**TIMP3 is a CLOCK-dependent diurnal gene that inhibits the expression of UVB-induced inflammatory cytokines in human keratinocytes**

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**ABSTRACT:** As the outermost physical barrier of an organism, the skin is diurnally exposed to UV radiation (UVR). Recent studies have revealed that the skin exhibits a circadian rhythm in various functions, and this oscillation is disturbed and reset via a strong environmental cue, the UVR. However, a molecular link between circadian perturbation by UVR and UVR-induced cellular responses has not been investigated. We identified tissue inhibitor of metalloproteinase (TIMP)-3 as a novel circadian locomotor output cycles kaput (CLOCK)–dependent diurnal gene by using a CLOCK–knockdown strategy in human keratinocytes. Among dozens of identified transcripts down-regulated by CLOCK knockdown, TIMP3 displayed a rhythmic expression in a CLOCK-dependent manner, in which the expression of matrix metalloproteinase (MMP)-1 and inflammatory cytokines, such as TNF-α, chemokine (C-X-C motif) ligand (CXCL)-1, and IL-8, were inversely regulated. Upon UVB exposure, the expression of CLOCK and TIMP3 was down-regulated, which led to an up-regulation of secretion of MMP1 and TNF-α proteins and in the transcription of CXCL1 and IL-8 via CCAAT-enhancer binding protein (C/EBP)–α. UVB-induced TNF-α secretion increased further or decreased by knockdown or overexpression of TIMP3, respectively, as well as by CLOCK. As a novel CLOCK-dependent diurnal gene, TIMP3 inhibits the expression of inflammatory cytokines that are up-regulated by UV irradiation in human keratinocytes. Thus, our work suggests a molecular link between circadian perturbation by UVR and UVR-induced inflammation.

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**KEY WORDS:** circadian rhythm · skin inflammation · MMP1 · TNF-α · chemokine

**ABBREVIATIONS:** ADAM, a disintegrin and metalloproteinase; ASAMTS, ADAM thrombospondin motif; BMAL1, brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like; C/EBP, CCAAT-enhancer binding protein; ChIP, chromatin immunoprecipitation; CLOCK, circadian locomotor output cycles kaput; CXCL, chemokine (C-X-C motif) ligand; EU, ethynyl uridine; KBM, keratinocyte basal medium; MIF, macrophage inhibitory factor; MMP, matrix metalloproteinase; NHEK, Neonatal human epidermal keratinocyte; qPCR, quantitative PCR; siRNA, small interfering RNA; TACE, TNF-α-converting enzyme; TIMP, tissue inhibitor of metalloproteinase; U2OS, unsynchronized human osteosarcoma cells; UVR, UV radiation; ZT, zeitgeber time

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Most mammals have a central clock system that is regulated by neurons in the suprachiasmatic nucleus and synchronized by light (1). This central clock orchestrates a daily oscillation of various physiologic functions, including body temperature, blood pressure, oxygen consumption, and metabolic rate, by coordinating clock systems located in peripheral tissues through hormones and neuronal signals (2–4). At the molecular level, circadian oscillation of core clock genes, including circadian locomotor output cycles kaput (CLOCK), brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like (BMAL1), period, cryptochrome, retinoid-related orphan receptor, and reverse orientation c-erbA (REV-ERB) gene, is regulated via autoregulatory transcriptional-translational feedback loops (1, 5). Circadian patterns can also be regulated by the phosphorylation, interactions, and subcellular localization of clock proteins (1, 6).

The skin has become a model system for studying circadian clock regulation of cell proliferation, stem cell differentiation, aging, tissue regeneration, hair growth, and pigmentation (7, 8). Human skin shows circadian
variations in many functions, such as transdermal water loss, pH, blood flow, barrier recovery, sebum secretion, itch, and irritation (9–12). Similar to other tissues, skin cells and skin tissue possess circadian machinery that work with the central clock, either cooperatively or autonomously (8, 12–16). Environmental cues to which the skin is exposed, such as UV radiation (UVR), pathogens, humidity, and temperature, can affect the skin’s circadian clock. Up to 10% of genes are estimated to show circadian expression in particular tissues, including the skin (14, 17, 18). Of those, Krüppel-like factor (KLF)-9 and aquaporin (AQP)-3 have been reported to be circadian regulatory factors that control epidermal proliferation and differentiation (19) and epidermal hydration (20), respectively. Psoriasis-like skin inflammation, diminished barrier function, and skin aging are also known to be worsened by disturbed circadian rhythms of core clock genes or clock-controlled genes (21, 22), suggesting a molecular link between the circadian clock and skin physiology.

