CaCl_2$. Cells were cultivated at 37 °C, 7% CO_2, and 95% relative humidity. Cells were trypsinized and subcultured by standard methods when approaching approximately 80% confluence.

HaCaT keratinocytes (Boukamp *et al.*, 1988) (kind gift from Dr Fusenig, Deutsches Krebsforschungsinstitut, Heidelberg, Germany) were cultured in DMEM (PAA, Pasching, Austria) supplemented with 10% fetal calf serum (PAA), 1% penicillin/streptomycin (PAA), and 25 mM HEPES (Roth, Karlsruhe, Germany) at 37 °C and 5% CO_2. For live-cell bioluminescence monitoring, cells were either seeded to 35-mm dishes (2×10^5 cells) or to white 96-well plates (2×10^4 cells). Following synchronization with 1 μM dex (Sigma-Aldrich, Hamburg, Germany) for 1 hour, medium was changed to phenol-red-free DMEM (PAA) containing 10% fetal calf serum, 1% penicillin/streptomycin, 25 mM HEPES, and 250 μM D-luciferin (Biotherma, Handen, Sweden). Bioluminescence recordings were performed using a LumiCycle (Actimetrics, Wilmette, IL) or light-tight boxes with single photonmultiplier tubes (Hamamatsu Photonics, Herrsching am Ammersee, Germany) for the 35-mm dish format. The 96-well plates were monitored in a plate luminometer (TopCount, PerkinElmer, Rodgau, Germany). After bioluminescence recording for 5–8 days, data were analyzed using ChronoStar software (Stephan Lovenzen, Institute of Theoretical Biology, Humbold-University, Berlin, Germany). Raw data were de-trended by dividing data points by the 24 hours running average. Maxima within 24 hours were selected for curve fitting using the non-linear least square method. Period, phase, amplitude, damping (i.e., amplitude half-life), and correlation coefficient to the fitted cosines curve were calculated as output (for details, see Maier *et al.*, 2009).

Lentivirus production, precipitation, and transduction

Lentiviruses containing the 0.9 kb *Bmal1* promoter-driven luciferase, shRNAmir knockdown constructs targeting *Fbxl3*, and a non-silencing control (Thermo Scientific, Huntsville, AL) were generated as described (Brown *et al.*, 2005). Lentiviral supernatant was concentrated by precipitation for 3 days using 8% polyethylene glycol 6000 (PEG600; Sigma-Aldrich) in 1.6 M NaCl at 4 °C. Lentiviral particles were harvested at 4,000 × *g*, 4 °C for 30 min and resuspended in 1/100 cold 1 × phosphate-buffered saline. HaCaT keratinocytes (80–90% confluent) were transduced with $\frac{1}{2}$ to $\frac{3}{4}$ final volume of lentiviral supernatant or 1/200 to 1/20 final volume of 100 × concentrated lentivirus and 8 μg ml^{-1} protamine sulfate (Sigma-Aldrich). Cells were selected for 24–72 hours after transduction. HaCaT cell lines with stably integrated *Bmal1* shRNA constructs were generated by Sirion GmbH (Martinsried, Germany).

Generation of HaCaT reporter cell lines

The lentiviral plasmid pABhygro expressing a firefly luciferase under the control of a 0.9-kb *Bmal1* promoter fragment (kind gift from Steven Brown, University of Zurich, Switzerland) was stably integrated into HaCaT keratinocytes. After hygromycin selection (0.1 μg ml^{-1}; Invitrogen, Karlsruhe, Germany) cells were diluted to 0.1, 1, and 10 cells and seeded into 96-well plates, supplemented with wild-type HaCaT serving as feeder cells. After 4 to 5 weeks of hygromycin selection, 17 single-cell-derived subclones were analyzed in online bioluminescence monitoring over 7 days following dex synchronization. HaCaT reporter clone 20 obtained best circadian characteristics with a period of 23.3 ± 0.07 hours, an amplitude of 1.0 ± 0.02, and a damping (i.e., amplitude half-life) of 40 ± 0.7 hours (SEM, $n = 4$), and was used for subsequent experiments.

