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Peroxiredoxin 2 nuclear levels are regulated by circadian clock synchronization in human keratinocytes

Q1 Daniele Avitabile[a,f,*,1], Danilo Ranieri[a,1], Arianna Nicolussi[b], Sonia D'Inzeo[b], Anna Laura Capriotti[c], Licia Genovese[a], Sara Proietti[a], Alessandra Cucina[d], Anna Coppa[b], Roberto Samperi[c], Mariano Bizzarri[b], Aldo Laganà[c], Maria Rosaria Torrisi[a,e]

[a] Istituto Pasteur-Fondazione Cenci Bolognetti, Dipartimento di Medicina Clinica e Molecolare, Sapienza Università di Roma, Italy
[b] Dipartimento di Medicina Sperimentale, Sapienza Università di Roma, Italy
[c] Dipartimento di Chimica, Sapienza Università di Roma, Italy
[d] Dipartimento di Chirurgia"P. Valdoni", Sapienza Università di Roma, Italy
[e] Azienda Ospedaliera S. Andrea, Rome, Italy
[f] Laboratiorio Biologia Vascolare e Medicina Rigenerativa, Centro Cardiologico Monzino – IRCCS, Milan, Italy

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ABSTRACT

Circadian rhythms are highly conserved time tracking systems regulating important biological processes at both systemic and cellular levels. The present study was aimed to identify proteins and biological functions circadian regulated in human keratinocytes.

HaCaT keratinocytes were entrained by temperature cycles, and a proteomic study was performed on cell fractions isolated under free running conditions at constant temperature. Bioinformatics analysis revealed that molecular clock entrainment was associated with changes in molecular components regulating cell proliferation, energy metabolism, transcription, translation and redox balance. Nuclear levels of the antioxidant enzyme Peroxiredoxin 2 (PRDX2) were found to oscillate rhythmically over two entire 24 h long cycles. Donwregulation of PRDX2 resulted in upregulation of the mitochondrion-specific Peroxiredoxin 3 (PRDX3), all other members of the Peroxiredoxin family remained unaltered. Furthermore, PRDX2 knockdown increased intracellular levels of reactive oxygen species (ROS) and impaired cell cycle progression and proliferation. HaCaT cells transduced with a scramble shRNA were used as control.

Our work is the first to show that nuclear levels of PRDX2 display circadian oscillation participating in the regulation of human keratinocytes redox balance.

1. Introduction

Circadian rhythms have evolved as an adaptive response to daily cyclic variations, such us changes in light/darkness or temperature oscillation, which can act as resetting stimulus (zeitgeber) inducing synchronization (entrainment) of several physiological and metabolic processes (Saini et al., 2012). In addition to central clock, mammals possess self-sustaining peripheral clocks exist in most body districts (Brown et al., 2012). Circadian rhythmicity coordination is achieved by an intricate network of transcription–translation feedback loops (TTFL) involving the transcriptional activators circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-like protein 1 (BMAL1) and their own repressors the Period (Per1, Per2 and Per3) and Cryptochrome (Cry1 and Cry2) genes (Bass, 2012).

Among peripheral tissues, skin is the most exposed to environmental changes, such as light, temperature and oxidant variations. A functional TTFL has been reported in several skin cell types (Sandu et al., 2012; Tanioka et al., 2009), including the spontaneously immortalized human keratinocyte HaCaT cell line (Sporl et al., 2011), widely used as cellular model to study keratinocyte cell physiology (Boukamp et al., 1988). However, an accurate analysis of the biological processes and molecular functions engaged in human keratinocytes upon clock synchronization is still lacking.

Abbreviations: TTFL, transcriptional/translational feedback loops; PRDX1–6, Peroxiredoxins 1–6; HaCaT, human adult low calcium temperature keratinocytes; L2N, Lists Two Network Software; nHPLC-MS/MS, nano liquid chromatography and mass spectrometry proteomic analysis; ROS, reactive oxygen species; ShRNA, short hairpin RNA; ΔMFI, difference of mean fluorescence intensity; RLU, relative light unit; RFU, relative fluorescence unit.

* Corresponding author at: Via di Grottarossa 1035, 00189 Rome, Italy. Tel.: +39 0633775995; fax: +39 0633775995.
E-mail address: daniele.avitabile@uniroma1.it (D. Avitabile).
[1] These authors contributed equally to this work.

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Interestingly, the existence of a transcriptional-independent metabolic clock has been described, involving the circadian changes in the oxidation states of the antioxidant enzymes peroxiredoxins (PRDXs) (O'Neill and Reddy, 2011). In mammals, six members of the PRDX family have been identified, divided into three classes: 2-Cys (PRDX1–4); atypical 2-Cys (PRDX5); and 1-Cys (PRDX6). Beyond their antioxidant activity, PRDXs regulate oxygen-peroxide mediated signal transduction and act as molecular chaperones when hyper-oxidized (Woo et al., 2010).

