

# Krüppel-like factor 9 is a circadian transcription factor in human epidermis that controls proliferation of keratinocytes

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**Circadian clocks govern a wide range of cellular and physiological functions in various organisms. Recent evidence suggests distinct functions of local clocks in peripheral mammalian tissues such as immune responses and cell cycle control. However, studying circadian action in peripheral tissues has been limited so far to mouse models, leaving the implication for human systems widely elusive. In particular, circadian rhythms in human skin, which is naturally exposed to strong daytime-dependent changes in the environment, have not been investigated to date on a molecular level. Here, we present a comprehensive analysis of circadian gene expression in human epidermis. Whole-genome microarray analysis of suction-blister epidermis obtained throughout the day revealed a functional circadian clock in epidermal keratinocytes with hundreds of transcripts regulated in a daytime-dependent manner. Among those, we identified a circadian transcription factor, Krüppel-like factor 9 (*Klf9*), that is substantially up-regulated in a cortisol and differentiation-state-dependent manner. Gain- and loss-of-function experiments showed strong antiproliferative effects of *Klf9*. Putative *Klf9* target genes include proliferation/differentiation markers that also show circadian expression in vivo, suggesting that *Klf9* affects keratinocyte proliferation/differentiation by controlling the expression of target genes in a daytime-dependent manner.**

glucocorticoids | skin cancer

**B**iological rhythms regulate cellular and physiological processes ranging from milliseconds to years. A well-studied timing system is the circadian (~24-h) clockwork that allows organisms to anticipate diurnal variations in environmental conditions such as light, food availability, oxidative stress, pathogen exposure, or temperature. In mammals, the central circadian pacemaker resides in the suprachiasmatic nucleus (SCN), located in the anterior hypothalamus. Oscillations of SCN neurons are cell-autonomous, self-sustained, and synchronized to external time cues (*Zeitgebers*) such as light (1). The SCN, in turn, synchronizes peripheral clocks by systemic time cues such as neuronal input, hormonal signaling (e.g., cortisol), body temperature, and possibly many others (2). Interestingly, most cells in peripheral tissues also possess cell-autonomous clockworks with a similar molecular makeup to SCN neurons. These peripheral clocks are thought to generate or amplify daytime-dependent physiological and metabolic functions in a tissue-specific manner by circadian regulation of clock-controlled genes (3, 4).

On a molecular level, circadian oscillation is generated by interlocked transcriptional-translational feedback loops. The transcription factor dimer CLOCK/BMAL1 drives expression of target genes such as *Periods* (*Per1-3*) and *Cryptochromes* (*Cry1-2*) by binding to E-box elements in their promoters. The negative feedback is formed by PER/CRY protein complexes that shuttle back into the nucleus, where they block CLOCK/BMAL1-mediated

transactivation, thereby inhibiting their own transcription (5). In a second feedback loop, the orphan nuclear receptor REV-ERB $\alpha$  rhythmically represses *Bmal1* transcription, presumably adding to the robustness of the circuitry. The molecular clock, but also rhythms of systemic cues such as hormones or temperature, can mediate circadian expression of tissue-specific output genes either directly or by controlling transcription factors that in turn drive rhythmic expression of target genes. Transcriptional analysis in mice revealed that large subsets of genes (2–10%) in a given tissue are rhythmically expressed (6). This allows tissue-specific circadian control of various physiological functions including cell growth, metabolic processes or immune function (7–9). Consequently, disruption of the circadian system can lead to severe pathological conditions such as metabolic syndrome (10), diabetes (11), and cancer (12). Although mouse models have greatly advanced our understanding of the connections between circadian rhythms, physiology and disease studies of the molecular mechanisms of circadian action in human tissues are still in its infancy.

In particular, circadian rhythms of human skin have received only little attention. Because the epidermis forms the outermost barrier between body and environment, it is naturally exposed to diurnal environmental variations such as UV radiation, temperature, or pathogen exposure. Accordingly, some skin functions such as barrier recovery and sebum secretion show circadian variations (13). However, on a molecular level, it is still unclear whether a functional circadian clock is operative in human epidermis and how this clock might contribute to rhythmic skin function.

