



# Population-level rhythms in human skin with implications for circadian medicine

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**Skin is the largest organ in the body and serves important barrier, regulatory, and sensory functions. The epidermal layer shows rhythmic physiological responses to daily environmental variation (e.g., DNA repair). We investigated the role of the circadian clock in the transcriptional regulation of epidermis using a hybrid experimental design, in which a limited set of human subjects ( $n = 20$ ) were sampled throughout the 24-h cycle and a larger population ( $n = 219$ ) were sampled once. We found a robust circadian oscillator in human epidermis at the population level using pairwise correlations of clock and clock-associated genes in 298 epidermis samples. We then used CYCLOPS to reconstruct the temporal order of all samples, and identified hundreds of rhythmically expressed genes at the population level in human epidermis. We compared these results with published time-series skin data from mice and found a strong concordance in circadian phase across species for both transcripts and pathways. Furthermore, like blood, epidermis is readily accessible and a potential source of biomarkers. Using ZeitZeiger, we identified a biomarker set for human epidermis that is capable of reporting circadian phase to within 3 hours from a single sample. In summary, we show rhythms in human epidermis that persist at the population scale and describe a path to develop robust single-sample circadian biomarkers.**

human skin | hybrid design | circadian medicine | population-level rhythms | biomarkers

Skin is the largest organ in the body, functioning in the biosynthesis of vitamins and protection from the environment (1, 2). Anticipated daily variation in stressors (e.g., temperature, humidity, UV light) is managed by corresponding changes in skin physiology, especially in the epidermal layer. For example, genetic programs regulating responses to transepidermal water loss, cell proliferation, and DNA repair all undergo daily change (3–5). These changes depend on an intact clock in mouse epidermis (5–7). Human epidermis is no exception, as a pilot study identified hundreds of transcripts that vary in the daytime (8).

In addition to knowledge gained in exploring the biology of human epidermis, it is also a potential source of circadian biomarkers. For circadian medicine to influence health, such as when to take a drug (9) or undergo a procedure (10), a practical measure of circadian phase is needed. The current gold standard tool for assessing human circadian phase is the dim-light melatonin-onset (DLMO) assay. This assay requires a subject to sit in a dim room for repeated saliva sample collection, a difficult practice to standardize and perform at scale and unsuitable for clinical practice. Furthermore, DLMO is a biomarker for the circadian phase of locomotor activity. This may or may not be aligned with clocks in the periphery regulating drug metabolism or action. Which is the best source of circadian biomarkers? What aspects of circadian biology should these biomarkers measure? These remain important and open questions.

Over the last decade, many groups have sought to develop more practical and informative circadian biomarkers. Originally, this research focused on whole blood transcriptomics and metabolomics; however, there is little evidence this work has influenced clinical care (11, 12). Moreover, whether whole blood is the best source for circadian biomarkers has been called into question. The composition of blood varies greatly over time and between subjects due to internal (e.g., endocrine, body temperature, individual variation) and external (e.g., diet, infection) factors. Recently, Wittenbrink et al. (13) explored a blood cell type, CD14<sup>+</sup> monocytes, as a source of circadian biomarkers in a two-stage process. The discovery study was longitudinal and included 12 healthy young people in a hospital environment. The validation study focused on 28 individuals, following gene expression measured in their home environment. These excellent studies showed accurate assignment of DLMO phase from a single sample to within 3 h; however, these studies remain relatively small scale (40 total individuals), the study population was generally young (18–41 y), and only part of the circadian cycle was predicted (morning and afternoon phases). Will these markers extend to larger populations? To different age groups? Ethnicities? Can they accurately report the entire circadian cycle?

Here we explore epidermis as an alternative source of robust biomarkers of circadian phase. We describe a hybrid experimental

## Significance

**We used a hybrid design combining epidermis samples from both deep phenotyping of 20 individuals and snapshot data from more than 200 other human subjects to find biomarkers of the human circadian phase. Using CYCLOPS to reconstruct the temporal order of all samples, we identified hundreds of genes cycling at population level and found that phase relationships of human skin epidermis output genes are conserved with the mouse skin. Finally, we showed that human epidermis has a stronger clock than blood and developed a panel of biomarkers that can phase individuals to within 3 hours from a single sample.**

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Conflict of interest statement: K.J.M., J.E.O., J.D.S., and R.T. are employees of the Procter & Gamble Company, which markets skin care products.