In the present study, in order to identify proteins and biological functions circadian regulated in human keratinocytes, we performed a proteomic study through liquid chromatography–tandem mass spectrometry, bioinformatics analysis, biochemical and molecular methods to confirm results. The nuclear and cytoplasmic levels of PRDX2 were monitored in temperature-entrained cells under free running conditions at constant temperature for two consecutive 24 h long cycles. This experimental approach revealed that nuclear PRDX2 levels change with circadian rhythmicity regulating the redox balance of entrained cells. Finally, we found that the loss of PRDX2 is associated with the upregulation of PRDX3, increased intracellular ROS levels and impairment of cell cycle progression and proliferation in human keratinocytes.

2. Materials and methods

2.1. Cells and treatments

The human keratinocyte cell line HaCaT, spontaneously immortalized from a primary culture of keratinocytes (Boukamp et al., 1988) and the Human Embryonic Kidney cell line HEK 293T were cultured as previously described (Avitabile et al., 2011; Belleudi et al., 2011). For proteomic studies HaCaT cells were seeded at a concentration of 1×10^5 ml^{-1} to obtain 40% of confluence 16 h later and entrained by two 24 h long temperature profiles as previously described (12-h 37 °C/12-h 33 °C) (Sporl et al., 2011). Samples were collected at the end of the second cycle (T0) and 8 h later (T8).

To study the circadian nuclear/cytoplasmic translocation of PRDX2, HaCaT cells were growth to confluence and then entrained by temperature as described above. Protein samples for cell fractionations were collected under free running conditions every 4 h starting from T24 and until T72. Further details are provided in Supplementary Methods.

Reactive oxygen species (ROS) were induced with 250 μM hydrogen peroxide (H_2O_2, 30% (v/v), Sigma–Aldrich) for 30 min at 37 °C. Ultrapure water ($\Omega = 18.2$) was used as negative control.

2.2. Viruses and cell transduction

HaCaT cells were transduced with lentiviral vectors expressing: scramble shRNA sequence (ShCtr, SHC016, Sigma–Aldrich), shRNA for human PRDX2 (ShPrdx2, TRCN0000064906, Sigma–Aldrich) or the luciferase gene under control of the Bmal1 promoter (BLuf vector, kindly provided by Prof. Steven A. Brown, Institute of Pharmacology and Toxicology, University of Zürich, Zürich, Switzerland) (Brown et al., 2008). Further details are provided in Supplementary Methods.

2.2.1. Protein extraction and Western Blot analysis

Nuclear, cytosolic and membrane fractions of HaCaT cells were extracted as previously described (D'Inzeo et al., 2010) from samples collected upon temperature synchronization, at the end of the second cycle (T0) and 8 h later (T8). Protein quantification and Western Blot analysis (WB) was performed as previously reported (Belleudi et al., 2011). The analysis of PRDX2 in non-reducing conditions was performed as reported elsewhere (Cox et al., 2010). Further details are provided in Supplementary Methods.

2.3. Flow cytometry analysis

ROS production was evaluated with CellROX Deep Red Reagent (CellROX, Life Technologies – Invitrogen, Carlsbad, CA, USA) by FACS analysis. Further details are provided in Supplementary Methods.

2.4. Luciferase and proliferation and assays

Bmal1 promoter activity was measured using the Luciferase Assay System kit (Promega) according to the manufacturer's instructions.

Cell proliferation was measured using CyQUANT (Life Technologies) reagent following the manufacturer's instructions. Further details are provided in Supplementary Methods.

2.5. Protein extraction and Western Blot analysis

Nuclear, cytosolic and membrane fractions of HaCaT cells were extracted as previously described (D'Inzeo et al., 2010) from samples collected upon temperature synchronization, at the end of the second cycle (T0) and 8 h later (T8). Protein quantification and Western Blot analysis (WB) was performed as previously reported (Belleudi et al., 2011). Western Blots in non-reducing conditions were performed with a SDS-sample buffer lacking 2-mercaptoethanol. Further details are provided in Supplementary Methods.

2.6. Proteomic and bioinformatics analysis

Proteomic analysis was performed as previously described (Capriotti et al., 2012). Only proteins identified in two independent experiments and displaying a coefficient of variation of their expression ($CV = \sigma/\mu \times 100\%$) < 40% were considered for subsequent bioinformatics analysis. Further details are provided in Supplementary Methods.

2.7. Immunofluorescence

Immunofluorescence was performed as previously described (Belleudi et al., 2011) using the rabbit anti-PRDX2 (Abcam, 1:100). CellROX was employed as fluorescent marker of intracellular ROS. Further details are provided in Supplementary Methods.

2.8. Statistical analysis

All experiments were repeated independently at least three times. All data are expressed as mean ± SEM. Luciferase assay data for long-term analysis of Bmal1 promoter activity were processed by linear detrend. Significance of time series data was established using 1-way ANOVA and followed by Newman–Keuls post hoc test. Comparisons and plots were performed using Prism 5.0 software analysis (GraphPad Software) choosing the most appropriated statistical test according to the experimental design. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Detection of nuclear PRDX2 in HaCaT keratinocytes upon clock entrainment

HaCaT cells were transduced with a lentiviral vector expressing the luciferase gene under control of the Bmal1 promoter (HaCaT-BLuf cells), cells were entrained by two 24 h long temperature

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