Pharmacological perturbation

For pharmacological inhibition studies, cells were cultured and bioluminescence was monitored in phenol red-free medium (as described above) containing 5, 10, or 15 μM DMAT (2-dimethyl-amino-4,5,6,7-tetrabromo-1H-benzimidazole; Sigma-Aldrich) or 50, 100, or 150 μM CKI-7 (N-(2-aminoethyl)-5-chloro-isoquinoline-8-sulfonamide; Biomol, Hamburg, Germany) or 0.2% DMSO (solvent; Merck, Darmstadt, Germany).

Temperature entrainment

For temperature entrainment with continuous bioluminescence recordings, cells were grown in 35-mm dishes, synchronized with dex, and prepared for online bioluminescent measurements as described above. Continuous bioluminescence recordings during temperature entrainment were obtained using photomultiplier tubes on top of light-tight boxes (Hamamatsu Photonics). Temperature cycles were applied using a heating plate at the bottom of individual boxes controlled by a Jumo Imago 500 control unit (Jumo, Fulda, Germany). This setup was built by the Technische Werkstätten Charité (Berlin, Germany).

For RNA isolation during temperature entrainment, cells were grown in 35-mm dishes, and temperature profiles (12-hour 37 °C/12-hour 33 °C temperature cycle with 2-hour transitions) were applied using a KBF115 incubator (Binder, Tuttlingen, Germany).

Real-time PCR

Cells were harvested and total RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Following reverse transcription-PCR (high-capacity complementary DNA reverse transcription kit; Applied Biosystems, Branchburg, NJ) complementary DNA was loaded on a custom-made TaqMan Custom Array containing primer pairs for 48 genes, which was obtained from Applied Biosystems. Quantitative real-time PCR was performed using the 7900HT Fast Real-Time-PCR System (Applied Biosystems). Expression data were normalized to ribosomal 18S RNA and relative quantification (RQ) of gene expression was assessed by comparative δ-CT analysis. Data sets from each individual gene were de-trended applying a linear fit (Excel, Microsoft) as follows:

$$y(t) = \frac{\mathrm{RQ}(t)}{f(t)}$$

where y is the de-trended value at a given time (t), RQ is the gene expression value (see above), and f the corresponding fit value at a given time (t). Statistical analysis for circadian gene expression was performed using CircWave software (Oster *et al.*, 2006).

Cell monolayer immunofluorescence

For immunostaining, cells were fixed on 35-mm dishes in 3% paraformaldehyde for 30 minutes at room temperature, washed with phosphate-buffered saline, and permeabilized with 0.5% Triton X-100 for 5 minutes, after which the cells were blocked with 3% BSA for 30 minutes. Cells were then incubated with primary antibodies (10 μg ml^{-1}) in 1% BSA for 1 hour. Antibody detecting PER1 and

CLOCK were purchased from Alpha Diagnostics (San Antonio, TX). After incubation, cells were washed with phosphate-buffered saline. Cells were then incubated for 1 hour with 1% BSA solution containing fluorescent-labeled secondary antibody (AlexaFluor 488 goat anti-mouse IgG, AlexaFluor 546 goat anti-rabbit IgG; Molecular Probes, Eugene, CA). After extensive washing with phosphate-buffered saline, cells were DAPI (4′-6-diamidin-2′-phenylindol-dihydrochlorid) stained and fluorescent images were obtained using a digital fluorescence microscope (BZ-8000; Keyence, Osaka, Japan).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Altenburger R, Kissel T (1999) The human keratinocyte cell line HaCaT: an in vitro cell culture model for keratinocyte testosterone metabolism. *Pharm Res* 16:766–71