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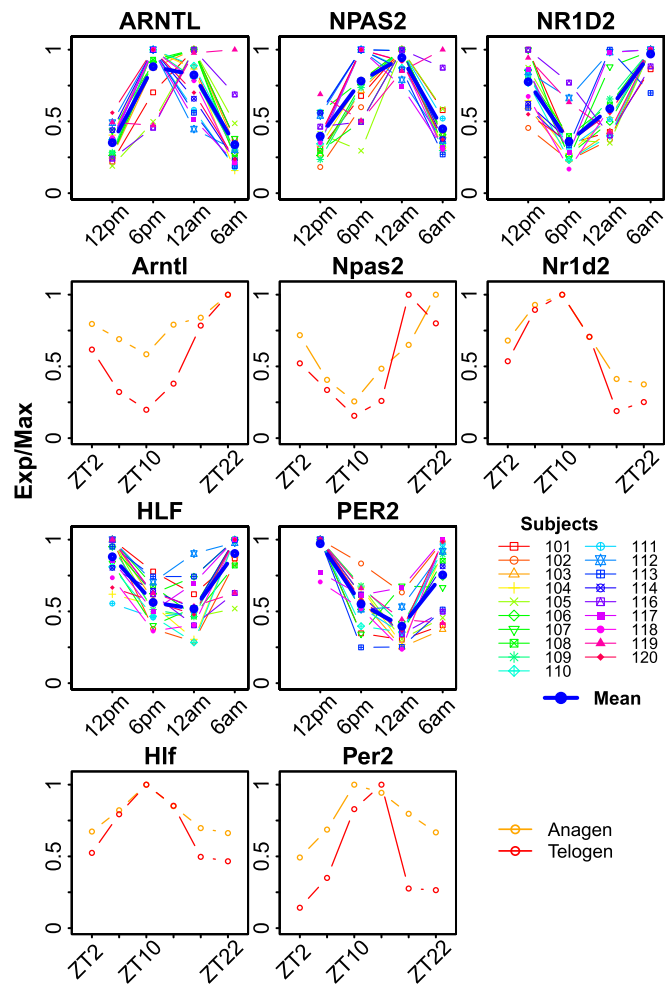
design in which a limited set of subjects ( $n = 20$ ; age 21–49 y) was sampled throughout the 24-h cycle and a larger population ( $n = 219$ ; age 20–74 y) was sampled once. Using a recently described algorithm, cyclic ordering by periodic structure (CYCLOPS) (14), we reconstructed the circadian transcriptome from human epidermis and found hundreds of clock-regulated genes with rhythms persisting at the population level. We compared these results with time-series data collected from mouse skin and found strong concordance in circadian phase across species at the transcript and pathway levels. Finally, we applied a second algorithm, ZeitZeiger, to these reconstructed time course data. We describe a biomarker set for human epidermis capable of reporting circadian phase to within 3 h from a single sample. In summary, we present a survey of circadian rhythms in human epidermis and point to skin as a source of robust single-sample circadian biomarkers suitable for population-scale research.

## Results

**Clock-Regulated Genes Identified from Time-Series Analysis of Human Epidermis.** To explore the potential of human skin as a source of circadian phase biomarkers, we performed a time-series analysis of human epidermis every 6 h across a 24-h sampling period from 19 individuals (subject 115 was excluded; *SI Appendix, Table S1*). In each subject, an epidermal biopsy specimen was collected every 6 h starting at 12 PM, for a total of four specimens. We used MetaCycle's meta3d function to analyze these data. We identified 110 genes ( $P < 0.05$ ; *SI Appendix, Fig. S1 A and B*) that varied with a rhythmic pattern over a 24-h period. These genes show a bimodal distribution, with peak phases clustered at 8–9 AM and 8–9 PM (*SI Appendix, Fig. S1C*). We reasoned that genes with a circadian pattern showing evolutionary conservation between mice and humans would represent a robust source for circadian biomarkers. If it was conserved across 80 My of evolution, it should be conserved across different populations of humans. To identify these genes using a common statistical framework, we applied MetaCycle's meta2d function to a recently published time-series dataset of mouse telogen skin (containing small and resting hair follicles) and anagen skin (containing large and growing hair follicles) (7). Our reanalysis found 1,280 and 294 circadian genes in telogen and anagen skin, respectively ( $P < 0.05$ ). Similar to our observations in human epidermis, we noticed a pronounced bimodal phase distribution from mouse telogen, as has been reported in analyses of many other mouse tissues (15) (*SI Appendix, Fig. S1D*).

