The discovery of peripheral intracellular clocks revealed circadian oscillations of clock genes and their targets in all cell types, including those in the lung, sparking exploration of clocks in lung disease pathophysiology. While the focus has been on the role of these clocks in adult airway diseases, clock biology is also likely to be important in perinatal lung development, where it has received far less attention. Historically, fetal circadian rhythms have been considered irrelevant owing to lack of external light exposure, but more recent insights into peripheral clock biology raise questions of clock emergence, its concordance with tissue-specific structure/function, the interdependence of clock synchrony and functionality in perinatal lung development, and the possibility of lung clocks in priming the fetus for postnatal life. Understanding the perinatal molecular clock may unravel mechanistic targets for chronic airway disease across the lifespan. With current research providing more questions than answers, it is about time to investigate clocks in the developing lung.
It’s about time: clocks in the developing lung

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The discovery of peripheral intracellular clocks revealed circadian oscillations of clock genes and their targets in all cell types, including those in the lung, sparking exploration of clocks in lung disease pathophysiology. While the focus has been on the role of these clocks in adult airway diseases, clock biology is also likely to be important in perinatal lung development, where it has received far less attention. Historically, fetal circadian rhythms have been considered irrelevant owing to lack of external light exposure, but more recent insights into peripheral clock biology raise questions of clock emergence, its concordance with tissue-specific structure/function, the interdependence of clock synchrony and functionality in perinatal lung development, and the possibility of lung clocks in priming the fetus for postnatal life. Understanding the perinatal molecular clock may unravel mechanistic targets for chronic airway disease across the lifespan. With current research providing more questions than answers, it is about time to investigate clocks in the developing lung.

Introduction

The circadian system enables adaptation to environmental stimuli and is evolutionarily conserved (1). In mammals, the suprachiasmatic nucleus (SCN) in the brain provides time cues that coordinate physiological and behavioral functions (e.g., sleep, alertness, eating, hormone levels) (2, 3). The SCN is entrained by light (4), although intrinsic SCN clock genes (5) and genomic oscillatory mechanisms also exist (6–10). However, 25 years after the SCN was celebrated as the “master clock,” peripheral cellular clocks, i.e., intracellular networks of transcription-translation feedback loops, were discovered in all tissues (11–14). Peripheral clocks are responsive to various synchronizing agents, such as glucocorticoids (15) and adenylyl cyclase activators (16), and, in vivo, circadian entrainment strategies such as light-dark cycles or non-photic cues like time-of-day feeding regimens and activity (17). The SCN can synchronize peripheral clocks across tissues and circadian physiological behaviors through neuronal (direct) or humoral (indirect) cues in response to external stimuli (18). Supporting this notion, a novel clock luciferase reporter mouse model established that the SCN was necessary for phase synchronization across tissues (19). However, mechanisms of circadian entrainment between external environment, SCN, and peripheral clocks vary by tissue type. Such heterogeneity underlines potential cell-, context-, and organ-dependent roles of peripheral clocks. Therefore, it has become critical to understand clock biology and its disruption in the specific context of any organ and its normal function or role(s) in disease.

Pulmonary function is known to vary diurnally in healthy individuals (20). Circadian variations in symptoms and treatment responsiveness for chronic airway diseases such as asthma were reported in the 1970s amid initial studies on clock biology (21, 22). Lung molecular clocks, first identified in 1998 in rats (23), have since been implicated in adult airway disease pathophysiology. However, clock biology in perinatal lung has received far less attention. While circadian rhythms were long considered irrelevant to the developing fetus given its erratic sleep patterns and lack of external light exposure, peripheral clock biology calls this assumption into question. This Review aims to bridge the gap between the clock and the developing lung to hopefully unravel mechanistic targets for chronic airway disease across the lifespan. With more questions than answers, it is nonetheless time to investigate clocks in the developing lung.

Basics of clock biology

The core clock oscillatory network (Figure 1) consists of BMAL1 (encoded by ARNTL); CLOCK (CLOCK); PER1, PER2, and PER3 (PER1, PER2, PER3); and CRY1 and CRY2 (CRY1, CRY2). BMAL1 and CLOCK oscillate anti-phase to PERs and CRYs in an approximately 24-hour day. BMAL1 and CLOCK form a DNA-binding complex that transcriptionally regulates PER1, PER2, PER3, CRY1, and CRY2 gene expression via E-box promoter elements. PER1, PER2, PER3, CRY1, and CRY2 proteins form cytoplasmic heterodimers and, following phosphorylation, translocate back to the nucleus to prevent BMAL1-CLOCK complex from regulating downstream targets, including transcription of PER and CRY genes themselves. Two notable nuclear receptors regulate timing and amplitude of BMAL1 and therefore stabilize the clock: Retinoic acid-related orphan receptor-α (RORa) binds to ROR response elements (ROREs) in promoters of ARNTL, driving BMAL1 expression, while REV-ERBa (NR1D1) competes with RORa at ROREs. Furthermore, the NR1D1 promoter contains an E-box element and ROREs, which drive gene expression under control of BMAL1-CLOCK (1). In addition to these core components, clock stabilization, timekeeping, and entrainment can involve other signaling molecules. For example, cAMP and Ca2+ target the feedback transcriptional loop via cAMP response elements (CREs) in Per1 and Per2 promoters to modulate clock amplitude, phase, and period (16, 24–26). In support of this notion, in the mouse SCN, cAMP/Ca2+ signaling is elevated.
For example, BMAL1-CLOCK can modulate HIF-1α— and BMP-key targets potentially relevant to the lung (as described below). Regulation of clock-controlled genes into how core clock components regulate downstream non-clock points to multiple paths for dysfunction. Heterogeneity in cell- and context-specific fashion, and conversely modulating intracellular clocks allows for substantial dynamics and all, complex regulation of genes/proteins involved in mediating and around dawn and decreases later in the day (16, 24, 27, 28). Overall, complex regulation of genes/proteins involved in mediating and modulating intracellular clocks allows for substantial dynamics and heterogeneity in cell- and context-specific fashion, and conversely points to multiple paths for dysfunction.