Aschoff J, Pohl H (1978) Phase relations between a circadian rhythm and its zeitgeber within the range of entrainment. *Naturwissenschaften* 65:80–4

Balsalobre A, Marcacci L, Schibler U (2000) Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Curr Biol* 10:1291–4

Boukamp P, Petrussevska RT, Breitkreutz D *et al.* (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 106:761–71

Brown SA, Fleury-Olela F, Nagoshi E *et al.* (2005) The period length of fibroblast circadian gene expression varies widely among human individuals. *PLoS Biol* 3:e338

Brown SA, Zumbrunn G, Fleury-Olela F *et al.* (2002) Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Curr Biol* 12:1574–83

Bunger MK, Wilsbacher LD, Moran SM *et al.* (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* 103:1009–17

Busino L, Bassermann F, Maiolica A *et al.* (2007) SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. *Science* 316:900–4

Calay D, Vind-Kezunovic D, Frankart A *et al.* (2010) Inhibition of Akt signaling by exclusion from lipid rafts in normal and transformed epidermal keratinocytes. *J Invest Dermatol* 130:1136–45

Dibner C, Schibler U, Albrecht U (2010) The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol* 72:517–49

Fonjallaz P, Ossipow V, Wanner G *et al.* (1996) The two PAR leucine zipper proteins, TEF and DBP, display similar circadian and tissue-specific expression, but have different target promoter preferences. *EMBO J* 15:351–62

Godinho SI, Maywood ES, Shaw L *et al.* (2007) The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. *Science* 316:897–900

Hughes ME, DiTacchio L, Hayes KR *et al.* (2009) Harmonics of circadian gene transcription in mammals. *PLoS Genet* 5:e1000442

Keller M, Mazuch J, Abraham U *et al.* (2009) A circadian clock in macrophages controls inflammatory immune responses. *Proc Natl Acad Sci USA* 106:21407–12

Kornmann B, Schaad O, Bujard H *et al.* (2007) System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. *PLoS Biol* 5:e34

Kurosawa G, Goldbeter A (2006) Amplitude of circadian oscillations entrained by 24-h light-dark cycles. *J Theor Biol* 242:478–88

Lamia KA, Storch KF, Weitz CJ (2008) Physiological significance of a peripheral tissue circadian clock. *Proc Natl Acad Sci USA* 105:15172–7

Lavery DJ, Lopez-Molina L, Margueron R *et al.* (1999) Circadian expression of the steroid 15 alpha-hydroxylase (Cyp2a4) and coumarin 7-hydroxylase (Cyp2a5) genes in mouse liver is regulated by the PAR leucine zipper transcription factor DBP. *Mol Cell Biol* 19:6488–99

Le Fur I, Reinberg A, Lopez S *et al.* (2001) Analysis of circadian and ultradian rhythms of skin surface properties of face and forearm of healthy women. *J Invest Dermatol* 117:718–24

Le Martelot G, Claudel T, Gatfield D *et al.* (2009) REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. *PLoS Biol* 7:e1000181

Liu AC, Tran HG, Zhang EE *et al.* (2008) Redundant function of REV-ERBalpha and beta and non-essential role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms. *PLoS Genet* 4:e1000023

Maier B, Wendt S, Vanselow JT *et al.* (2009) A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock. *Genes Dev* 23:708–18

Meng QJ, Logunova L, Maywood ES *et al.* (2008) Setting clock speed in mammals: the CK1 epsilon tau mutation in mice accelerates circadian pacemakers by selectively destabilizing PERIOD proteins. *Neuron* 58:78–88

Micallef L, Belaubre F, Pinon A *et al.* (2009) Effects of extracellular calcium on the growth-differentiation switch in immortalized keratinocyte HaCaT cells compared with normal human keratinocytes. *Exp Dermatol* 18:143–51

Nagoshi E, Brown SA, Dibner C *et al.* (2005) Circadian gene expression in cultured cells. *Methods Enzymol* 393:543–57