Expression patterns for five genes—*ARNTL*, *NPAS2*, *NR1D2*, *HLF*, and *PER2*—were conserved across all three datasets (Fig. 1 and *SI Appendix, Fig. S1E*). We compared the expression profiles of each of these five genes across all human subjects. The patterns of expression for these five genes generally followed the same phase relationship (Fig. 1); however, there were clear interindividual differences. For example, subjects 116 and 119 show delayed clock phases of genes, salivary melatonin, and cortisol (*SI Appendix, Fig. S2*). These five clock genes were also robustly rhythmic in mouse telogen and anagen, albeit with a nocturnal pattern of expression (Fig. 1). As noted previously, the phase of clock gene expression reflects locomotor activity; that is, *ARNTL* peaks at 8–9 PM in humans and ZT23 in mouse, predicting the sleep phase in both species. The phase of clock gene expression matches the established phase relationships: *ARNTL* precedes *NR1D2*, which precedes *PER2* (16).

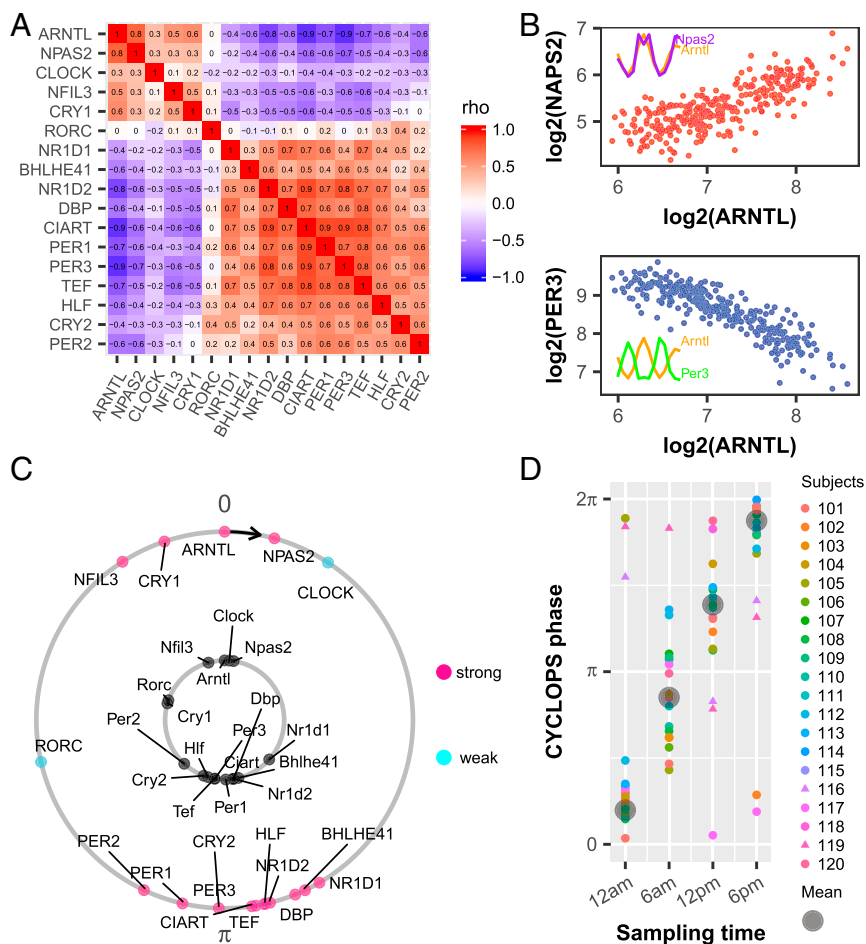
**Reproducing Human Epidermis Phase Without Wall Time.** For skin epidermis to be a good source for determining human circadian phase, clock gene phase relationships should be conserved at a population scale. In other words, the circadian variation in clock gene expression must exceed interindividual variation. We analyzed a gene expression dataset from human epidermis from 20 subjects sampled around a 24-h clock and 219 subjects each sampled once irrespective of wall time (*SI Appendix, Table S1*). This hybrid design (*SI Appendix, Fig. S3*) captures advantages of both longitudinal and population-based studies while mitigating disadvantages. Using a set of clock and clock-associated genes (*Materials and Methods*) (17–20), we applied Spearman's rho to evaluate the