As the outermost barrier of an organism, the skin is diurnally exposed to UVR, which is the most characterized causative factor for inducing DNA mutations and altered gene expression patterns. Upon exposure to UVR, human keratinocytes up-regulate the expression of inflammation- and stress-related genes, but down-regulate metabolism- and adhesion-related transcripts (23). Moreover, circadian clock genes have been shown to be initially down-regulated by a low dose of UVB but to recover after certain time intervals (24), suggesting that UVR-induced alteration of clock genes affects the expression of clock-controlled genes in human keratinocytes and promotes UVR-initiated disturbances in normal functions. Therefore, we searched for clock-controlled genes using a CLOCK knockdown strategy and 2-time-point sampling in 50% horse serum–synchronized human keratinocytes. We identified dozens of transcripts that were down-regulated by CLOCK knockdown. Among them, tissue inhibitor of metalloproteinases (TIMP)-3 was selected as a CLOCK-regulated and UVR-down-regulated gene. TIMP3 displayed a rhythmic expression in a CLOCK-dependent manner, in which the expression of MMP1 and inflammatory cytokines, such as TNF-α, CXCL1, and IL-8, was inversely regulated. CLOCK and TIMP3 proteins were decreased by UVR irradiation in a reconstituted human skin model, and UBV-induced TNF-α secretion was inversely regulated by the expression levels of TIMP3, as a CLOCK-dependent diurnal gene. Therefore, TIMP3 may be an important factor for maintaining epidermal homeostasis under UVR conditions by down-regulating the expression of inflammatory factors.

MATERIALS AND METHODS

Cell culture, synchronization, and UV irradiation

Neonatal human epidermal keratinocytes (NHEKs) were purchased from Lonza (Walkersville, MD, USA) and maintained in keratinocyte growth medium (KBM Gold) supplemented with BulletKit (Lonza). The cells were cultured at 37°C in a humidified incubator containing 5% CO2. For synchronization, NHEKs were grown until they became confluent. The growth medium was exchanged with serum-rich medium [i.e., keratinocyte basal medium (KBM) supplemented with 50% horse serum (Thermo Fisher Scientific, Waltham, MA, USA)]. The cells were incubated for 2 h, and the medium was subsequently replaced with KBM supplemented with antibiotics GA-1000. Cells were collected every 4 or 12 h after synchronization to examine the RNA and protein expression of each gene. For UV irradiation, cells were treated with 20 mJ/cm² UVB, with Bio-Sun UV-H (Vilber Lourmat, Marne-la-Vallée, France) and then returned to fresh KBM for culture. The cells were harvested every 4 or 12 h after UVB irradiation to analyze the mRNA and protein expression of each gene.

Quantitative PCR

Total RNA was isolated with Trizol reagent (Thermo Fisher Scientific), and 2 μg total RNA was used to synthesize cDNA with a reverse transcription kit (Thermo Fisher Scientific). Gene expression analyses were performed with TaqMan Universal Master Mix and TaqMan Gene Expression assays (Thermo Fisher Scientific) in a 7500 Fast Real-Time PCR system (Thermo Fisher Scientific) according to the manufacturer’s instructions. The 60S ribosomal protein L13a was used to normalize variations of cDNA quantities synthesized from different samples. Relative differences in gene expression were calculated from Ct values (25). The genes and corresponding Taqman probes used in the Taqman Gene Expression assays were as follows: ribosomal protein L13a: Hs04194366_g1, CLOCK: Hs00231857_m1, BMAL1: Hs00154147_m1, TIMP3: Hs00165949_m1, CXCL1: Hs00605382_gH, IL-8: Hs00174103_m1, thrombospondin (THBS)-1: s00962908_m1, late endosomal/lysosomal adaptor, MAPK, and MTOR activator -1: Hs00215524_m1, salt-inducible kinase-1: Hs00545020_m1, open reading frame kinase-3: Hs01566923_m1, TIMP1: Hs00171558_m1, TIMP2: Hs00234278_m1, and TIMP4: Hs00162784_m1.

Nascent RNA synthesis assay

A nascent RNA synthesis assay was performed using the Click-iT Nascent RNA Capture Kit (Thermo Fisher Scientific). Cells were cultured at 50–60% confluence in 6-well plates and synchronized with 50% horse serum–rich medium for 2 h. At 20 h after synchronization [at zeitgeber time (ZT) 20], the cells were treated with 20 mJ/cm² UVB. To label the nascent mRNAs, 0.2 mM 5-ethyluridine (EU) was added to the cell medium and incubated for 4 h. EU-total RNA was isolated with Trizol reagent, and 10 μg EU-total RNA was reacted with 1 mM biotin azide for the click reaction, according to the manufacturer’s instructions. Biotinylated RNA was captured with Dynabeads MyOne Streptavidin T1 magnetic beads from the Click-iT Nascent RNA Capture Kit (Thermo Fisher Scientific). Then, cDNA synthesis was performed by capturing nascent mRNA for further assays.

siRNA transfection

NHEKs in one well of a 6-well plate were transiently transfected with 25 pmol of Silencer Select small interfering RNA (siRNA) (Thermo Fisher Scientific) with Lipofectamine RNAiMax (Thermo Fisher Scientific), according to the manufacturer’s instructions. After incubation for 8 h, the cells were incubated in the growth medium overnight, synchronized for 2 h with serum-rich medium, and collected for the analysis of RNA or protein expression every 4 or 12 h.