Here, we report genome-wide identification of rhythmic gene expression in human epidermis using a three-point sampling strategy. We find significant daytime-dependent gene expression in hundreds of epidermal transcripts. In vitro studies further revealed that many of these genes including canonical clock genes are driven by a keratinocyte clock. We identified Krüppel-like factor 9 (*Klf9*) as a circadian transcription factor that regulates the rhythmic expression of several output genes. We found that *Klf9* is expressed in a cortisol dependent and differentiation-specific manner. Gain-

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and loss-of-function experiments revealed a strong antiproliferative effect of *Klf9* in keratinocytes in vitro. Thus, our results demonstrate a cell autonomous circadian clock in human epidermis and point to a role for the circadian transcription factor *Klf9* for rhythmic modulation of target genes with implications for dermatological homeostasis and disease.

## Results

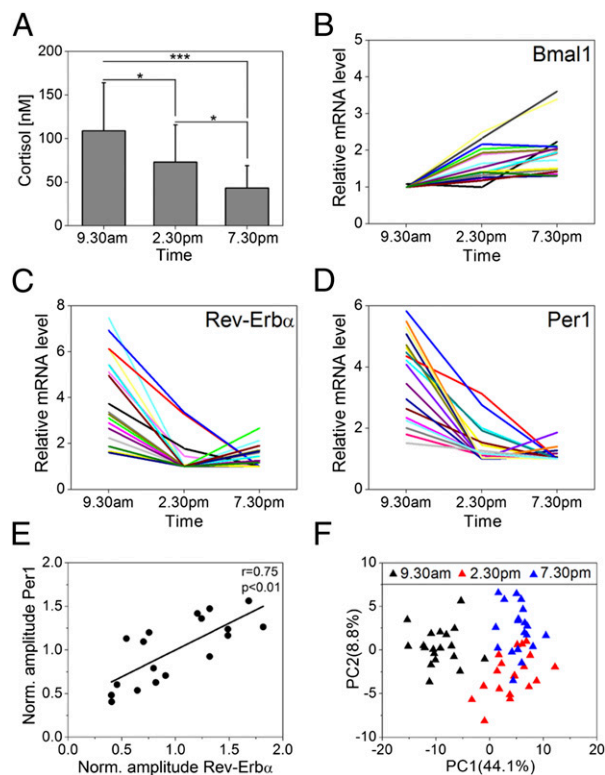
**Functional Circadian Clock in Human Epidermis.** Circadian rhythms in peripheral tissues are largely dependent on local molecular clocks driving rhythmic gene expression that result in timed cellular and physiological functions. To test whether such a clock is operative in human epidermis in vivo, we obtained epidermal biopsies from 20 healthy human subjects (see Fig. S1A and B for control parameters) throughout the activity phase (0930 hours to 1930 hours) using the suction-blister method (see *Material and Methods*).

For each subject, we determined cortisol levels in saliva samples and found, as expected, significant diurnal variations (Fig. S1C). Cortisol is known to contribute to the synchronization process of peripheral oscillations (14, 15). To test whether cortisol may exert a similar function in the epidermis, we analyzed cortisol levels in epidermal suction-blister fluids (SBF) collected at 0930 hours, 1430 hours, and 1930 hours from 20 subjects. Again, we detected a strong decrease in suction-blister cortisol and cortisone levels throughout the day (Fig. 1A and Fig. S1D, H, and I). To assess potential rhythmic gene expression in the epidermis, we performed whole genome microarray analysis of suction-blister epidermis samples from 19 subjects. In total 18,224 annotated genes were found to be expressed with highly significant diurnal regulation of *Bmal1*, *Per1*, and *Rev-Erba*: essential genes of the core clock circuitry (Fig. 1B–D). *Per1* and *Rev-Erba* displayed high expression levels in the morning compared with midday and evening in all subjects, whereas *Bmal1* expression increases during the day. This phase relation was as expected from the molecular makeup of the circadian clockwork. In general, substantial interindividual variances were detected in “amplitudes” (defined as maximum minus minimum expression in the analyzed time frame) of daytime-dependent genes similar to other studies using human blood mononuclear cells or oral mucosa as human tissue source (16–18). This is likely primarily attributable to interindividual differences in the epidermal oscillator, because we find a strong correlation of *Per1* and *Rev-Erba* amplitudes between individual subjects (Fig. 1E and Fig. S1F and G).

