Expression of the Circadian Clock Genes *clock* and *period1* in Human Skin

Susana B. Zanello, David M. Jackson, and Michael F. Holick

Vitamin D, Bone and Skin Laboratory; Departments of Medicine, Physiology and Dermatology; Division of Endocrinology, Diabetes and Metabolism; Boston University School of Medicine, Boston, Massachusetts, U.S.A.

The circadian clock is a cellular machine composed of proteins with regulated expression that gives rise to circadian rhythms. Two main new concepts have arisen from recent research in the field in the last few years: (i) at least three to five key genes are involved in maintaining the basic circadian cellular rhythms, and (ii) their expression is fairly ubiquitous, extending beyond the traditionally considered pacemaker in mammals, the suprachiasmatic nucleus. We have demonstrated the expression of two circadian clock genes, *clock* and *period1*, in human skin cells. Reverse transcriptase polymerase chain reaction revealed the presence of *clock* and *period1* mRNA in cultured human keratinocytes, melanocytes, and dermal fibroblasts, as well as in the human keratinocyte cell line HaCaT and the human melanoma line A375. In addition, antibodies to these two proteins produced immuno-positive staining in these cell types. Our investigations demonstrate for the first time that skin cells express circadian clock proteins constitutively although regulation of their expression and activity has not been elucidated. These proteins may have a role in cutaneous and/or systemic circadian biology and the skin and skin cells may provide an attractive model for the study of circadian rhythms.
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In the last few years there has been a renewed interest in circadian biology leading to the discovery of genes that appear to comprise the basic cellular machinery of circadian rhythms in a wide range of organisms. In mammals, the genes that constitute this internal timekeeping system or molecular clock include, among others, *period1 (per1)*, *period2 (per2)*, *period3 (per3)*, *clock*, and *b-mal* (King *et al*, 1997; Sun *et al*, 1997; Tei *et al*, 1997; Gekakis *et al*, 1998; Zylka *et al*, 1998; for a review see Weaver, 1998).

As elucidated in *Drosophila* sp., where homologs of these genes exist, the circadian clock's basic mechanism relies on negative feedback gene regulation (Darlington *et al*, 1998; Gekakis *et al*, 1998; Reppert, 1998; Schibler, 1998). The proteins CLOCK and bMAL form a complex that activates *per* and *timeless (tim)* transcription through binding to the E-box response element in their promoters, and PER and TIM proteins negative regulate their own gene expression. This sets a fundamental oscillation that, by secondary pathways, elicits cyclic events in other cellular targets.

The anatomic locus of the circadian oscillator in mammals is the suprachiasmatic nucleus (SCN) and all the clock genes have been shown to be expressed in the few thousand neurons that compose it. Ocular information is transmitted to the SCN via the retinohypothalamic tract, constituting the way in which the environmental cues may reach the pacemaker to entrain the circadian oscillator. Recent research suggests that the light receptors involved in this sort of transduction are independent from the visual pathways (Yang *et al*, 1998). Two blue-light photoreceptors, cryptochromes 1 and 2 (CRY1 and CRY2), have been proposed to function as circadian photopigments (Miyamoto and Sancar, 1998; Thresher *et al*, 1998).

Interestingly, the genes that compose the circadian clock have recently been found not only in the SCN in mammals or anterior ganglia in insects (Reppert *et al*, 1994; Sauman *et al*, 1996), but in peripheral tissues as well. The expression of *clock* has been demonstrated in mammalian (mouse) hypothalamus, brain (without the hypothalamus), eye, heart, lung, liver, kidney, testis, and ovary (King *et al*, 1997). *Per1* has been found expressed in brain, heart, lung, liver, skeletal muscle, kidney, and testis (Sun *et al*, 1997; Tei *et al*, 1997). PER2 and PER3 are also widespread (Albrecht *et al*, 1997; Zylka *et al*, 1998) and similar observations have been made for the cryptochrome genes *cry1* and *cry2* (Miyamoto and Sancar, 1998). Moreover, in *Drosophila*, the expression of the gene period has been shown to oscillate independently in explanted parts of the insect's body (Plautz *et al*, 1997). Thus, even though region(s) of the central nervous system have long been recognized as the master controller(s) of circadian rhythms, now evidence suggests that additional pacemakers are located in non-neural tissues.