**Fig. 1.** Expression profiles of conserved circadian genes in human and mouse skin. Epidermis from forearms of human subjects ( $n = 19$ ) was sampled every 6 h for one circadian cycle starting at 12 PM. Expression profiles from all 19 human subjects along with the mean (blue) are shown (rows 1 and 3). Expression profiles for mouse anagen (orange) and telogen (red) skin samples are shown (rows 2 and 4). Mouse data are from Geyfman et al. (7) (*SI Appendix, Table S1*). Exp/Max indicates the expression value at each time point normalized to the maximum expression across the time series.

correlation of each gene against all others (21) (Fig. 2A). If the clock is intact, then *ARNTL* should correlate positively with its partner transcriptional activator, *NPAS2*, and negatively with its target, *NR1D1*, which represses *ARNTL*. As expected, we saw strong positive correlations among ROR/REV-ERB-response element (RORE) targets *ARNTL*, *NPAS2*, and *CLOCK* and strong negative correlations between these genes and their E-box targets *DBP*, *NR1D1*, and *PER3* (Fig. 2A and B).

To reconstruct the daily rhythm of genome-wide gene expression in human epidermis, we applied CYCLOPS (*Materials and Methods*). We first evaluated the accuracy of the reconstructed sample order by comparing clock gene phase order between humans and mice (Fig. 2C). By both visual inspection and statistical analysis (Fisher's circular correlation, 0.715), the reconstruction of the human circadian cycle (Fig. 2C, outer circle) matched that of the mouse (Fig. 2C, inner circle). In addition, we compared CYCLOPS-predicted sample phase for the 20 subjects for which sampling time was available. The strong linear relationship between the mean CYCLOPS-predicted phase and sampling time suggests accurate sample ordering (Fisher's circular correlation, 0.955; Fig. 2D). As expected, the interindividual circadian phase variability was also reflected in the CYCLOPS-predicted phase (e.g., samples



**Fig. 2.** Evaluation of circadian function in human epidermis. (A) Heat map of Spearman's  $\rho$  for clock and clock-associated genes from ordered data ( $n = 20$ ; *SI Appendix, Table S1*) and unordered data ( $n = 219$ ; *SI Appendix, Table S1*) showing a conserved correlation structure. (B) Examples of clock genes with positively correlated (*ARNTL* and *NPAS2*, red) and negatively correlated (*ARNTL* and *PER3*, blue) expression. Each point represents one human sample. (Inset) Expression profiles of corresponding clock genes from mouse telogen. (C) CYCLOPS was used to order all 298 human samples. In an intact clock, ROR-phased genes (e.g., *ARNTL*, *NPAS2*, *CLOCK*) always peak before E-box-phased genes (e.g., *NR1D1*, *DBP*, *PER1*). Conserved phase relationships are shown for clock and clock-associated genes (internal circle, mouse; external circle, human). For human genes, pink indicate strong cycling ( $P < 0.01$ ,  $r_{AMP} > 0.1$ ,  $rsq > 0.1$ ) and cyan depicts weaker cycling. The phase of *Arntl* (mouse) or *ARNTL* (human) is set as 0 to facilitate comparison. (D) CYCLOPS accurately recalls circadian phase from 20 subjects. Different colors indicate different subjects, and the circular average phases for all samples is shown in gray. Samples from subjects 116 and 119 are indicated by triangle points.

from subject 116 and 119 were separated from the mean phase at each time point; Fig. 2D).

**Human Transcriptional Rhythms Are Evolutionarily Conserved.** We expanded our analysis of rhythms in human epidermis to the broader transcriptome from 298 samples collected from 238 donors. Using the CYCLOPS modified cosinor regression algorithm, we selected 188 circadian genes [ $P < 0.01$ , relative amplitude ( $r_{AMP}$ )  $> 0.1$ , goodness-of-fit ( $rsq$ )  $> 0.1$ ,  $fitmean > 16$ ] (Fig. 3A, *SI Appendix, Fig. S4*, and *Dataset S1*). While RORE and E-box phases had dominant signatures, other phases were represented as well (Fig. 3A). Our central hypothesis is that high-amplitude oscillatory genes with conserved phase relationships across species will be the most robust biomarkers. Comparing periodic genes from mouse telogen, we defined five categories of genes, including those with (i) no mouse homologs, (ii) low amplitude and no significant cycling in mouse, (iii) high amplitude and no significant cycling, (iv) low amplitude and cycling, and (v) high amplitude and cycling (Fig. 3B). For those genes that were robustly cycling in mouse telogen, we compared their phases and found a strong linear correlation for most (Fig. 3C). These evolutionary conserved clock and clock output genes suggest the improved power of a hybrid design compared with a longitudinal design.