To identify putative clock-controlled genes, we performed statistical analysis of microarray data from 19 subjects revealing 294 annotated genes that showed significantly ( $q < 0.05$ ) different expression levels throughout the day (Fig. S2 and Table S1B). Principal component analysis between microarrays led to a clear clustering according to daytime (Fig. 1F). Most regulated genes showed peak expression in the morning (Fig. S1E), similar to *Per1* and *Rev-Erba* (E-box phase), suggesting similar regulatory mechanisms. These data (together with theoretical considerations; see Fig. S1J and Table S2) clearly demonstrate that our three-time-point experimental setup allows identifying daytime-dependent variations in gene expression in human epidermis. Among the genes ranking highest in the statistical analysis, we found several core clock genes such as *Bmal1* and *Npas2*, as well as E-box driven genes *Per1-3*, *Cry2*, *Rev-Erba*, and *Rev-Erbβ* [Fig. S3A; for quantitative (q)PCR validation, see Fig. S3D and E].

In addition, a set of transcription factors, rate limiting enzymes and cell cycle regulators was identified within the top ranking genes (Fig. S3B and C). Kyoto Encyclopedia of Genes and Genomes pathway analysis identified highly significant daytime-dependent cellular processes, including “circadian rhythms,” “DNA replication,” “metabolic processes,” and “cell cycle” (Table S1A), indicating that a substantial part of epidermal physiology is likely under circadian control.

To further validate our three-time-point sampling strategy, we performed two additional studies. First, suction blisters from six



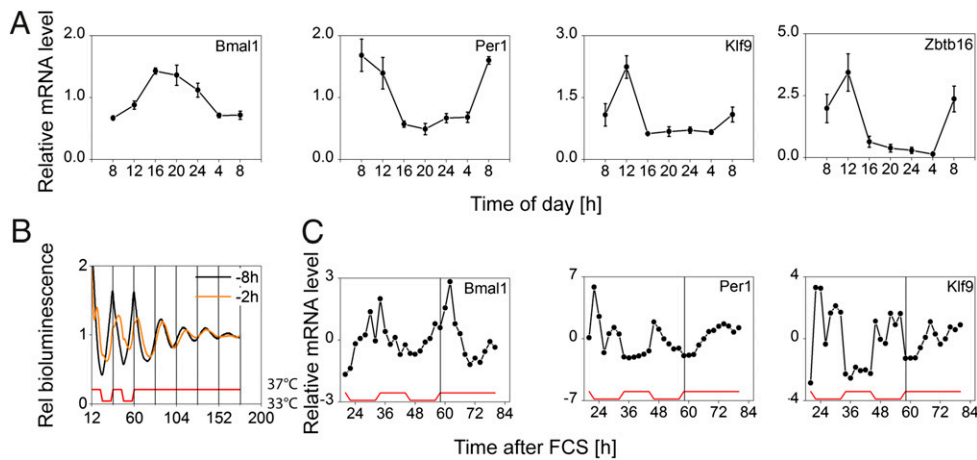
**Fig. 1.** Circadian gene expression in human epidermis. (A) Cortisol levels in suction-blister liquid obtained at indicated times were determined in 20 individuals. Mean values  $\pm$  SD are given.  $*P < 0.05$ ;  $***P < 0.001$  (Mann-Whitney  $U$  test). (B–D) Genome-wide microarray analysis was performed using suction-blister epidermis of 19 subjects at indicated times. *Bmal1* (B), *Rev-Erba* (C), and *Per1* (D) expression of each subject are shown relative to minimum. (E) Normalized (relative to mean of all subjects) amplitudes (maximum minus minimum expression) of *Per1* and *Rev-Erba* were correlated. The Pearson correlation coefficient ( $r = 0.75$ ) and the corresponding  $P$  value ( $P < 0.01$ ) indicate a strong linear correlation. (F) Principal component analysis (PCA) of microarrays was performed for the 250 top-ranking genes according to  $q$  value. The relative variance in gene expression (relative to total variance) comprised in PC1 and PC2 is indicated.

subjects were obtained in 12-h intervals over 2 consecutive days (0930 hours and 2130 hours each day). In this setup, we obtained similar results for the expression dynamics of clock and clock-controlled genes (including the transcription factors *Klf9* and *Zbtb16*) (Fig. S4A). Moreover, we obtained punch biopsies from six subjects every 4 h for a full 24-h cycle. Again, we found highly rhythmic transcript levels for clock and clock-controlled genes with a similar phase compared with the three-point sampling strategy (Fig. 2A and Fig. S4B and C).