More controversial, however, is the physiologic evidence supporting the above-mentioned hypothesis. There are observations that the human circadian response to light can be mediated through an extraocular phototransduction. According to one study, phase shifts in the circadian pattern of body temperature and melatonin levels seem to correlate with the exposure to certain light pulses restricted to the skin (Campbell and Murphy, 1998). Other attempts to show significant phase changes after irradiation of the skin have not been successful, however (Lamber, 1998), and light exposure in the popliteal region was unable to suppress the nocturnal plasma melatonin rhythm (Lockley *et al*, 1999). This notwithstanding, the skin is the organ that receives the greatest exposure to light, the main environmental stimulus for the

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Reprint requests to: Dr. Michael F. Holick, 715 Albany St. M-1013, Boston, MA 02118. Email: mfholick@bu.edu

Abbreviation: SCN, suprachiasmatic nucleus.

entrainment of the circadian clock, which added to the ubiquity of circadian clock proteins poses the possibility of a role for the skin in circadian rhythms. Although several studies have been done on the tissue distribution of clock genes, none have been reported for skin or skin cells.

We provide evidence for the expression of two circadian clock genes in cultured human skin cells. *Clock* and *per1* mRNAs were detected in keratinocytes, melanocytes, and dermal fibroblasts, as well as the corresponding gene products by immunocytochemical methods. Therefore, the skin is an important candidate for harboring a timekeeping mechanism that might act locally or interact with the central pacemaker.

MATERIALS AND METHODS

Cell culture Human keratinocytes from neonatal foreskins were cultured in serum-free conditions as previously described (Chen *et al*, 1995). Dermal fibroblasts from neonatal foreskins, the keratinocyte cell line HaCaT (human, high calcium, high temperature), COS-1 fibroblasts (monkey, renal), and the A375 melanoma cell line were cultured and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Primary human melanocytes were kindly provided by the Dermatology Department of Boston University School of Medicine.

Reverse transcription (RT) and polymerase chain reaction (PCR) Cells grown to 70%–80% confluency were lyzed in RNAzol reagent (Test-Tel., Friendswood, TX) according to the manufacturer's procedure to obtain total RNA. Four micrograms of total RNA was reverse transcribed at 42°C for 50 min plus 10 min at 50°C using oligo(dT) primer and Superscript II reverse transcriptase (Life Technologies, Rockville, MD) in a final volume of 20 μl. A 3 μl aliquot of this incubation mixture was taken for a 40 μl PCR using AmpliTaq Gold polymerase (Perkin Elmer, Wellesley, MA) under the conditions recommended by the manufacturer. The cycling pattern was as follows: a single initial 9 min incubation at 94°C; 30 cycles of (i) 45 s at 94°C, (ii) 45 s at 60°C, (iii) 80 s at 72°C; and one final extension step of 15 min at 72°C.

Cloning of PCR products was performed with the TOPO-Cloning kit according to the manufacturer's instructions (InVitrogen, Carlsbad, CA). Sequencing was carried out at the DNA and Protein Core Facility of Boston University School of Medicine.

Primers were selected to amplify specific regions of the human *clock* and *period1* genes. PER and CLOCK belong to the bHLH-PAS family of transcription factors, characterized by the PAS dimerization domain (Kay, 1997), and primers were chosen to encompass this region. Clock primers spanned a region of 728 bp from nucleotide 1321 to nucleotide 2049 of the *Homo sapiens* CLOCK sequence (accession number AF011568): (forward) 5′-GCAAAATGTCATGAGCACTTAATG-3′; (reverse) 5′-CTGCAG-CCCCTGACCATGGACC-3′. A second set of clock primers spanned a 138 bp fragment corresponding to the PAS B domain, from nucleotide 1185 to nucleotide 1363 of the *Mus musculus* CLOCK sequence (accession number AF000998): (forward) 5′-TGTGTACTGTTGAAGAACCAAA-TGAAGAGTT-3′; (reverse) 5′-GTGACATTTTGCCAGATTTTCTA-GGTCATG-3′. *Period1* primers comprised a region of 544 bp from nucleotide 582 to nucleotide 1126 of the *Homo sapiens* PERIOD1 sequence (accession number AF022991): (forward) 5′-CAAGGACTCAGAAGGA-ACTCATGACAG-3′; (reverse) 5′-GTACCGAGGCCCTGGATCCCG-GTCAG-3′. For control experiments, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used that spanned a 253 bp segment from nucleotide 548 to nucleotide 801 of the *Homo sapiens* GAPDH sequence (genebank number X01677): (forward) 5′-GTCATC-CATGACAACTTTGGTATCG-3′; (reverse) 5′-GCAGGTCAGGTCC-ACCACTG-3′.