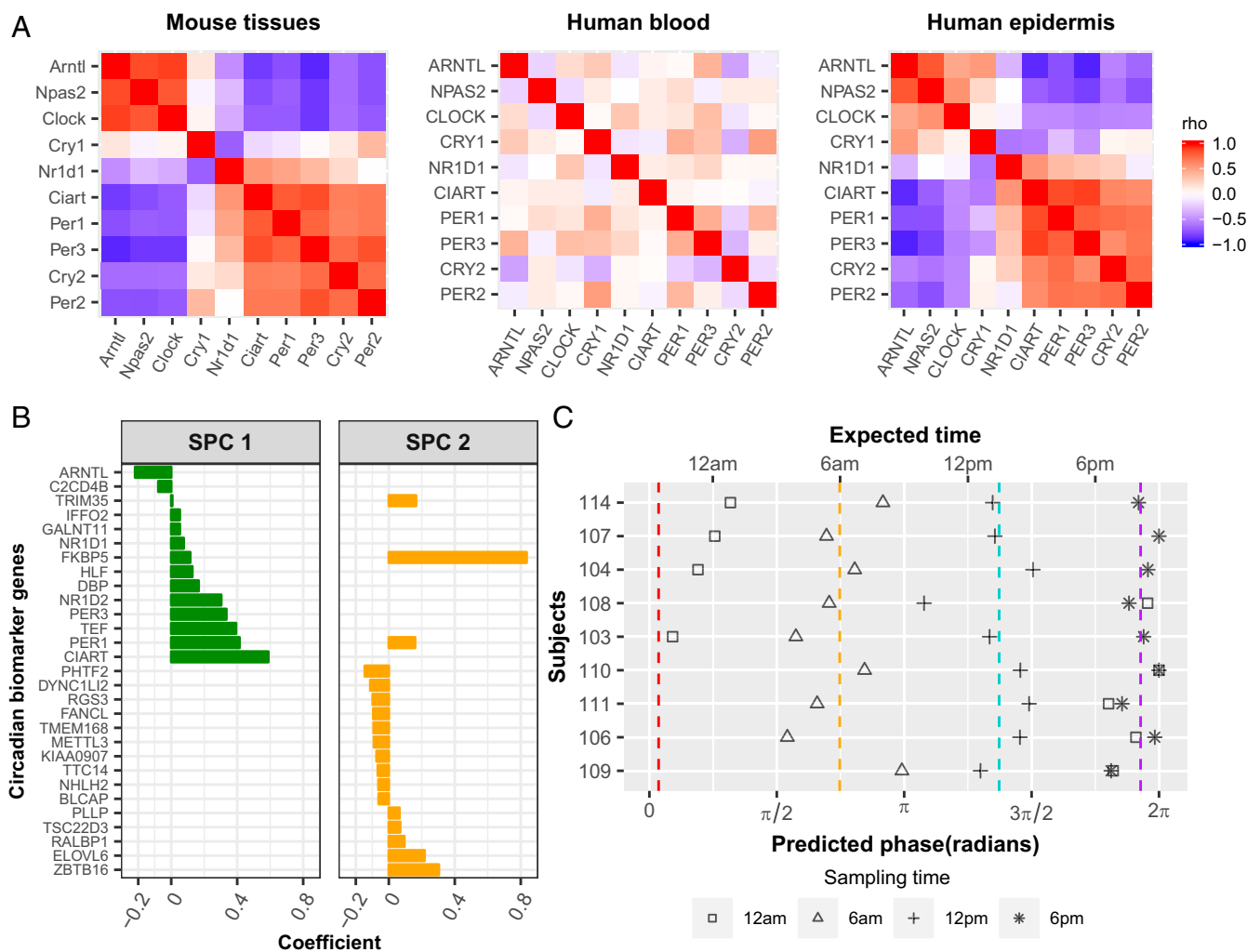
We investigated the underlying biology using phase set enrichment analysis (22) and identified temporal regulation of eight significantly enriched pathways at the population level (*SI Appendix, Fig. S5A*). Three of these pathways are also enriched in mouse telogen skin (*SI Appendix, Fig. S5B and C*), including pathways related to the cell cycle, adaptive immune system, and matrisome. Interestingly, the phase order of the pathways is also conserved (*SI Appendix, Fig. S5C*), demonstrating the orderly progression of the skin's circadian gene expression program.