Together, these data indicate a functional circadian clock in human epidermis that likely controls various output genes with implications for cellular functions. Moreover, circadian gene expression in human epidermis can be accurately determined using a simple three-point sampling strategy.

### Cell-Autonomous Circadian Rhythms in Cultured Primary Keratinocytes.

In mouse liver, a small subset of rhythmic genes is not (or not only) driven by the local liver clock but by systemic factors, such as temperature or cortisol (19). To discriminate these possibilities for some of our identified rhythmic genes in keratinocytes, we established a circadian in vitro model system using primary neonatal human epidermal keratinocytes (NHEKs). To this end, we used a luciferase reporter for *Bmal1* promoter activity stably integrated into NHEK cells. Serum shock combined with temperature cycles led to



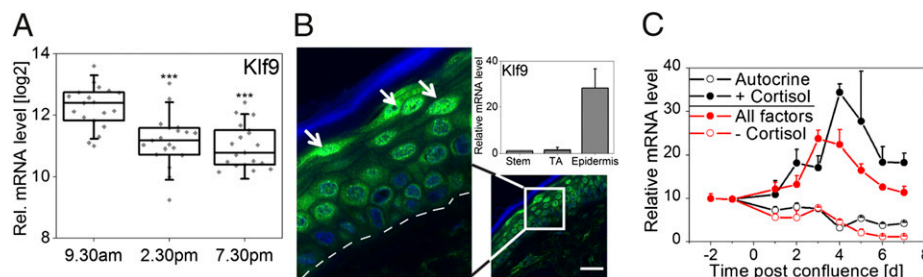
**Fig. 2.** Epidermis clock in vivo and in vitro. (A) Gene expression in punch-biopsy epidermis obtained at indicated times is shown relative to 24-h mean expression (mean of six subjects  $\pm$  SEM; except *Zbtb16*:  $n = 5$ ). *Bmal1*, *Per1*, *Zbtb16*:  $P < 0.001$ ; *Klf9*:  $P = 0.001$ . (B) Circadian rhythms of *Bmal1* reporter activity in primary keratinocytes. Note that instant entrainment is achieved if FCS is administered 8 h before the temperature cycles (black curve). This synchronization paradigm was used for subsequent experiments. (C) Cells were harvested in regular 2-h intervals starting 24 h after FCS for 60 h and mRNA levels of depicted genes were determined. Expression levels are given relative to mean expression. *Bmal1*, *Per1*:  $P < 0.001$ ; *Klf9*:  $P = 0.001$ . (For  $P$  values during entrainment and free run, see Table S1C). Statistical analysis for circadian gene expression was performed using CircWave software (43).

persistent circadian rhythms in *Bmal1* promoter activity (Fig. 2B). As reported for human adult low calcium temperature (HaCaT) keratinocytes earlier (20) circadian rhythms showed strong dampening after release into constant conditions. Of note, circadian period in HaCaT keratinocytes was temperature compensated (Fig. S5A) similar to other cellular clocks (21). Next, we tested whether canonical clock genes and clock-controlled genes identified from our microarray analysis can be entrained to temperature cycles. To this end, we harvested synchronized NHEKs in regular 2 h intervals. Similar to *Bmal1* promoter activity *Bmal1* mRNA abundance was highly rhythmic with a peak at the beginning of the warm phase during entrainment and persistent (albeit damped) oscillation in free running conditions (Fig. 2C). Other clock and clock-controlled genes also showed significant circadian rhythms (Fig. 2C and Fig. S5B and C). For instance, *Klf9* gene expression [which is circadian in vivo (Fig. 2A)] was highly rhythmic during temperature entrainment but not unambiguously in constant conditions (Fig. 2C) suggesting that *Klf9* is at least in part driven by systemic cues (such as temperature or others). Together, our NHEK model system demonstrates a cell autonomous circadian clock in primary keratinocytes consistent with our in vivo results.

### **Klf9 Is a Cortisol-Dependent Marker for Epidermal Differentiation.**

Among the clock-controlled genes in epidermis a large subset of transcription factors was found that possibly mediate timed transcription of downstream genes thereby mediating circadian control of cellular functions.