Immunocytochemistry Cells were seeded on plastic multiwell slides (Labtex, Nunc, Naperville, IL) and subsequently fixed and permeabilized in formaldehyde/glutaraldehyde/Triton X-100 in stabilization buffer as previously described (Holleran *et al*, 1996). A wash with 0.5 mg per ml sodium borohydride in phosphate-buffered saline (PBS) was included to eliminate auto-fluorescence due to the glutaraldehyde. The preparations were blocked in 5% goat serum/2% bovine serum albumin in PBS for 30 min. All incubations were performed at room temperature. The following affinity purified antibodies were purchased from Alpha Diagnostic, San Antonio, TX: (i) CLO12-A was raised to a 20 amino acid peptide sequence near the C-terminus of human CLOCK; (ii) PER12-A was raised to a 20 amino acid peptide sequence within the C-terminus of human PER1. These rabbit primary antibodies were used in 1:50 dilution. Goat secondary biotinylated antibodies to rabbit IgGs (Molecular Probes, Eugene, OR) were used at a dilution of 1:400. Finally, streptavidin coupled to Oregon Green 514 (Molecular Probes) was added at a final concentration of 10 μg per ml in PBS, and 5 μg per ml propidium iodide (Molecular Probes) was used to stain the nuclei. The specimens were mounted with Prolong Antifade (Molecular Probes) to preserve fluorescence and were visualized under a Meridian Insight Plus confocal microscope (Genomic Solutions, Ann Harbor, MI) with the 488 nm argon laser line.

RESULTS

***Clock* and *per1* genes are expressed in human skin cells: RT-PCR results** Three different primary cell types from human skin were examined and mammalian cell line counterparts of each kind were chosen to extend the observations to transformed cells, i.e., HaCaT keratinocytes, COS-1 fibroblasts, and A375 melanocytes. RT-PCR of various RNA samples yielded expected 728 bp and 544 bp products corresponding to human *clock* and *per1* cDNAs, respectively, in primary human keratinocytes, dermal fibroblasts, and melanocytes cultured from neonatal human foreskins, and in HaCaT keratinocytes, COS-1 fibroblasts, and the A375 melanoma cell line (**Fig 1**). Direct PCR of a human keratinocyte cDNA library (Stratagene, La Jolla, CA) confirmed these results, obtaining a 138 bp cDNA fragment when using the primer pair corresponding to the PAS B domain of the clock protein, and the 544 bp fragment corresponding to *per1* cDNA (data not shown). Subcloning and partial sequencing of all three products corroborated their identity. The identity of the 544 bp cDNA fragment with the human *per1* cDNA sequence was 96% along 329 nucleotides sequenced. The identity of the 728 bp cDNA fragment with the human clock cDNA sequence was 98.8% along 604 nucleotides sequenced. The identity of the 138 bp cDNA product with the human clock cDNA was 87.6% along 121 nucleotides sequenced.

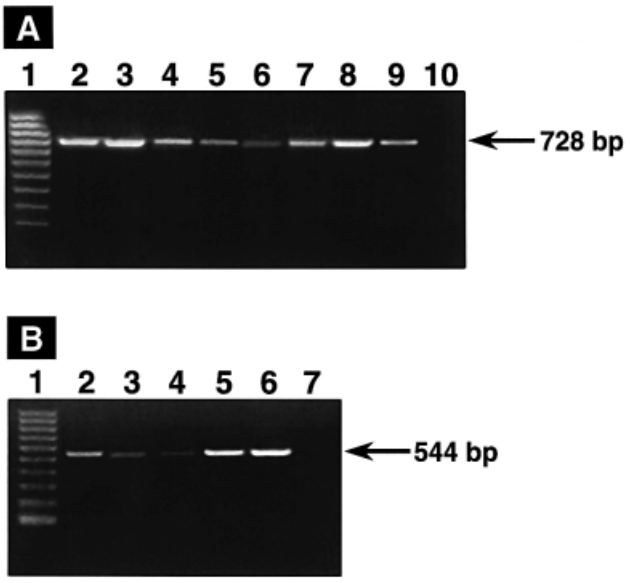

Figure 1. *Clock* and *per1* expression. RT-PCR of various cell type RNA samples using specific primers as detailed in *Materials and Methods*. (*A*) The 728 bp fragment corresponds to *clock* cDNA; (*B*) the 544 bp is the *per1* cDNA. *Lanes, panel* (*A*): (1) 100 bp DNA molecular weight ladder; (2) human primary keratinocytes poly-(A^+) mRNA; (3) human primary keratinocytes; (4) human primary dermal fibroblasts; (5) human primary melanocytes; (6) HaCaT keratinocytes; (7) COS-1 monkey fibroblasts; (8) A375 human melanoma; (9) human kidney (positive control); (10) non-reverse-transcribed, RNAsed RNA (negative control). *Lanes, panel* (*B*): (1) 100 bp DNA molecular weight ladder; (2) human primary keratinocytes; (3) human primary dermal fibroblasts; (4) human primary melanocytes; (5) COS-1 monkey fibroblasts; (6) A375 human melanoma; (7) non-reverse-transcribed, RNAsed RNA (negative control).

