



Temperature synchronisation of circadian rhythms in primary human airway epithelial cells from children

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ABSTRACT

Introduction Cellular circadian rhythms regulate immune pathways and inflammatory responses that mediate human disease such as asthma. Circadian rhythms in the lung may also contribute to exacerbations of chronic diseases such as asthma by regulating observed rhythms in mucus production, bronchial reactivity, airway inflammation and airway resistance. Primary human airway epithelial cells (AECs) are commonly used to model human lung diseases, such as asthma, with circadian symptoms, but a method for synchronising circadian rhythms in AECs has not been developed, and the presence of circadian rhythms in human AECs remains uninvestigated.

Methods We used temperature cycling to synchronise circadian rhythms in undifferentiated and differentiated primary human AECs. Reverse transcriptase-quantitative PCR was used to measure expression of the core circadian clock genes *ARNTL*, *CLOCK*, *CRY1*, *CRY2*, *NR1D1*, *NR1D2*, *PER1* and *PER2*.

Results Following temperature synchronisation, the core circadian genes *ARNTL*, *CRY1*, *CRY2*, *NR1D1*, *NR1D2*, *PER1* and *PER2* maintained endogenous 24-hour rhythms under constant conditions. Following serum shock, the core circadian genes *ARNTL*, *NR1D1* and *NR1D2* demonstrated rhythmic expression. Following temperature synchronisation, *CXCL8* demonstrated rhythmic circadian expression.

Conclusions Temperature synchronised circadian rhythms in AECs differentiated at an air–liquid interface can serve as a model to investigate circadian rhythms in pulmonary diseases.

INTRODUCTION

Circadian rhythms are hypothesised to play an important role in complex functions exhibited at cellular, tissue, organ and organism levels.¹ For example, circadian rhythms in the lung may contribute to exacerbations of chronic diseases such as asthma by regulating observed rhythms in mucus production, bronchial reactivity, airway inflammation and airway resistance.^{1,2} A series of transcription/translation feedback loops in core circadian genes such as *ARNTL* (encoding *BMAL1*), *CLOCK*, *PER1-3*, *CRY1-2* and

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Circadian rhythms in the human airway epithelium have been hypothesised play a role in regulating immune pathways and contribute to night/day differences in chronic inflammatory diseases, such as asthma, based on animal models. Airway epithelial cell cultures are commonly used as an *ex vivo* for experimental studies of human respiratory diseases, but circadian rhythms had not been described in the airway epithelial culture.

WHAT THIS STUDY ADDS

⇒ Here, we present a method for synchronising circadian rhythms in human airway epithelial cell cultures using temperature and confirm the presence of an endogenous circadian clock in the human airway epithelium.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This model will allow for future studies investigating the role of the circadian clock in human respiratory and airway diseases.

NR1D1/REV-ERBa generate molecular circadian rhythms.¹ In mice, circadian genes in airway epithelia regulate cyclical expression of inflammatory genes and susceptibility to viral infections such as influenza A.^{3,4} Investigation of molecular circadian rhythms in humans is limited by the need for repeated measurements within 24 hours. We developed a model of synchronised circadian rhythms in human airway epithelial cells (AECs) differentiated at an air–liquid interface (ALI) to investigate whether human AECs demonstrate similar circadian rhythms to those seen in mice models.

Differentiated primary human AECs are used as an organotypic *ex vivo* model to perform molecular characterisation of the human airway since ALI cultures demonstrate all the major cell types, basal-apical organisation and intact host inflammatory responses to viral infections exhibited *in vivo*.⁵ Prior