In particular, *Klf9* raised our interest as it has been implicated in differentiation and proliferation of epithelial tissues (22), both essential processes in epidermal homeostasis. We found robust diurnal oscillations of *Klf9* mRNA in epidermal suction blister, as well as punch-biopsy samples in vivo (Figs. 2A and 3A) with a peak around noon. Of note, *Klf9* mRNA is also circadian in mouse liver (<http://bioinf.itmat.upenn.edu/circa>) and peritoneal macrophages (8). We found that (i) an E-box downstream of the *Klf9* transcription start site could be mildly activated by CLOCK/BMAL1 (Fig. S5E); and (ii) BMAL1 binds to this and a second further upstream E-box in a daytime-dependent manner (Fig. S5E), suggesting that although *Klf9* mRNA does not exhibit robust oscillations in vitro, the keratinocyte clock might contribute to *Klf9* oscillation in vivo. Notably, *Klf9* knockdown did not significantly affect circadian rhythms in U2OS cells (Fig. S5D) indicating that

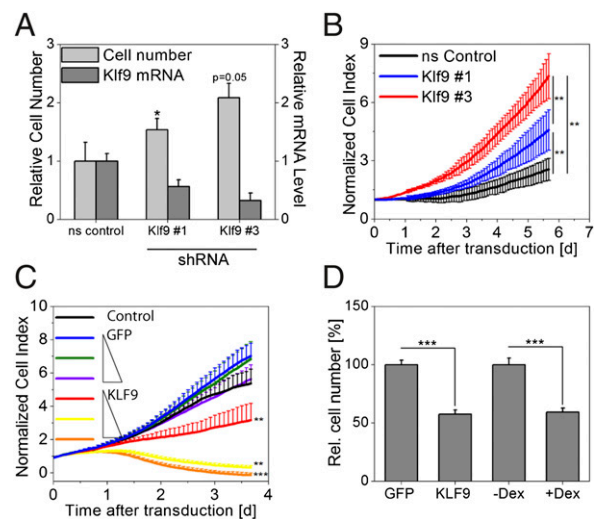


**Fig. 3.** *Klf9* is an epidermal differentiation marker. (A) Log<sub>2</sub> *Klf9* mRNA levels (background-corrected signal intensity) in epidermis of 19 subjects is shown in a box plot. Box, 25–75 percentile; vertical bar, median value; error bars, SDs. Individual signal intensities are shown in gray rectangles. \*\*\* $P < 0.001$  ( $t$  test). (B) KLF9 protein levels in human skin section determined by immunofluorescence staining. KLF9 immunoreactivity is shown in green and nuclei are stained with DAPI (blue). The stratum corneum layer (outermost epidermal layer) is indicated in blue, and the basal membrane is indicated by a white dashed line. Arrowheads show strong staining in differentiated cells in upper epidermal layers (stratum granulosum). (Scale bar: 50  $\mu$ m.) (Inset) *Klf9* mRNA levels in validated cell fractions (23) of stem cells, transient amplifying keratinocytes (TA Cell), and fully differentiated epidermis (Epidermis) and given relative to the stem cell fraction (four subjects; mean  $\pm$  SD). (C) Keratinocytes were differentiated by cultivating cells into postconfluence using different culture conditions: full growth medium  $\pm$  cortisol (All factors and -Cortisol, respectively) or autocrine growth conditions  $\pm$  cortisol (Autocrine and +Cortisol, respectively). *Klf9* mRNA levels of two experiments (mean  $\pm$  maximum or minimum) are shown relative to minimum expression.

*Klf9* functions as clock output gene rather than being part of the core clock machinery.