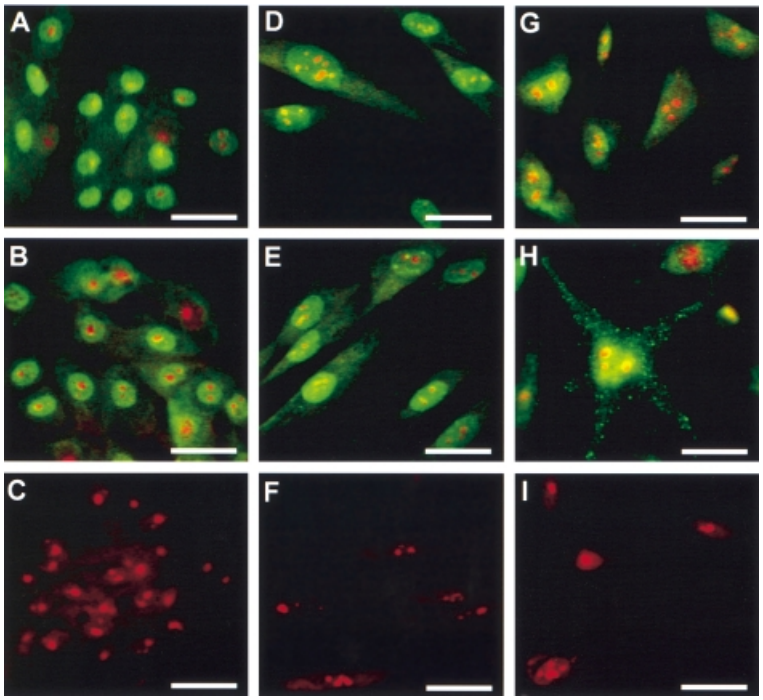

Figure 2. Immunostaining of primary skin cells for CLOCK and PERIOD1. Cell preparations were done as described in *Materials and Methods*. These confocal micrographs show immunoreactivity (green) for CLOCK (first row) and PER1 (second row) and DNA nuclear staining (red); coincident staining appears yellow. *Scale bar*: 50 μm. The third row shows negative controls (no incubation with primary antibody). (*A*, *B*, *C*) Keratinocytes; (*D*, *E*, *F*) dermal fibroblasts; (*G*, *H*, *I*) melanocytes.