RNA sequencing data analysis

The cells were transfected with scrambled, CLOCK, or BMAL1 siRNAs for 8 h and incubated overnight in growth medium. The
cells were then synchronized with serum-rich medium for 2 h and harvested at ZT 32 and 44. Total RNAs were extracted with Trizol reagent (Thermo Fisher Scientific), and poly-A-containing mRNAs were purified and converted into a cDNA library for subsequent cluster generation and DNA sequencing, according to the instructions of the TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA). Quality control checks on raw sequence data were performed by the FastQC program. For the data analysis, TopHat, Cufflinks, BWA, SamTools, Annovar, and DeFuse programs were used.

**Overexpression vector transfection**

NHEKs in one well of a 6-well plate were transiently transfected with 1 µg of pcDNA3.1 vectors with Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer’s instructions. After incubation for 6 h, the medium was replaced with the growth medium, and the cells were incubated overnight. The cells were synchronized in serum-rich medium, UVB irradiated at ZT 20 by using Bio-Sun UV-H (Vilber Lourmat), and collected for the analysis of protein expression at various time points.

**Western blot analysis and zymography**

The cells were lysed with RIPA lysis buffer (Millipore-Sigma, Billerica, MA, USA) containing protease inhibitors (MilliporeSigma). Protein concentration was determined by BCA assays, and 30 µg cell lysates were resolved by SDS-PAGE on 4-12% gradient Bio-Tris gels, transferred to nitrocellulose membranes (Thermo Fisher Scientific), and probed with each of the following antibodies: anti-CLOCK, anti-BMAL1, anti-TIMP3, anti-C/EBP-α, anti-C/EBP-β, anti-TNF-α, anti-NF-kB, anti-phospho-NF-kB, and anti-IκB (Cell Signaling Technology, Beverly, MA, USA); anti-MMP-1 (courtesy provided by Prof. Jin Ho Chung, Seoul National University College of Medicine, Seoul, South Korea); and GAPDH and horseradish peroxidase–conjugated secondary anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA). Western blotting luminol reagent (Santa Cruz Biotechnology) was used to develop the signals. For zymogram analysis, the cultured medium was harvested every 4 or 12 h after synchronization or UV irradiation and loaded on 10% Zymogram (gelatin) Protein Gels (Thermo Fisher Scientific). The gels were incubated at 37°C overnight and stained with 0.5% Coomassie blue (Millipore-Sigma) according to the manufacturer’s instructions.

**TCA precipitation**

To concentrate the proteins in cultured medium, 0.1 volume of 6.1 N trichloroacetic acid (Millipore-Sigma) was added to the culture medium. After incubation on ice for 30 min, the medium was centrifuged at 13,000 rpm for 15 min. The supernatant was removed, and the pellet was resuspended with 1× sample buffer (a mixture of SDS-PAGE staining buffer and 1 M Tris-HCl (pH 8.0)) and subjected to immunoblot analysis.

**Cytokine array and ELISA**

Culture medium was collected at various ZT points and centrifuged at 10,000 rpm for 3 min. The supernatants were used for multiple cytokine measurements (36-plex panel) with a Human Cytokine Array Panel (R&D Systems) according to the manufacturer’s instructions. ELISA assays for MMP-1, CXCL1, and IL-8 were performed with a DuoSet ELISA system (R&D Systems).

**Construction of a reconstituted human skin model**

A customized skin equivalent consisting of dermal and epidermal layers was developed. The major fabrication steps of engineered skin include the construction of the cellular layer containing fibroblasts for a dermal layer, seeding of keratinocytes on the dermal layer, and proliferation and differentiation of the epidermal layer. Through the above procedure, the dermal layer was cultured in a medium for 7 d at 37°C in 5% CO2 and the epidermal layer was seeded with human epidermal neonatal keratinocytes on the dermal layer. To induce keratinocyte proliferation, the skin equivalents were then submerged in a culture medium for 2 d. The skin equivalents were completed by air exposure to induce epidermal differentiation. Before UVB irradiation, the skin equivalents were exposed to air for 14 d and then irradiated with a UVB dose of 30 or 60 mJ/cm². The dermal tissues were harvested 24 h after UVB irradiation for histochemical staining.

**Immunohistochemistry**

Unfixed reconstituted human skin samples were embedded in resin (Shandon Cryomatrix; Thermo Fisher Scientific), frozen in liquid nitrogen, and sectioned at 10 µm thickness (CM1950; Leica, Wetzlar, Germany). Replicated cryosections were stained by immunohistochemistry with rabbit antibodies: CLOCK, TIMP3, C/EBP-α (all from Abcam, Cambridge, United Kingdom), as the primary antibody at 4°C overnight. Then, horseradish peroxidase–conjugated donkey anti-rabbit IgG (Abcam) or Alexa Fluor 488-conjugated goat anti-Rabbit IgG (Thermo Fisher Scientific) was applied as the secondary antibody for 1 h at room temperature. Brown immunoreactivity was visualized with 3, 3′-diaminobenzidine as a chromogen, and fluorescent reactivity was evaluated under a microscope (BX53; Olympus, Tokyo, Japan) equipped with a reflected-fluorescence system. Photomicrographs were taken with the microscope’s digital camera (DP72; Olympus).

