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 NR1D1/REV-ERBα 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.3 Prior
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.
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. 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. 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 RNAqueous 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.

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...
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
house dust mites, our work suggests that cultured human AEC demonstrate similar molecular pathways. 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.

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. 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-epithelial 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. As an alternative explanation, as rhythmic expression of CLOCK is found in many tissues in CircaDB (a database of circadian expression profiles), 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.

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. 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. 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. 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. 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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