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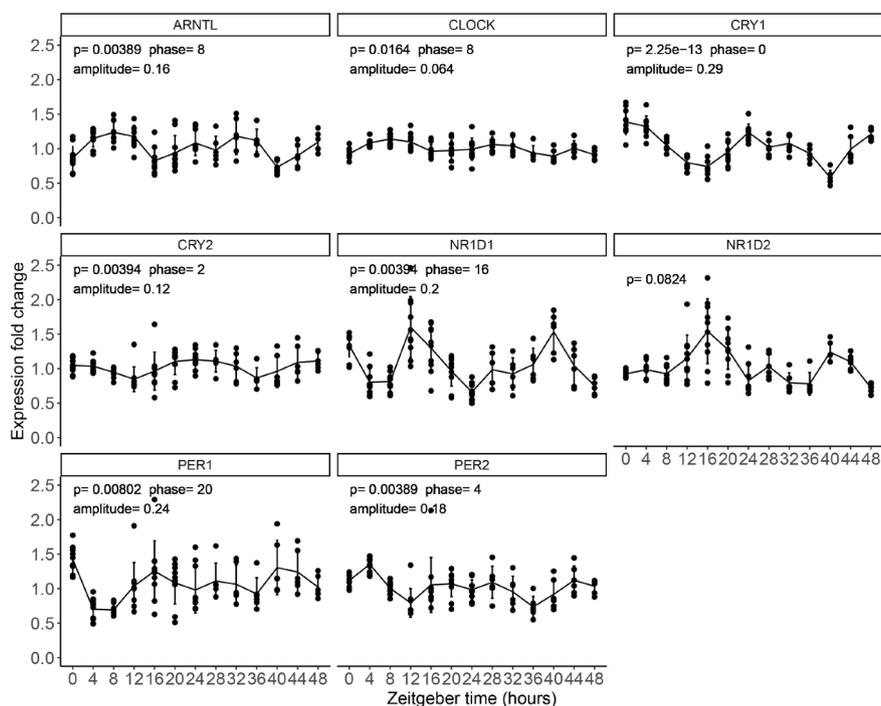


Figure 1 RT-qPCR analysis of core circadian gene expression normalised to *GAPDH* in undifferentiated airway epithelial cells following 6 days of temperature cycling. Individual biological replicates plotted as fold expression change relative to all time points with line plot of mean expression at each time point and SD error bars. Zeitgeber time 0=end of 6 days temperature synchronisation. RT-qPCR, reverse transcriptase-quantitative PCR.

work has investigated glucocorticoid or serum shock synchronisation of circadian rhythms in immortalised lung cells and mouse AECs; however, such methods

limit the investigation of innate inflammatory responses due to induction of widespread transcriptional changes by serum shock and glucocorticoid administration.