**Transcriptomic and bioinformatics analysis**

The Protein Atlas database (http://www.proteinatlas.org) was used to select target genes showing ample expression of mRNA in the transcriptomic data (Supplemental Table 2). Bioinformatics analysis was performed using the GeneCard database (http://www.genecards.org).

**Statistics analysis**

The single cosinor method was used to analyze the circadian rhythm of the core clock genes (CLOCK and BMAL1) and TIMP3 (26). We used Cosinor software (v.3.1; Refinetti R. Circadian Software: http://www.ciradiang.org/software.html). Each dataset was tested for circadian rhythm by the fit of a 24-h single-cosine model. Resulting values, such as the P value, mesor, amplitude, and acrophase from each dataset were used to detect and compare the rhythmicity. Each value for CLOCK/BMAL1/TIMP3 was as follows: mesor, 1.0338/0.4324/5.437; amplitude, 0.1445/3.142/2.67; and degrees, 11.68/9.45/17.78 h (Figs. 1A, B, and 2B). Significance values for the rest of the data, except the cosinor analysis, were calculated with the 2-sample Student’s t test.

**RESULTS**

**Steady-state expression of clock genes is altered by UVB irradiation**

The skin is diurnally exposed to UVR, which damages the human epidermis, leading to various cellular responses,
including inflammation. We examined the expression of clock genes in human epidermal keratinocytes upon UVB exposure, to test whether UV-induced inflammatory responses are regulated by local clock genes. Keratinocytes were synchronized by incubation in a medium containing 50% horse serum for 2 h (27), where this time point was designated ZT 0. Next, cells were harvested at various ZT points, before or after UVB exposure. Under normal conditions, CLOCK mRNA was stably expressed over time (cosinor; \( P = 0.124787 \)) (Fig. 1A). However, the CLOCK mRNA expression pattern was disturbed by UVB irradiation, and this pattern was reflected by altered CLOCK

![Graphs showing CLOCK and BMAL1 mRNA expression](image1)

**Figure 1.** The steady-state expression of clock genes is altered by UVR. Human epidermal keratinocytes were synchronized with serum-rich medium for 2 h, irradiated with UVB (20 mJ/cm²) at the indicated ZT points, and harvested every 4 h, along with the nontreated control. A, B) The mRNA expression of CLOCK (A) and BMAL1 (B) was examined by qPCR. C) The cells were irradiated with UVB at ZT 20 and harvested at the indicated time points, along with the nontreated control. The protein expression of CLOCK and BMAL1 was examined by Western blot analysis with anti-CLOCK and -BMAL1 antibodies. Two independent Western blot analyses were performed, and representative images are shown. GAPDH was used as a loading control. D) Nascent mRNA synthesis of CLOCK and keratin 10 (K10) during ZT 20–24 was analyzed by qPCR. The data are presented as means ± SD of 2 independent experiments.
Figure 2. TIMP3 expression is regulated by CLOCK. A) The cells were transfected with CLOCK siRNAs for 8 h then stabilized in growth medium overnight. Then, cells were synchronized in serum-rich medium for 2 h and harvested at ZT 32 and 44. The down-regulated transcripts were analyzed through RNA-Seq. After considering basal mRNA expression and denoted protein expression (Protein Atlas), 11 transcripts were selected.

B) The synchronized keratinocytes were treated with scrambled control or CLOCK siRNA (si-CLOCK) and harvested every 4 h for the analysis of TIMP3 mRNA expression by qPCR (B) or every 8 h for the analysis of CLOCK and TIMP3 by Western blot (C). Densitometry analyses of TIMP3 protein expression over GAPDH (continued on next page)
protein levels (Fig. 1C and Supplemental Fig. 1D). CLOCK transcript levels were immediately and significantly down-regulated, regardless of time and repeated UVB treatment, but they eventually recovered to baseline expression levels (Fig. 1A and Supplemental Fig. 1A, B). Another representative clock gene, BMAL1, showed a clear circadian rhythm in both mRNA (cosinor, $P = 0.000224$) and protein expression under normal conditions (Fig. 1B, C and Supplemental Fig. 1D). Upon UVB exposure, BMAL1 expression patterns were different, depending on the treatment scheme (ZT 8/32 vs. ZT 20/44) (Fig. 1B and Supplemental Fig. 1C). Treatment with UVB at ZT 20 and 44 caused a shift in the peak time of mRNA expression (Fig. 1B) and a severe reduction in protein levels (Fig. 1C and Supplemental Fig. 1D). Given the maximum effects of UVB on CLOCK and BMAL1 expression, the experimental conditions of UVB treatment at ZT 20, as well as consecutive harvesting of cells every 4 or 12 h, were selected to study the effects of clock or clock-controlled genes on UV-induced cellular responses. Compared with keratin 10 (K10) expression, which did not display de novo transcript levels altered by UVB (Fig. 1D), CLOCK mRNA was dramatically reduced because of the lack of nascent RNA synthesis after UVB treatment.