### Population-Level Biomarkers of Circadian Phase in Human Epidermis.

In addition to analyzing clock-regulated biology, these datasets provide an opportunity to discover robust biomarkers of circadian phase that persist at the population level. To date, studies performed on human whole blood suggest high variability and low-amplitude rhythms from mixed cell types (23). Furthermore, when Brown et al. (24) analyzed single cell types from blood, they found a significantly weaker autonomous oscillator in blood monocytes than in fibroblasts. To clarify which human tissue is a better source of circadian biomarkers, we compared the expression correlation matrices of 10 core clock genes (18–20, 25) from time-series samples of human whole blood ( $n = 201$ ) (26), skin epidermis ( $n = 79$ ), and multiple mouse tissues ( $n = 144$ ) (15). The coexpression pattern of these 10 core clock genes across 12 mouse tissues is similar (Mantel test,  $P = 3e-06$ ) to human epidermis, but not blood (Mantel test,  $P = 0.897$ ) (Fig. 4A). At population level, the coexpression pattern of clock genes is also stronger in epidermis (Fig. 2A) than in isolated monocytes or T cells collected from hundreds of individuals (*SI Appendix, Fig. S6 A and B*). This suggests that skin epidermis is a strong alternative tissue for identifying robust circadian biomarkers at the population level.

We used ZeitZeiger (27) to identify biomarkers that can accurately report circadian phase. ZeitZeiger was trained with CYCLOPS-assigned phase from all 219 samples without sampling time and 43 samples with sampling time (*Materials and Methods*). This resulted in a set of 29 candidate biomarkers (Fig. 4B), with approximately one-half expressed in two different phases, represented by two sparse principal components, SPC1 and SPC2 (Fig. 4B). To validate these biomarkers, we analyzed a set of nine human subjects each measured at four separate times of day. The average predicted phase was within 3 h at all four time points compared with the expected time (Fig. 4C). For six of the subjects,





**Fig. 4.** Population-level biomarkers of circadian phase from a single human epidermal sample. (A) Evaluation of circadian clock function from 12 mouse tissues (Left), human blood (Middle), and human epidermis (Right). The mouse data are from Zhang et al. (15) and are included as a benchmark. Red and blue indicate positive and negative Spearman's  $\rho$ , respectively. (B) ZeitZeiger (27) was used to select a set of 29 circadian marker gene candidates. (C) Validation of predicted markers. Using the circadian marker set and ZeitZeiger, we analyzed samples collected every 6 h over a circadian day for nine subjects who were excluded from the training set. Average predicted phases of samples collected at 12 AM, 6 AM, 12 PM, and 6 PM are indicated with red, orange, cyan, and purple dashed lines, respectively.

division. This process is key to epidermal homeostasis, as new cells replace those lost during turnover or injury (31).

Biomarkers capable of reporting circadian phase are essential for circadian medicine. Our data showing strong population-level rhythms in human epidermis suggest the epidermis as a source for single-sample circadian biomarkers suitable for population scale research. Recent studies have shown that the timing of medical interventions can impact patient response (10; reviewed in ref. 9). The current standard in the field, DLMO, is impractical and burdensome. The identification of a biomarker set that can accurately predict circadian phase to within 3 h from a single sample is a major step toward translating circadian medicine.

Numerous groups have sought circadian biomarkers from whole blood, including analysis of metabolites and gene expression (11, 12, 32). However, whole blood is a heterogeneous mixture that changes composition in response to diet, infection, exercise, and many other factors, likely explaining the weak rhythmic expression of clock genes in time-series whole blood samples (23). Analysis of publicly available datasets including hundreds of subjects showed that the oscillator is far stronger in human epidermis than in isolated T cells or monocytes (SI Appendix, Fig. S6A–C); however, an

excellent recent study identified promising circadian biomarkers from isolated monocytes from young subjects (13). This raises the possibility that certain blood cell types may be a useful source of biomarkers. There are several important differences between our present study and that previous study, however, including varying gene expression platforms and experimental designs. We analyzed a larger population (239 subjects vs. 40 subjects) with a wider age range (20–74 y vs. 18–41 y), and validated the biomarker set against time points covering the entire circadian cycle (morning/noon/afternoon/midnight vs. morning/afternoon). Head to head comparisons are needed to define the best source of biomarkers for each application of circadian medicine.