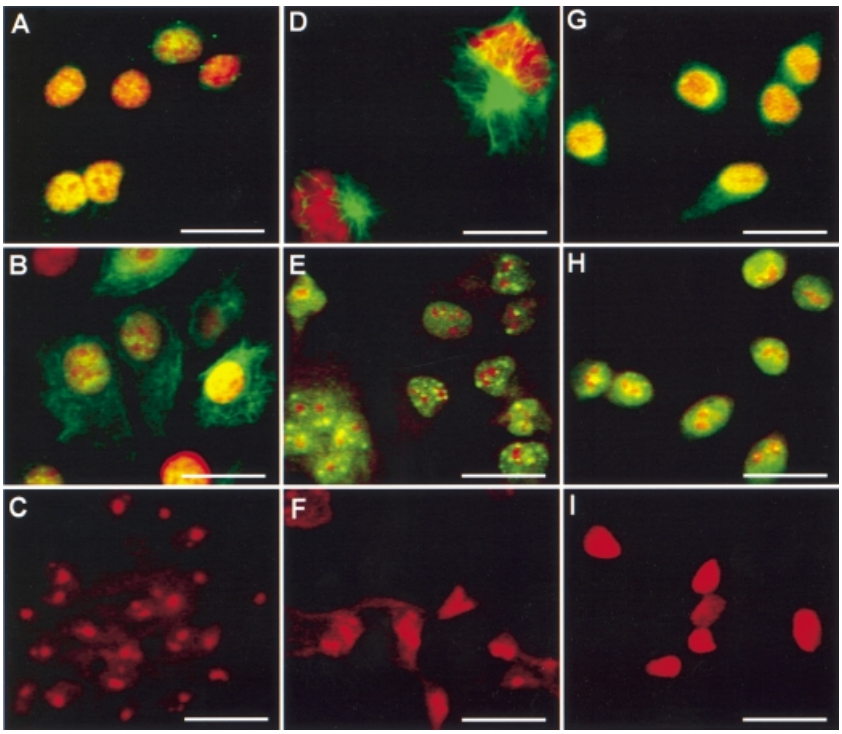

Figure 3. Immunostaining of various cell lines for CLOCK and PERIOD1. Cell preparations were done as described in *Materials and Methods*. These confocal micrographs show immunoreactivity (green) for CLOCK (first row) and PER1 (second row) and DNA nuclear staining (red); coincident staining appears yellow. *Scale bar*: 50 μm; 30 μm for (*D*). The third row shows negative controls (no incubation with primary antibody). (*A*, *B*, *C*) HaCaT keratinocytes; (*D*, *E*, *F*) COS-1 monkey kidney fibroblasts; (*G*, *H*, *I*) A375 human melanoma cells.

Clock and per1 proteins are present in human skin cells: immunocytochemistry results **Figure 2** shows the micrographs corresponding to CLOCK and PER1 immuno-reactivity in primary human skin cells. Both CLOCK and PER1 showed nuclear localization in keratinocytes, fibroblasts, and melanocytes, although levels of immunoreactivity were significantly lower in fibroblasts and melanocytes. The micrographs in *panels D*, *E*, *G*, and *H* were obtained with a longer exposure (2–3-fold) than those corresponding to keratinocytes, evidencing a lower level of expression and more variability of expression among these cells. PER was also apparent as speckled immunoreactivity in the nucleus and cytoplasm of all cell types.

In **Fig 3**, CLOCK and PER1 immunoreactivity is shown for several cell lines: human HaCaT keratinocytes, COS-1 fibroblasts, and human melanoma A375 cells. Both HaCaT and A375 cells showed similar nuclear staining to that observed for their primary cellular counterparts (**Fig 2**) for CLOCK and PER1. The staining pattern for CLOCK in COS-1 cells markedly differed from the others. This consisted of a strong immunoreactivity with structures associated with the centrosome and microtubules, judging by their

morphology. In addition, this staining was observed only in approximately 50% of the cells.

DISCUSSION

We present molecular and immunologic evidence of the constitutive expression of circadian clock-related genes in the skin, although we present no evidence of their regulation. The presence of *clock* mRNA and CLOCK protein was very consistent in the primary skin cells and cell lines examined, according to RT-PCR and immunohistochemical analysis. The fluorescence intensity in PER1-immunoreactive cells was more variable across cell fields.

Confocal microscopy revealed nuclear CLOCK and PER1 immunoreactivity, colocalized with DNA staining. CLOCK possesses the typical bHLH-PAS domain characteristic of transcription factors, and therefore a nuclear localization was expected. The *period* gene has been previously shown to encode a predominantly nuclear protein in *Drosophila*, with a fraction of it existing in the cytoplasm (Liu *et al*, 1992; Saez and Young, 1996). In agreement, our results evidenced a similar nuclear staining pattern in mammalian cells. Interestingly, a speckled distribution was seen in the cytoplasm and the nuclei. These may represent aggregates formed by mammalian PER homolog complexes, which should be tested with studies of colocalization when PER2 and PER3 antibodies are available.

A most curious staining pattern with CLOCK antibodies was observed in COS-1 cells, corresponding to an aster-like structure. This centrosomal staining was not apparent in all cells but in a considerable percentage of them, approximately 50%, suggesting that it was not due to an artifact or nonspecific staining. Even though the exact identity of the immunoreactive species is to be determined, we can speculate that CLOCK antibodies reacted to a clock-related protein associated to microtubules, and the different sizes of the aster structure could reflect a different degree of aggregation of the antigen.

The demonstration of *clock* and *per1* expression in human skin cells supports the notion of the ubiquity of the circadian clock machinery in the different tissues of an organism (King *et al*, 1997; Sun *et al*, 1997; Tei *et al*, 1997). A main topic of discussion is the synchrony of the central and peripheral timekeepers. One idea proposes that humoral factors delivered by the SCN or ganglia - in invertebrates - target the various tissues, supporting the concept of a predominant central control from these central nervous system structures. Important evidence advocating this hypothesis came with the induction of circadian gene expression cycles in established tissue culture lines, fibroblasts, and hepatocytes, after a serum shock (Balsalobre *et al*, 1998). The skin may very well be another tissue in which the existence of a running clock may prove beneficial to adaptively anticipate daily changes that might influence the local physiology of the skin. In addition, as the skin is an active endocrine tissue, it is conceivable that this tissue may contribute to the humoral signals to synchronize the rhythms of the rest of the organism.

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