To test whether *Klf9* is differentially expressed during epidermal differentiation in vivo we compared validated cell fractions (23) of epidermal stem cells, transit amplifying (TA) keratinocytes (i.e., cell fraction with high proliferative capacity) and fully differentiated non proliferating epidermis. *Klf9* mRNA levels were highly elevated in differentiated epidermis compared with stem cells and TA cells (Fig. 3B). In addition, KLF9 protein was strongly expressed in nuclei of suprabasal epidermal layers (Fig. 3B) whereas only weak KLF9 immunoreactivity was observed in the basal cell layer comprising epidermal stem cells and TA cells. To confirm these findings in vitro *Klf9* mRNA levels were monitored in cultured keratinocytes during confluence induced differentiation (24). As *Klf9* expression is influenced by serum factors and cortisol in other tissues (25, 26) *Klf9* expression was assessed in growth conditions with and without growth factors and cortisol. We found strong induction of *Klf9* expression during keratinocyte differentiation in a cortisol dependent manner. When cells were cultured in growth factor supplemented medium *Klf9* gene expression gradually increased after cells reached confluence (Fig. 3C). Peak expression was observed 4 d postconfluence, correlating with induction of the early differentiation marker *Keratin10* (*Krt10*) and the differentiation associated cell cycle inhibitor *p21* (compare Fig. 3C and Fig. S6A and B). In contrast, in autocrine culture conditions lacking growth factors no differentiation dependent induction of *Klf9* was detected, which was not due to impaired differentiation in general as differentiation markers showed similar (albeit not identical) induction kinetics (Fig. S6A–C). Importantly, supplementing autocrine medium with cortisol restored *Klf9* induction during keratinocyte differentiation. Accordingly, removing cortisol from the growth factor mix led to similar abrogation of *Klf9* induction as removing all growth factors (Fig. 3C). This shows that cortisol is a necessary and sufficient factor in the differentiation dependent induction of *Klf9* expression. Notably, cortisol sensitivity during epidermal differentiation was specific for *Klf9*, whereas other *Klf* genes (as well as clock genes) did not - or only weakly - respond to cortisol (Fig. S6D). To monitor *Klf9* induction kinetics autocrine keratinocyte cultures were treated with cortisol or the synthetic glucocorticoid dexamethasone (dex). Both cortisol and dex induced *Klf9* expression in a time dependent manner with strong (>two-fold) induction occurring already 3 h after treatment (Fig. S6E). This suggests that *Klf9* is a fast responding gene in keratinocytes similar to observations in vertebrate tissues (25). In line with this, we found a significant correlation between cortisol levels in suction-blister fluid and *Klf9* transcript abundance in suction-blister epidermis throughout the day (Fig. S6F). Thus, *Klf9* oscillation in vivo seems to be mainly driven by rhythmic systemic cortisol levels.

**Klf9 Regulates Proliferation in Primary Keratinocytes.** Keratinocyte differentiation is associated with cell cycle withdrawal, and forced *Klf9* expression has been reported to cause growth arrest in mouse cancer stem cells (27). Together with the differentiation dependent induction of *Klf9* expression reported here, it is conceivable that *Klf9* exerts a similar role in human keratinocytes. To test this, we down-regulated *Klf9* mRNA using stably integrated microRNA-adapted short hairpin RNA (shRNAmir) constructs and subsequently assessed keratinocyte proliferation. Both flow cytometry as well as real-time proliferation studies revealed a significant increase in cell proliferation in *Klf9*-deficient cells. This effect was concentration dependent with lower *Klf9* levels resulting in a stronger increase in cell growth (Fig. 4A and B). Ectopic, stable KLF9 overexpression in primary keratinocytes (Fig. S7A and B) induced the opposite effect: a concentration-dependent decrease in cell growth was observed in cells overexpressing KLF9 whereas no effect was observed in GFP expressing control cells (Fig. 4C and D). Interestingly, dex treatment of keratinocyte cultures resulting in *Klf9* induction led to a similar attenuation of



**Fig. 4.** *Klf9* attenuates keratinocyte proliferation. (A) *Klf9* transcript was down-regulated in primary keratinocytes using stably integrated shRNAmir constructs (#1 and #3), and cell numbers were assessed 5 d after transduction. *Klf9* mRNA levels and cell numbers are given relative to cells transduced with a nonsilencing (ns control) vector (mean  $\pm$  SD of three experiments; \* $P$  < 0.05,  $t$  test). (B) Cells were treated similar to A, and cell growth was assessed using real-time impedance measurements (mean value of six wells  $\pm$  SD; representative plot of three experiments is shown). (C) Primary keratinocytes were transduced with increasing (1 $\times$ , 2 $\times$ , and 4 $\times$ ) viral titers carrying GFP or KLF9 expression constructs or left untreated (Control). Cell growth was subsequently analyzed in real-time impedance measurements (mean value of six wells  $\pm$  SD; representative plot of three experiments is shown). \*\*\* $P$  < 0.01; \*\*\*\* $P$  < 0.001 (Mann-Whitney  $U$  test). (D) Cells were treated similar to C, and a different set of cells was treated with dex. Cell number was analyzed by cell counting 4 d after transduction/treatment [1 $\times$  virus; mean  $\pm$  SEM of 9 wells (virus) or 24 wells (dex treatment)] of two pooled independent experiments; \*\*\*\* $P$  < 0.01,  $t$  test).

proliferation (Fig. 4D) confirming results reported earlier (28). Together these data suggest that cortisol dependent *Klf9* expression may modulate proliferation of differentiating keratinocytes.