**TIMP3 expression is regulated by CLOCK**

To search for the genes affected by CLOCK expression levels in human keratinocytes, we introduced siRNAs against CLOCK and harvested the cells at 2 time points, ZT 32 and 44, when BMAL1 expression was highest (ZT 32) and lowest (ZT 44) (Fig. 1B). We speculated that those genes that were down-regulated at both ZT points must be influenced by CLOCK expression levels. We performed a transcriptomic analysis of genes through RNA-sequencing and identified 61 transcripts that were down-regulated at both ZT points (ZT 32 and 44) (Fig. 1B). We speculated that those genes that were down-regulated at both ZT points must be influenced by CLOCK expression levels. We performed a transcriptomic analysis of genes through RNA-sequencing and identified 61 transcripts that were down-regulated at both ZT points (Fig. 2A and Supplemental Table 1). Among the 61 common genes, we selected 11 target genes that showed ample expression of mRNA in the transcriptomic data and in the Protein Atlas database (Supplemental Table 2). Excluding keratins and low-expressing genes, the down-regulation of mRNA levels of putative CLOCK-controlled genes upon CLOCK siRNA treatment was confirmed by real-time quantitative PCR (qPCR) (Supplemental Fig. 2). Of those, only TIMP3 mRNA showed a periodic expression pattern over time (cosinor, $P = 0.002399$), which disappeared with CLOCK siRNA treatment (cosinor, $P = 0.069285$; Fig. 2B).

Other TIMP family members, including TIMP1 and 2, did not display rhythmic expression and were not down-regulated by CLOCK siRNA treatment (Supplemental Fig. 3). Although TIMP4 expression seemed to be rhythmic and was down-regulated by CLOCK siRNA treatment, the expression level was very low, so that it was not conclusive (Supplemental Fig. 3). These findings indicate that TIMP family members exhibited distinct expression patterns and that TIMP3 was a potent candidate regulated by CLOCK in human keratinocytes. Consistent with the down-regulated TIMP3 mRNA levels over time, TIMP3 protein levels were obviously decreased by CLOCK siRNA treatment, showing the enhanced effects over time (Fig. 2C). We examined whether TIMP3 mRNA expression was modified by UVB treatment, whereas the mRNA and protein expression of CLOCK was decreased (Fig. 1A, C). We confirmed that TIMP3 expression was reduced by UVB treatment (Fig. 2D) and that the reduction was associated with the lack of synthesis of new mRNA (Fig. 2E).

**Down-regulation of TIMP3 enhances the expression of MMP1 and inflammatory cytokines**

TIMP3 has a broad inhibition profile for matrix metalloproteinases (MMPs) and is also a specific inhibitor of a disintegrin and metalloproteinase (ADAM)-17, which is known as a TNF-α converting enzyme (TACE) and cleaves membrane-bound TNF-α into its active soluble form (28–30). MMPs and TNF-α have been implicated in UV-induced photoaging and inflammatory skin disorders, respectively (12, 28). Therefore, we examined whether TIMP3 regulates the expression and secretion of MMPs and TNF-α. Endogenous TIMP3 expression was efficiently knocked down with siRNAs for TIMP3, as validated by qPCR (Supplemental Fig. 4A). When TIMP3 was knocked down, secreted MMP1 protein levels, but not those of MMP2, increased significantly, compared with those of the scrambled control at each ZT point, as validated by Western blot analysis (Fig. 3A) and ELISA (Fig. 3B). Secreted TNF-α was also increased by TIMP3 siRNA treatment (Fig. 3A), suggesting that TACE is active because of the reduction of its inhibitor, TIMP3. TIMP3-dependent expression and secretion of MMP1 and TNF-α was also phenocopied by the knockdown of CLOCK (Supplemental Fig. 4B).

In addition to the induction of MMPs and TNF-α, UVB provokes inflammation by inducing inflammatory cytokines (29). To examine whether TIMP3 regulates the expression of inflammatory cytokines, we performed a cytokine array analysis using conditioned medium from scrambled control- or TIMP3 siRNA–treated keratinocytes. Five cytokines were detected in the conditioned media: chemokine (C-X-C motif) ligand 1 (CXCL1), IL-8, macrophage migration inhibitory factor (MIF), IL-1ra, and serpin E1 (Fig. 3C). Three of these cytokines were influenced by TIMP3 knockdown: CXCL1 and IL-8 were increased ~2.8- and 2-fold, respectively, whereas MIF was decreased 1.3-fold compared with the scrambled control (Fig. 3D). We examined the expression of the cytokines under normal conditions and confirmed that CXCL1 and IL-8 displayed expression patterns opposite that of TIMP3.

Control were performed with ImageJ, and the quantified data are presented as means ± SD of 3 independent experiments. $D, E$) The cells irradiated with UVB (20 mJ/cm²) at ZT 20 were harvested every 4 h, along with the nontreated control cells. The mRNA expression of TIMP3 was analyzed by qPCR (D). Nascent mRNA synthesis of TIMP3 during ZT 20–24 was analyzed with qPCR (E). The qPCR data are presented as means ± SD of 2 independent experiments.