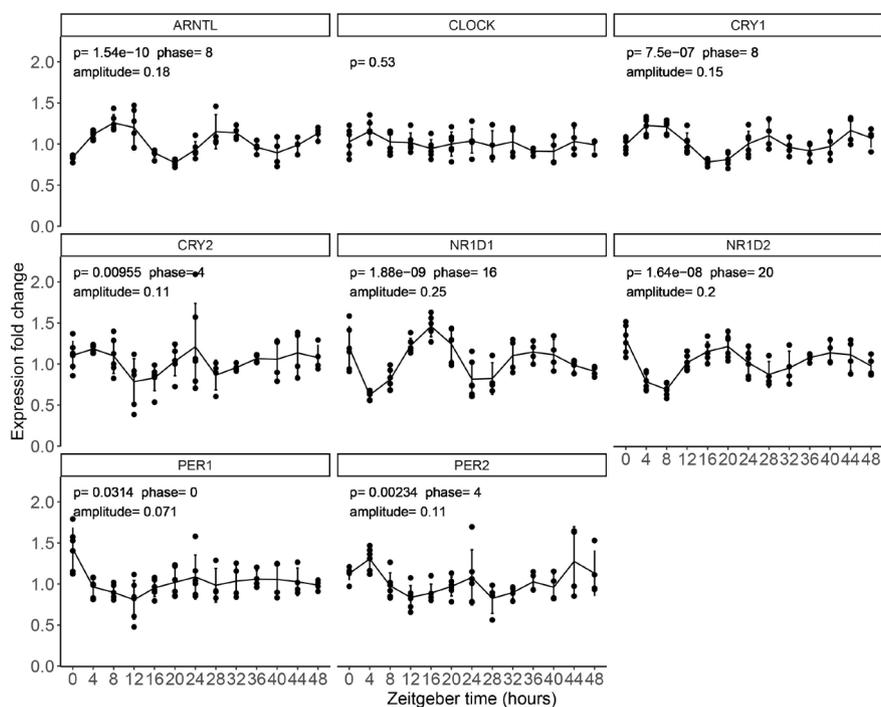


Figure 2 RT-qPCR analysis of core circadian gene expression normalised to *GAPDH* in differentiated airway epithelial cells following 6 days of temperature cycling. Individual biological replicates plotted as fold expression change relative to all time points with line plot of mean expression at each time point and SD error bars. Zeitgeber time 0=end of 6 days temperature synchronisation. RT-qPCR, reverse transcriptase-quantitative PCR.

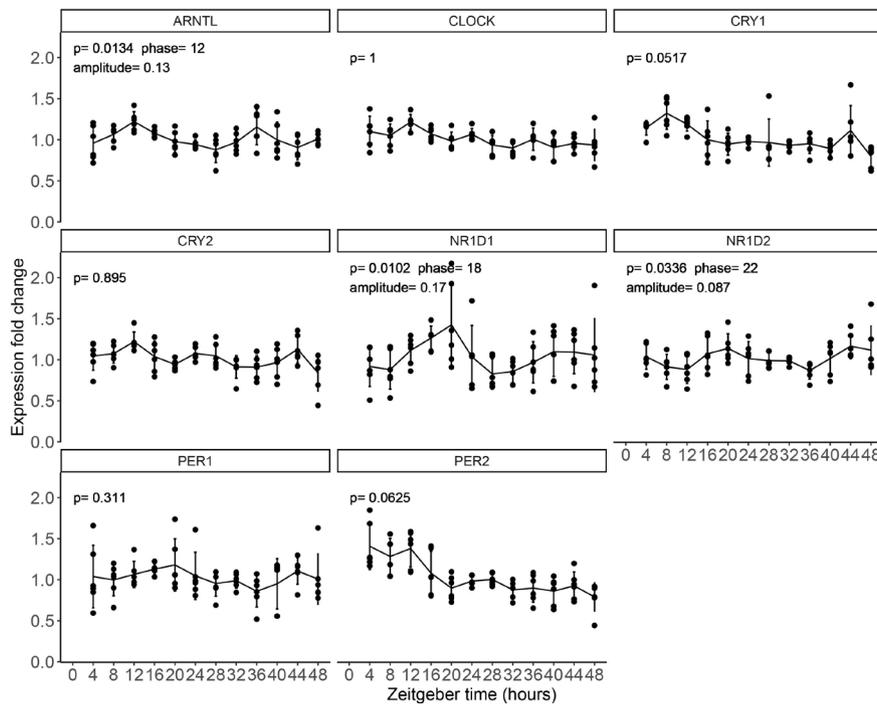


Figure 3 RT-qPCR analysis of core circadian gene expression normalised to *GAPDH* in differentiated airway epithelial cells following serum shock. Individual biological replicates plotted as fold expression change relative to all timepoints with line plot of mean expression at each time point and SD error bars. Zeitgeber time 0=serum shock. RT-qPCR, reverse transcriptase-quantitative PCR.

Alternatively, temperature has also been used in immortalised cell lines *in vitro* to synchronise circadian rhythms but has not been investigated in the organotypic AEC-ALI model.⁶ Use of temperature as a synchronising signal mimics *in vivo* temperature rhythms and follows existing *ex vivo* protocols for viral infections performed at 34°C. To develop a model for future *ex vivo* studies of human lung and airway diseases, we temperature synchronised human AECs in ALI culture and measured rhythmic expression in core circadian genes over 48 hours.

METHODS

Cell culture

AECs from healthy children were differentiated at an ALI in PneumaCult ALI media (Stemcell) at 37°C as we have previously described, producing an organotypic differentiated epithelial culture with mucociliary morphology.⁵ Following at least 35 days of differentiation, temperature cycling occurred for 6 days (12 hours at 37°C, 12 hours at 34°C) before cultures were maintained at a constant 37°C for 48 hours during the period of RNA isolation. For serum shock experiments cells were placed in Pneumacult ALI media supplemented with 50% fetal bovine serum for 1 hour at Zeitgeber 0.