Is KLF9 function rhythmic in the epidermis? To address this question, we identified putative KLF9 target genes via transcriptome microarray analysis of KLF9 over-expressing keratinocytes. Within the set of genes down-regulated upon KLF9 overexpression, we found significantly more genes, whose expression is also daytime-dependent in vivo, indicating that KLF9-mediated transcriptional regulation indeed conveys circadian rhythmicity to KLF9 target genes (Fig. S7D). Supporting the hypothesis that (at least a subset) of these genes are direct targets of KLF9 we found a significant enrichment of KLF9 binding sites in the corresponding promoter regions (Fig. S7D). Interestingly, gene ontology analysis of putative KLF9 target genes points to biological processes such as ‘epidermal development’ and ‘pigment biosynthetic process’ (Table S1D). In fact, putative KLF9 target genes include proliferation/differentiation markers [such as *Ell3* (29)], as well as genes implicated in glucocorticoid signaling [such as *Fkbp5* (30)]. These putative KLF9 targets also showed significant rhythmic expression in the epidermis, with a phase predicted from KLF9 being a repressor of these genes (Fig. S7C), and have putative KLF9 binding motifs in their promoters (Fig. S7E).

In summary, these data suggest that circadian *Klf9* gene expression translates into rhythmic repression of putative target genes, including proliferation markers.

## Discussion

**Human Epidermis Harbors a Molecular Circadian Clock.** Accumulating evidence points to a central role of the circadian system for cellular

and organ homeostasis. Although circadian rhythms have been detected in virtually all tested tissues in mice, studies in humans addressing the molecular mechanisms of circadian rhythms have been hindered mostly by accessibility of sample material. To our knowledge, no genome-wide transcriptional profiling for daytime-dependent variations in gene expression has been reported in a human organ so far. In this study, we present an easy yet robust tool to study circadian gene expression in human epidermis. Collecting samples during the day, we identified about 300 genes with significant daytime-dependent expression. Validation experiments using more time points, as well as computer simulations, show that our three-time-point sampling strategy is a very good alternative to sampling over the whole 24-h cycle in humans. Clock gene transcripts with robust diurnal variations in epidermis samples *in vivo* also showed circadian expression in keratinocytes *in vitro*. Other rhythmic genes such as *Klf9* seem to require systemic signals for strong oscillation. Thus, we believe our suction-blister approach is feasible to identify daytime-dependent-regulated genes in human epidermis *in vivo*. By collecting suction-blister fluid together with epidermis samples, it is possible to directly correlate systemic rhythms (such as cortisol) to circadian gene expression.

Skin forms the natural barrier between body and environment and, as such, is strongly exposed to diurnal changes in environmental conditions; however, studies of the circadian systems of the skin are surprisingly rare. Circadian gene expression has been shown in mouse skin, and some skin functions (e.g., barrier recovery, sebum secretion, pH) show diurnal variations (13). Only one study has reported circadian gene expression of (only two) clock genes *in vivo*, however, without differentiating between dermal and epidermal expression (31). Our approach allows studying genome wide circadian gene expression specifically in the human epidermis. This is crucial because physiology of epidermal and dermal compartments vary considerably. Functional grouping of rhythmic transcripts in the epidermis suggested circadian control of various pathways ranging from cell cycle control to metabolic processes. Future studies may address the question whether and how circadian gene expression is translated into rhythmic skin function.

***Klf9* Is an Epidermal Circadian Transcription Factor Regulating Keratinocyte Proliferation.** The epidermis undergoes a constant process of self-renewal in which epidermal stem cells gradually commit to a complex program of terminal differentiation (32). A key element in this process is precise spatial and temporal control of keratinocyte proliferation and cell cycle withdrawal (33). Disruption of this tightly regulated homeostatic process leads to severe skin diseases such as psoriasis, dermatitis, and nonmelanoma skin cancer (34). The spatiotemporal organization of keratinocyte cell cycle events, however, is still not completely understood. This study offers molecular insights into a role of the circadian system in controlling keratinocyte proliferation. We identified *Klf9* as a circadian transcription factor in human epidermis that seems predominantly driven by rhythmic systemic cortisol and possibly also temperature cycles. *Klf9* expression is highly sensitive to glucocorticoids and shows diurnal expression patterns *in vivo* with a similar phase to systemic cortisol rhythms in suction-blister fluid. However, we also observed *Klf9* oscillations *in vitro* during temperature entrainment with a similar phase to E-box driven genes. Because we found that CLOCK/BMAL1 can activate E-boxes in the *Klf9* gene and BMAL1 binds to these E-boxes in a daytime-dependent manner, it is conceivable that the local keratinocyte clock also contributes to *Klf9* oscillation *in vivo*.