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Figure 3. Down-regulation of TIMP3 enhances the expression of MMP1 and inflammatory cytokines. The cells were transfected with scrambled or TIMP3 siRNAs and harvested at the indicated ZT points. A) The secreted MMP1 and TNF-α were analyzed by Western blot after precipitation into the culture medium. MMP2 activity was analyzed by zymography and used as a control for MMP activity. Three independent analyses were performed, and representative images are shown. B) The secreted MMP1 was analyzed by ELISA. C) The blot image of cytokine array. The culture media were harvested at ZT 20 for cytokine array analysis. D) Each spot on the blot was analyzed by densitometry and quantified by percentage relative to the reference spot (RS). E) The cells were harvested at the indicated ZT points, and the mRNA expression of CXCL1 and IL-8 was analyzed by qPCR. The data are presented as means ± SD of 3 independent experiments. *P < 0.05, **P < 0.01.
showing the highest expression levels at ZT 32, a time point when TIMP3 expression levels were lowest (Fig. 2B and Supplemental Fig. 5A). Although MIF was affected by TIMP3 siRNA treatment, the decrease in its expression was small, and its expression pattern was not related to that of TIMP3 (Fig. 3D and Supplemental Fig. 5A). We further validated that the released protein levels of CXCL1 and IL-8 were increased by TIMP3 siRNA treatment at all ZT points, even though IL-8 showed only a mild effect at the protein level (Supplemental Fig. 5B). In agreement with the increased protein levels, CXCL1 and IL-8 mRNAs were significantly up-regulated by TIMP3 siRNA treatment at all ZT points tested (Fig. 3E). These results suggest that expression of CXCL1 and IL-8 in human keratinocytes could be negatively regulated by TIMP3 at the transcription level.

**Regulation of CXCL1 and IL-8 by TIMP3 is mediated by C/EBP-α**

Because TIMP3 is a secretory protein and not a transcription factor, we searched for mediators that could regulate mRNA expression of both cytokines in a TIMP3-dependent manner. We performed bioinformatics analyses by using the GeneCard database and selected NF-κB, NF-κB1, and C/EBP-α as the putative transcription factors for CXCL1 and IL-8 (Supplemental Fig. 6A). Among these, NF-κB and NF-κB1, especially the phosphorylated forms of each, were not affected by treatment with TIMP3 siRNAs (Supplemental Fig. 6B), indicating that these transcription factors did not mediate the increased expression of CXCL1 and IL-8 observed upon TIMP3 knockdown. In contrast to NF-κB and -B1, C/EBP-α was up-regulated by treatment with TIMP3 siRNAs, as well as with CLOCK siRNAs (Fig. 4A and Supplemental Fig. 6C). Moreover, when C/EBP-α was knocked down, the levels of released CXCL1 and IL-8 proteins were decreased 3- and 3.8-fold, respectively, compared with the scrambled control in the cytokine array analysis (Fig. 4B, C). These results indicate that C/EBP-α is a transcription factor with the potential to drive expression of CXCL1 and IL-8 upon TIMP3 knockdown and is negatively regulated by TIMP3 in human keratinocytes.

**TIMP3 and its downstream inflammatory factors are inversely expressed in UVB-irradiated conditions**

MMP1, TNF-α, and the cytokines CXCL1 and IL-8, which were defined as regulatory targets of TIMP3 in our study, have been implicated in the inflammatory responses of cells to UVR. We examined whether TIMP3 and its downstream regulatory targets display altered expression upon UVB exposure. Consistent with an earlier result from our study (Fig. 2D), the protein levels of TIMP3, as well as of CLOCK, were considerably decreased, but C/EBP-α levels were increased by UVB treatment compared with the nonirradiated control in a reconstituted human skin model and in human keratinocytes (Fig. 5A, B). Corresponding to the down-regulation of TIMP3, the protein levels of C/EBP-α, MMP1, and secreted TNF-α were increased by UVB treatment (Fig. 5B). The increase in C/EBP-α was accompanied by the up-regulated mRNA expression of CXCL1 and IL-8 (Supplemental Fig. 7), which led to the increase in protein levels of each (Fig. 5C, D, respectively). These results suggest that the expression profile of TIMP3 and its upstream CLOCK can be perturbed by UVB irradiation and that this perturbation is associated with the increased expression of its downstream inflammatory factors in cultured keratinocytes and in a reconstituted human skin model.

**The expression of UVB-induced inflammatory cytokines is regulated in a TIMP3-dependent manner**

To investigate whether the expression of UVB-induced inflammatory cytokines could be directly regulated by TIMP3 expression levels, we introduced siRNAs or overexpression plasmids for TIMP3 and determined the secreted levels of inflammatory cytokines under UVB conditions. UVB treatment significantly induced the secretion of TNF-α, CXCL1, and IL-8 proteins, and, compared with the nontreated control and UVB-induced secretion of these cytokines, they were increased further by knockdown of TIMP3 (Fig. 6A, C, E), but decreased by overexpression of TIMP3 (Fig. 6B, D, F). The expression of CXCL1 and IL-8 in nonirradiated conditions was also inversely regulated by TIMP3 levels (Fig. 6C–F). In addition, TIMP3-dependent regulation of UVB-induced cytokine release was phenocopied by the knockdown or overexpression of CLOCK and UVB-reduced TIMP3 levels and had a tendency to be further decreased by CLOCK knockdown or was rescued by its overexpression (Fig. 7), suggesting that TIMP3 and its upstream CLOCK predominantly participate in the expression regulation of inflammatory factors.