RNA isolation

RNA was isolated using RNeasy total RNA isolation kit (Invitrogen AM1912) and two transwell cultures were pooled per replicate.

Reverse transcriptase-quantitative PCR

Reverse transcriptase-quantitative PCR using Taqman probes (*CRY1* Hs00172734_m1, *CRY2* Hs00901393_m1, *PER1* Hs00242988_m1, *PER2* Hs00256143_m1, *CLOCK* Hs00231857_m1, *ARNTL* Hs00154147_m1, *NR1D1* Hs00253876_m1, *NR1D2* Hs00233309_m1, *IL6* Hs00174131_m1, *CXCL5* Hs01099660_g1, *CCL5* Hs00982282_m1, *CXCL8* Hs00174103_m1, *TP63* Hs00978340_m1, *TUBB4A* Hs00760066_s1, *GAPDH* Hs02758991_g1) was performed. Expression was normalised to *GAPDH* and relative expression at all time points.

Statistics

Data analysis was performed using R, and rhythms identified using the R package MetaCycle JTK_cycle with Bonferroni correction.⁷

Patient and public involvement

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

RESULTS

Following 6 days of temperature synchronisation, undifferentiated AECs (figure 1) and differentiated AECs (figure 2) demonstrated rhythms in core circadian genes for 48 hours under constant temperature conditions. Appropriate phase relationships between genes

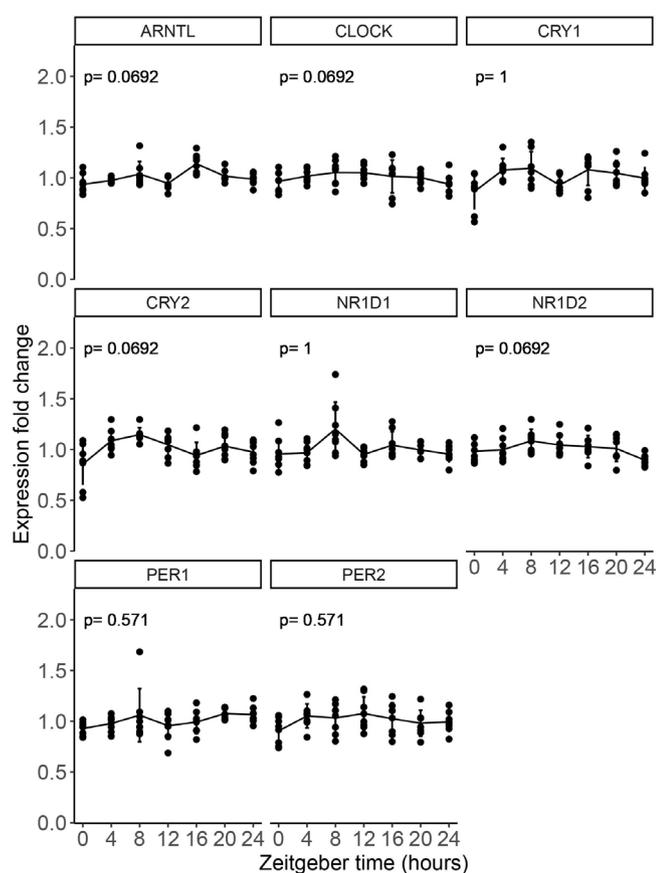


Figure 4 RT-qPCR analysis of core circadian gene expression normalised to *GAPDH* in differentiated airway epithelial cells under constant temperature conditions. Individual biological replicates plotted as fold expression change relative to all timepoints with line plot of mean expression at each time point and SD error bars. Zeitgeber time 0=media change and start of sampling. RT-qPCR, reverse transcriptase-quantitative PCR.

were maintained and period length was 24 hours in both conditions. The 48-hour amplitude of rhythms as measured by JTK varied from 0.071 arbitrary units in *PER1* to 0.25 arbitrary units in *NR1D1* with dampening of the amplitude in the second 24-hour period under constant conditions.