Nakahata Y, Sahar S, Astarita G *et al.* (2009) Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1. *Science* 324:654–7

Oster H, Damerow S, Hut RA *et al.* (2006) Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleosome assembly genes. *J Biol Rhythms* 21:350–61

Radhakrishnan A, Ikeda Y, Kwon HJ *et al.* (2007) Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig. *Proc Natl Acad Sci USA* 104: 6511–8

Rejnmark L, Lauridsen AL, Vestergaard P *et al.* (2002) Diurnal rhythm of plasma 1,25-dihydroxyvitamin D and vitamin D-binding protein in postmenopausal women: relationship to plasma parathyroid hormone and calcium and phosphate metabolism. *Eur J Endocrinol* 146:635–42

Reppert SM, Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* 418:935–41

Roenneberg T (1995) The effects of light on the Gonyaulax circadian system. *Ciba Found Symp* 183:117–27

Schibler U (2006) Circadian time keeping: the daily ups and downs of genes, cells, and organisms. *Prog Brain Res* 153:271–82

Schmidt R, Parish EJ, Dionisius V *et al.* (1991) Modulation of cellular cholesterol and its effect on cornified envelope formation in cultured human epidermal keratinocytes. *J Invest Dermatol* 97:771–5

Siepka SM, Yoo SH, Park J *et al.* (2007) Circadian mutant overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. *Cell* 129:1011–23

Sporl F, Wunderskirchner M, Ullrich O *et al.* (2009) Real-time monitoring of membrane cholesterol reveals new insights into epidermal differentiation. *J Invest Dermatol* 130:1268–78

Storch KF, Lipan O, Leykin I *et al.* (2002) Extensive and divergent circadian gene expression in liver and heart. *Nature* 417:78–83

Tanioka M, Yamada H, Doi M *et al.* (2009) Molecular clocks in mouse skin. *J Invest Dermatol* 129:1225–31

Vallett SM, Sanchez HB, Rosenfeld JM *et al.* (1996) A direct role for sterol regulatory element binding protein in activation of 3-hydroxy-3-methylglutaryl coenzyme a reductase gene. *J Biol Chem* 271:12247–53

Vanselow K, Vanselow JT, Westermark PO *et al.* (2006) Differential effects of PER2 phosphorylation: molecular basis for the human familial advanced sleep phase syndrome (FASPS). *Genes Dev* 20:2660–72

Watt FM, Frye M, Benitah SA (2008) MYC in mammalian epidermis: how can an oncogene stimulate differentiation? *Nat Rev Cancer* 8:234–42

Wells J, Lee B, Cai AQ *et al.* (2009) Ovol2 suppresses cell cycling and terminal differentiation of keratinocytes by directly repressing c-Myc and Notch1. *J Biol Chem* 284:29125–35

Wuarin J, Schibler U (1990) Expression of the liver-enriched transcriptional activator protein DBP follows a stringent circadian rhythm. *Cell* 63:1257–66

Yabe D, Brown MS, Goldstein JL (2002) Insig-2, a second endoplasmic reticulum protein that binds SCAP and blocks export of sterol regulatory element-binding proteins. *Proc Natl Acad Sci USA* 99:12753–8

Yosipovitch G, Sackett-Lundeen L, Goon A *et al.* (2004) Circadian and ultradian (12 h) variations of skin blood flow and barrier function in non-irritated and irritated skin-effect of topical corticosteroids. *J Invest Dermatol* 122:824–9

Yosipovitch G, Xiong GL, Haus E *et al.* (1998) Time-dependent variations of the skin barrier function in humans: transepidermal water loss, stratum corneum hydration, skin surface pH, and skin temperature. *J Invest Dermatol* 110:20–3

Zanello SB, Jackson DM, Holick MF (2000) Expression of the circadian clock genes clock and period1 in human skin. *J Invest Dermatol* 115:757–60