*Klf9* has been associated with differentiation and proliferation in mouse uterus epithelial tissue (35). However, a role for *Klf9* in epidermal homeostasis has not yet been reported in mice nor in humans. Here, we find highly elevated *Klf9* mRNA and protein levels late in the multistep differentiation program of human epidermis. We validated these findings using a confluence induced

differentiation model in primary keratinocyte *in vitro*. Interestingly, however, differentiation associated induction of *Klf9* was strongly dependent on glucocorticoids. Previously, it has been shown that topical or systemic administration of glucocorticoids as well as stress induced increase in systemic cortisol levels causes decreased keratinocyte proliferation in human and mouse epidermis (36, 37). In line with this, we observed a strongly attenuated keratinocytes proliferation upon either forced *Klf9* expression or glucocorticoid treatment, whereas depletion of *Klf9* led to hyperproliferation. Moreover, putative *Klf9* target genes (including proliferation/differentiation markers) also show rhythmic expression in the epidermis. It remains to be elucidated in future studies to what extent the glucocorticoid phenotype is *Klf9*-dependent. In fact, it is conceivable that glucocorticoid-dependent mechanisms independent of *Klf9* contribute to the inhibition of keratinocyte proliferation. However, our data support the hypothesis that *Klf9* might be involved in glucocorticoid-dependent modulation of the complex program of differentiation-associated cell cycle exit in epidermal keratinocytes. It is possible that this effect is daytime-dependent because our data suggest a rhythmic function of *Klf9* in the epidermis; however, whether this is dependent on the local keratinocyte clock or systemic time cues remains to be investigated. Such a daytime-dependent mechanism might be important for chronotherapeutic applications because glucocorticoids are commonly used for treatment of various skin diseases such as dermatitis and psoriasis. However, long-term glucocorticoid treatment results in severe adverse effects, particularly in the skin (38). Optimizing glucocorticoid administration for treating dermatological diseases according to daytime might ultimately improve efficiency and reduce side effects, as recently shown for rheumatoid arthritis (39).

Future studies could address the precise role of *Klf9* in keratinocyte proliferation control and its possible involvement in skin cancer. This might open avenues for chronotherapeutic approaches in treating skin diseases, as well as help understanding the consequences of disrupted circadian rhythms (e.g., by shift work) for epidermal homeostasis.

## Materials and Methods

Epidermal suction blister samples were collected according to the recommendations of the Declaration of Helsinki as well as applicable laws for a nondrug study. All donors provided written, informed consent. Suction-blister samples were obtained at the study center of Beiersdorf AG and approved by the Beiersdorf AG Legal Review Board. Ten male and 10 female volunteers aged  $28.35 \pm 4.36$  y (mean  $\pm$  SD) participated in the study. Individual chronotypes were assessed using the Munich Chronotype Questionnaire (MCTQ) (40). During the study equicaloric food was provided, and blood glucose levels were determined at 0730 hours, 1000 hours, 1230 hours, 1500 hours, and 1730 hours using the Accu Check system (Roche Diagnostics). Saliva samples were obtained at similar times and cortisol levels were determined by Demeditec Diagnostics. Suction-blister procedure was started at 0700 hours, 1200 hours, and 1700 hours, as described previously (41). Briefly, epidermis samples were detached by applying a negative pressure of 180–320 mmHg for  $\sim 2.5$  h. Consequently, suction blisters were harvested at 0930 hours, 1430 hours, and 1930 hours, respectively. Suction-blister fluid was removed and stored at  $-20$  °C, and suction-blister epidermis was snap frozen in liquid nitrogen and stored at  $-80$  °C. Cortisol/cortisone levels in suction-blister fluid were determined by ultrahigh pressure liquid chromatography–tandem mass spectrometry as described previously (42).

See *SI Material and Methods* for a full description of methods.

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