To compare temperature synchronisation to alternate methods for circadian synchronisation, serum shock was performed on differentiated AEC cultures from three donors. Following serum shock, *ARNTL*, *NR1D1* and *NR1D2* demonstrated rhythms with comparable phase relationships to temperature synchronisation, but with decreased amplitude (figure 3). Differentiated AECs under constant temperature conditions did not demonstrate rhythms in core circadian genes (figure 4).

To investigate the functional output of the core clock, expression analysis of *IL6*, *CCL5*, *CXCL5* and *CXCL8* was performed with rhythmic expression demonstrated in *CXCL8* (figure 5). *CXCL8* demonstrated phase matching with *BMAL1* and an amplitude of 0.25 arbitrary units as measured by JTK with amplitude decay in the second 24 hours. Negative control genes *TP63* (basal cell marker) and *TUBB4A* (ciliated cell marker) did not demonstrate circadian rhythmicity (figure 5).

DISCUSSION

Cycling incubator temperature leads to synchronisation of circadian rhythms in differentiated primary human AECs, with sustained rhythms for at least 48 hours under subsequent constant temperature. These results confirm the presence of endogenous, self-sustaining rhythms in core circadian genes. As circadian rhythms in mice have been shown to gate lung inflammatory responses to viruses such as influenza, and those to allergy-mediated

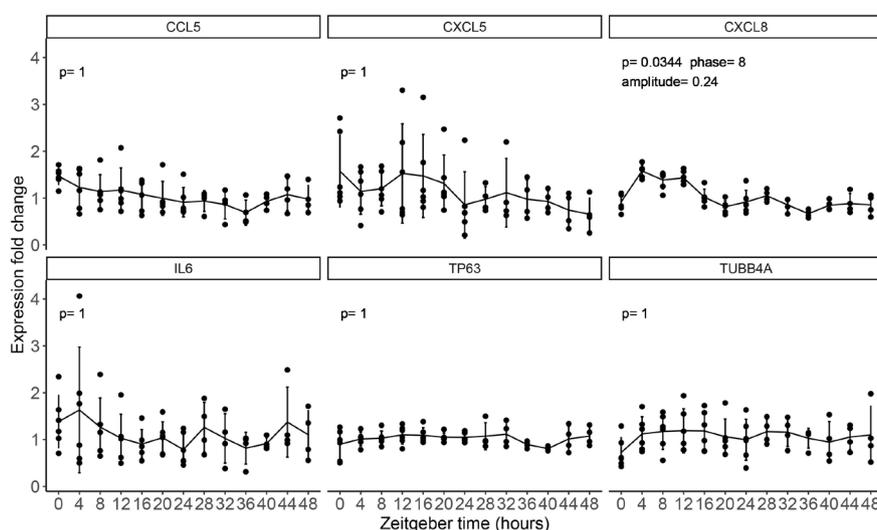


Figure 5 RT-qPCR analysis of *IL6*, *CXCL5*, *CCL5*, *CXCL8*, *TP63* and *TUBB4A* normalised to *GAPDH* in differentiated airway epithelial cells following 6 days of temperature cycling. Individual biological replicates plotted as fold expression change relative to all timepoints with line plot of mean expression at each time point and SD error bars. Zeitgeber time 0=end of 6 days temperature synchronisation. RT-qPCR, reverse transcriptase-quantitative PCR.

house dust mites, our work suggests that cultured human AEC demonstrate similar molecular pathways.^{3 4} While the core circadian machinery is conserved evolutionarily, translating molecular studies from animal to human samples is critical to advance our understanding of the relationship between circadian rhythms and human disease. Our work mirrors recent studies that identified the presence of molecular circadian rhythms in human primary pericytes and gut epithelial cells, without using serum shock or glucocorticoids as a synchronising signal.^{8 9}