Finally, we propose a model of the relationships among CLOCK, TIMP3, and downstream effector molecules and the regulatory modes under the conditions of normal vs. UVB irradiation (Fig. 8): Normally, TIMP3 with CLOCK acts as an anti-inflammatory element by inhibiting the secretion of MMP1 and TNF-α and down-regulating CXCL1 and IL-8 via C/EBP-α. Upon UVB irradiation, the anti-inflammatory regulation mode was suppressed due to the down-regulation of CLOCK and TIMP3, which results in the up-regulation of inflammatory factors and would in part participate in UVB-induced skin inflammation. Therefore, TIMP3, as a CLOCK-dependent diurnal gene and a key mediator between core clock genes and cellular responses, could be an important regulatory factor in maintaining human epidermal homeostasis.

**DISCUSSION**

The transcription of core clock genes has been reported to be down-regulated by UVB exposure (24). This change has been thought to lead to alterations in the expression of their downstream gene targets. An oligonucleotide microarray
analysis showed that transcriptional responses of genes to UVR vary, depending on their function (23). For example, some genes related to cell adhesion and metabolism were down-regulated by UV irradiation, whereas genes involved in stress and inflammatory responses, mRNA splicing, proteasome function, and translation were up-regulated. We hypothesized that some of the UVR-altered gene profiles and consequent cellular responses were induced by the alteration of core clock and clock-controlled gene expression. Upon UVB exposure, constitutively expressed CLOCK mRNA was disrupted (Fig. 1A). The genes whose expression was disrupted by the condition that disrupts CLOCK expression may be mediators of the CLOCK signal. We determined that TIMP3 is a new CLOCK-dependent diurnal gene in human epidermal keratinocytes. TIMP3 inhibited the expression and secretion of MMPs, TNF-α, and the inflammatory cytokines CXCL1 and IL-8. In the skin, it is possible that UVR induces epidermal damage, including photoaging and inflammation, by inhibiting CLOCK and its downstream mediator TIMP3. This potential mechanism suggests the presence of a process in which UVB affects certain physiologic functions of skin cells through CLOCK-initiated and -controlled gene-mediated signal cascades. Based on

**Figure 4.** The regulation of CXCL1 and IL-8 by TIMP3 is mediated by C/EBP-α. A) The cells were treated with siRNAs against TIMP3 and harvested at the indicated ZT points. TIMP3 and C/EBP-α were analyzed by Western blot with specific antibodies. GAPDH was used as the loading control. Densitometry analyses of each protein expression over GAPDH control were performed, and the quantified data are presented as means ± sd of 3 independent experiments. B) Blot image of the cytokine array. The cells were treated with scrambled or CEBPA siRNAs, and the culture media were harvested at ZT 20 for cytokine array analysis. C) Each spot on the blot was analyzed by densitometry and quantified by the percentage (above each histogram) relative to the reference spot (RS).
Figure 5. TIMP3 and its downstream inflammatory factors are inversely expressed in UVB-irradiated conditions. A) A reconstituted human skin model was irradiated with UVB and fixed at 24 h for immunohistochemical analyses. The protein levels of CLOCK, TIMP3, and C/EBP-α were determined with each antibody. Scale bars, 25 μm. Control, nonirradiated samples. B–D) The cells were treated with UVB (20 mJ/cm²) at ZT 20 and harvested at the indicated ZT points. B) The protein expression of TIMP3 and C/EBP-α was analyzed by Western blot with specific antibodies. The secreted MMP1 and TNF-α proteins were analyzed by Western blot after precipitation into the culture media. MMP2 activity in zymography was used as a control for MMP activity. Densitometry analyses of each protein expression over GAPDH control were performed with the ImageJ program, and the quantified data are presented as the means ± SD of 3 independent experiments. The secreted protein levels of CXCL1 (C) and IL-8 (D) were measured with each ELISA kit. The data are presented as means ± SD of 3 independent experiments. *P < 0.05, **P < 0.01.
A rhythmic expression observed in human keratinocytes (Fig. 2B), TIMP3 is expressed to a high degree during the daytime when skin is frequently exposed to UVR. This expression pattern indicates that circadian regulation via CLOCK and TIMP3 is important for maintaining skin homeostasis in UVR exposure conditions.