Clinical use of circadian biomarkers will require fast, cost-effective, and noninvasive sampling techniques (e.g., tape strip-based, hair follicle- or oral mucosa-based) (33) and standardization across platforms (e.g., qPCR or NanoString). Larger studies would also benefit from a comparison with other standardized techniques (e.g., DLMO, temperature, actigraphy). Finally, the consistency of results should be confirmed across a range of disease states and pathologies. Solving these problems will help unlock the potential of circadian medicine.

## Materials and Methods

**Human Clinical Experimental Design and Sample Collection.** Our clinical studies adhered to the guidelines of the International Council on Harmonization of Good Clinical Practices and the principles expressed in the Declaration of Helsinki. Associated protocols were approved by Aspire's Institutional Review Board, and each subject provided informed consent. For collecting the ordered samples, 20 healthy Caucasian male subjects, aged 21–49 y, were recruited and housed in a facility that specializes in sleep studies (Community Research). Subject inclusion criteria and rules of Community Research are described in detail in *SI Appendix, SI Methods*. Saliva samples were collected every 3 h over the same 24-h period to confirm normal cycling of cortisol and melatonin (*SI Appendix, Figs. S8 and S9*). Full-thickness (2 mm) punch biopsy specimens were collected from sites on the forearms of each subject at 6-h intervals over a 24-h period (6 AM, 12 PM, 6 PM, and 12 AM). The biopsy specimens were processed for separation into epidermal and dermal compartments by laser capture microdissection (*SI Appendix, SI Methods*). Collection of the unordered samples from 152 Caucasian and 67 African-American females has been described previously (34). The mRNA target labeling and processing steps are described in detail in *SI Appendix, SI Methods*.

**Time-Series Analysis with MetaCycle.** The RMA algorithm was used to extract expression profiles from the raw CEL files. MetaCycle (35) was used to detect circadian genes ( $P < 0.05$  and  $rAMP > 0.1$ ) from time-series expression profiles of human epidermis samples and mouse anagen and telogen samples (7), as detailed in *SI Appendix, SI Methods*.

**Ordering Human Epidermal Samples with CYCLOPS.** Before CYCLOPS ordering, we verified the presence of an intact circadian clock in human epidermis. To do this, we constructed pairwise gene correlation matrices for 17 clock and clock-associated genes, using a method similar to that described by Shilts et al. (21). Our criteria for selecting clock and clock-associated genes were high-amplitude rhythms in most (8 of 12) mouse tissues from Zhang et al. (15) and

coverage over multiple phases of expression (driven by E-box, D-box, and RORE elements) (17–20). Next, all skin samples (298 in total) were ordered with CYCLOPS (Julia version 0.3.12) (14). To select eigengenes for CYCLOPS, we incorporated features of a second algorithm, Oscope (36), to filter periodic out-of-phase eigengenes. The implementation of CYCLOPS and Oscope are described in detail in *SI Appendix, Methods*. Using the best sample ordering, modified cosinor regression was applied to each expressed gene. Genes with  $P < 0.01$ ,  $fitmean > 16$ ,  $rAMP > 0.1$ , and  $rsq > 0.1$  were designated as circadian.

**Using ZeitZeiger to Identify Epidermal Biomarkers of Circadian Phase.** ZeitZeiger (27) was used to identify circadian biomarkers from CYCLOPS-ordered human epidermis samples. All samples were divided into two datasets: testing and training. The testing set included 36 time-stamped samples from nine subjects (each subject with four samples). Clock genes in these nine subjects followed the mean expression profile of all subjects (Fig. 1). These nine subjects were selected for testing because they had CYCLOPS-predicted phases surrounding the mean phase at each time point (Fig. 2D), enhancing the ability to evaluate prediction accuracy. The training set included all 219 samples without sampling time and the remaining 43 time-stamped samples collected from 11 subjects. Circadian biomarkers were selected from the training dataset and used for predicting sample phases in the test set, as detailed in *SI Appendix, Methods*.

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