In contrast to prior work, we were able to synchronise circadian rhythms in the ex vivo AEC-ALI model using temperature cycling within the temperature range commonly used for cell culture and viral-response studies in primary human AECs.⁵ Therefore, the temperature-synchronised model avoids induction of transcriptional responses to glucocorticoid stimulation and facilitates future studies on circadian regulation of viral response pathways. Furthermore, we demonstrate that temperature cycling leads to higher amplitude rhythms suggesting more robust synchronisation as compared with serum shock synchronisation. As AECs grown at an ALI demonstrate basal-apical polarity with only the basal layer directly exposed to media, we hypothesise that temperature synchronisation may have been more effective by stimulating cells throughout the epithelial culture rather than serum shock exposure at the basal layer. Furthermore, temperature synchronisation occurred over 6 days, which may also have led to more robust synchronisation. We did not find rhythmic expression in the core clock gene *CLOCK* which may be consistent with early findings that in some mammalian systems *CLOCK* is constitutively expressed and cyclic binding to BMAL1 and other core clock proteins regulates circadian rhythmicity.¹⁰ As an alternative explanation, as rhythmic expression of *CLOCK* is found in many tissues in CircaDB (a database of circadian expression profiles),¹¹ a low amplitude rhythm (as was found in the undifferentiated cultures) may be obscured in the differentiated cultures by noise or variable expression. Extensive regulation at the post-transcriptional and post-translational level of the core circadian clock may also account for why rhythmic expression is seen in the other core circadian genes, but not *CLOCK*, in our model.¹²⁻¹⁴

While we synchronised circadian rhythms in the core circadian transcriptional machinery, our study reflected a relatively targeted measurement of specific genes, and future work will need to characterise the circadian transcriptome in human AECs. While *CXCL5* has been shown to be rhythmic in mouse lung and regulate inflammatory responses, we did not observe rhythmicity in *CXCL5* in differentiated AECs, however, further investigation is necessary to elucidate whether *CXCL5* induced expression has circadian gating as has been shown in mice.¹⁵ Similarly, a circadian epithelial clock has been shown to regulate macrophage *IL-6* response following endotoxin stimulation in the lungs, but we did not observe

rhythmicity in differentiated AECs.^{15 16} The absence of rhythmicity in *IL6* or *CXCL5* may reflect the conditions of our experiments (unstimulated cultures from healthy donors) and future studies will need to investigate the circadian regulation of infections in the AEC model system. We did observe rhythmic expression in *CXCL8* indicating a functional core clock, as *CXCL8* rhythmicity has been reported in mast cells and eosinophils.¹⁷ As cells were propagated ex vivo prior to differentiation, our study was also unable to relate ex vivo molecular circadian rhythms to in vivo measurements of human circadian rhythms such as body temperature, activity, melatonin or cortisol secretion. While we hypothesise that the cycled incubator temperature mirrors in vivo temperature cycles as a cue for synchronisation, in vivo epithelial clocks likely have further neurohormonal inputs from the suprachiasmatic nucleus and nutrients.^{6 18} It remains unknown whether other disease mechanisms previously identified in the AEC-ALI model exhibit circadian regulation. Furthermore, whether airway epithelial circadian rhythms modulate or contribute to dysregulated pathways in lung diseases such as asthma remains uninvestigated.

Our results highlight the potential of the AEC-ALI model for investigating circadian regulation of molecular pathways in human lung and airway diseases. Future studies in this circadian AEC-ALI model with associated clinical phenotyping will unveil the role molecular circadian rhythms have in pathogenesis of chronic inflammatory disease such as asthma and in clinical presentations such as nighttime cough, day-night variation in airway responsiveness and airway resistance.

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Contributors WP conceptualised and designed experimental protocols, performed experiments, conducted data analysis and reviewed and revised the manuscript. WP acts as guarantor, accepts full responsibility for the finished work and/or the conduct of the study, had access to the data, and controlled the decision to publish. LR, EV and MW designed experimental protocols, performed experiments and reviewed and revised the manuscript. JSD conceptualised and designed experimental protocols, and reviewed and revised the manuscript.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by AECs from children were obtained under study #12490 approved by the Seattle Children's Hospital Institutional Review Board with informed consent from parents and assent from children.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information.

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