Among 11 candidate genes down-regulated after CLOCK siRNA treatment, only TIMP3 showed a rhythmic expression pattern with a ~2-fold difference in amplitude between peak and trough, and this difference was significant, according to cosinor analysis (cosinor; $P = 0.002399$; Fig. 2B), suggesting rhythmic expression of TIMP3.
However, the oscillation amplitude of TIMP3 expression seemed to be relatively small when compared with that of BMAL1, showing more than a 3-fold difference between peak and trough in our experimental conditions (Fig. 1B), and with the previously reported clock genes BMAL1, period, cryptochrome, REVERBs, and o-box binding proline and acidic-rich basic leucine zipper transcription factor, which showed ~4-fold greater amplitude (30). According to a previous study in which unsynchronized human osteosarcoma cells (U2OS) were analyzed with a
chromatin immunoprecipitation (ChIP) assay, only 58 were rhythmically expressed among the thousands of genes containing circadian regulator binding sites on their promoters, and their oscillation amplitudes were mostly small (14, 30). The small amplitude of gene expression was associated with many factors, such as the expression level, the ratio of clock components, and the activity of noncircadian transcription factors. Even core clock genes display different oscillation amplitudes of expression, depending on the cellular state (14, 30) and other genes, except core clock genes, with rhythms oscillating with smaller amplitudes than those of core clock genes (29). TIMP3 was expressed at intermediate levels in human keratinocytes (Supplemental Table 2), which may be a reason why TIMP3 belongs to those with small amplitudes of oscillation. Consistent with our result showing that TIMP3 is a CLOCK-dependent diurnal gene, it was listed as one of the genes with a circadian expression pattern in telogen mouse skin, was down-regulated in Bmal1−/− mouse skin, and was observed among the circadian transcriptomes of other tissues (7, 14), supporting that TIMP3 in human epidermal keratinocytes could also be a diurnal gene sensitive to circadian regulation, possibly by CLOCK:BMAL1.

We further tested whether CLOCK could directly regulate the expression of TIMP3 at the transcriptional level. According to a report of ChIP sequencing performed in U2OS cells, CLOCK did not bind to the TIMP3 promoter (30). Consistent with this report, we did not detect a TIMP3 promoter sequence from CLOCK-immunoprecipitated chromatin complexes where the promoter sequence of the D-box binding proline and acidic-rich basic leucine zipper transcription factor, a representative clock-controlled gene, was detected (Supplemental Fig. 8A, B). Although we demonstrated a close relationship between CLOCK and TIMP3 by showing up- or down-regulated expression of TIMP3 by over- or reduced-expression of CLOCK, respectively (Figs. 2B, C, and 7), this regulation does not seem to be based directly on the ChIP assay (Supplemental Fig. 8A). With the result that TIMP3 was also down-regulated by BMAL1 knockdown (Supplemental Fig. 8C), we speculated that the rhythmic expression of TIMP3 is regulated through the circadian clock machinery, including a CLOCK:BMAL1 heterodimer or other regulatory loops affected by disrupted CLOCK activity (for example, in UV-irradiated conditions), rather than by CLOCK itself.

The TIMP family is composed of four members: TIMP-1, -2, -3, and -4. Although these belong to the same family, they exhibit distinct structural features, biochemical properties, and expression patterns (31). Their structural similarity is ~40%, and they have broader MMP inhibition profiles affecting matrix remodeling. However, their inhibition ability for ADAMs and disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) is more specific. Contrary to other TIMPs, TIMP3 has a broader inhibition profile for ADAM-10, -12, and -17 and ADAMTSs and can be sequestered to the extracellular matrix by binding to various proteoglycans (31, 32). In our study, TIMP3 was the only TIMP family member controlled by the CLOCK gene in human keratinocytes. The broad specificity and distinctive localization of TIMP3 may allow for it to have important roles in these tissues, and these unique features could be attributable to exclusive regulation by CLOCK, unlike other family members.

MMP1 and TNF-α/TACE were verified in our study, as direct inhibitory targets of TIMP3, and the cytokines CXCL1 and IL-8, as inhibitory transcriptional targets of TIMP3 through C/EBP-α down-regulation. The mechanism by which C/EBP-α was down-regulated by TIMP3 was not examined. The transcription of CXCL1 and IL-8 has been shown to be stimulated by primary cytokines, such as IL-1 or TNF-α (33, 34), suggesting that the up-regulation of CXCL1 and IL-8 is, in part, attributable to increased TNF-α under UVB conditions via TIMP3 down-regulation. IL-1 and TNF-α are increased in inflammatory human skin disorders, such as psoriasis, and induce a large set of cytokines, including CXCL1 and IL-8 (34). Thus, TIMP3 could be involved in inflammatory skin disorders in addition to UVB-induced inflammation by mediating circadian anti-inflammatory responses initiated by CLOCK. According to two reports, the defective Clock mutation caused conflicting effects on skin inflammation in two mouse models—an allergic contact dermatitis model and psoriasis-like skin inflammation model—with increased and reduced inflammation associated with a Clock mutation, respectively (21, 35). These results suggest that there could be different influences of Clock on inflammatory skin disorders, depending on their pathophysiology. In our experimental system, UVB-induced transcriptional alteration of CLOCK led to an increased inflammatory response, possibly through TIMP3.

In summary, we identified TIMP3 as a novel CLOCK-dependent diurnal gene that inhibits the expression of
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