Emerging data from multiple cell systems are providing insights into how core clock components regulate downstream non-clock genes and proteins (29–33). Regulation of clock-controlled genes is also cell- and context-specific (34–36). Nonetheless, there are key targets potentially relevant to the lung (as described below). For example, BMAL1-CLOCK can modulate HIF-1α- and BMP-regulated genes, while REV-ERBs negatively regulates BMP target genes. CRY1 and CRY2 modulate β-catenin nuclear localization and thus indirectly drive Wnt target genes, while nuclear localiza-

Clocks in the adult lung

Peripheral clock oscillations synchronize to signals from SCN through vagal innervation (ref. 37 and reviewed in refs. 38, 39). Lung physiology exhibits functional circadian rhythmicity in normal individuals, and a growing body of evidence suggests that clock disruption profoundly affects lung function and disease pathophysiology (refs. 39–46 and Table 1). Animal models with genetic deletion of clock genes support a functional role for lung clocks (Table 2). Jet-lag models (altered light-dark cycles), cigarette smoke, and viral or bacterial infections have been established as deleterious to lung clocks (43, 47–50). The relationship between clock disruption and chronic airway disease (chronic obstructive pulmonary disease, asthma, fibrosis) in adults reveals key mechanisms and provides insight into potential chronotherapeutic strategies (treatment based on endogenous circadian biology and time-of-day variation in pharmacological efficacy; ref. 51). Tables 1 and 2 depict only a fraction of the complex and diverse nature of lung clocks and emphasize the importance of precise cell-specific clock regulation.

With emerging recognition of clocks in adult lung physiology and disease, several questions become relevant for lung development and perinatal diseases:

A. When do lung clock pathways appear during development?

B. How does maternal circadian rhythmicity regulate fetal clocks and lung development?

C. What role, if any, do clocks play in the embryonic lung, which does not have a respiratory function in utero?

D. What is the functional status of lung clocks at birth, and do they play a role in perinatal and postnatal lung growth? Indeed, is an underdeveloped clock important in the context of premature birth and subsequent postnatal growth?

E. What effects do perinatal insults such as infection, inflammation, or iatrogenic factors in the context of ICU care of premature infants such as light, oxygen, and mechanical ventilation have on postnatal lung growth?

F. Are there heterogeneity and synchrony in clocks across lung cell types?
G. Does modulating clocks in developing lung limit the impact of detrimental factors in the perinatal period to improve outcomes for lifelong diseases?

An overview of fetal lung development

Understanding of lung development is largely derived from mouse models that are amenable to genetic manipulation and have a short period of embryonic lung growth (10–14 days). In spite of structural differences between mouse and human lung (52, 53), molecular factors coordinating lung developmental stages overlap (52, 53). Briefly, the endodermal transcription factor NKK2.1 (TTF1) initiates lung development (embryonic stage: E9.5–E12.5 in mice, 4–7 post-conception weeks [pcw] in humans), dependent on mesodermal Wnt signaling and inhibition of SOX2 (NKK2.1 inhibitor) by BMP4 (54–56), to establish ventral-dorsal patterning of the anterior foregut. Branching morphogenesis generates airways via epithelial FGF, SHH, and BMP4 (57–60), while SOX2 or SOX9 and 1D2 drive proximal or endodermal progenitors, respectively, to give rise to multiple airway cell types (61, 62) (pseudoglandular stage: E12.5–E16.5, 5–17

<table>
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<tr>
<th>Table 1. Clocks in the lung: establishing a relationship between circadian rhythms and lung structure/function in adults</th>
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<td><strong>Normal lung function and the clock</strong></td>
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<td>No disease/healthy individuals</td>
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<td><strong>Circadian clock disruption and the lung</strong></td>
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<td>Circadian clock disruption (gene/protein expression, amplitude, period, or phase)</td>
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<td><strong>Lung disease and the clock</strong></td>
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<td>Clock and lung disease via antioxidant defense pathway</td>
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<td>Clock and lung disease via inflammatory response</td>
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Clusters of epithelial sacs begin to form as the branches narrow (canalicular stage: E16.5–E17.5, 16–26 pcw; and saccular stage: E17.5–P5, 26–36 pcw), fully maturing into alveoli (alveolarization stage: P0–P14, 36 pcw–3 years) (52, 53).

The mouse model is advantageous for understanding neonatal/pediatric human disease (63–66). The mouse postnatal day 0 (P0) lung roughly correlates to that of a premature infant at about 32 weeks gestation, a 1-week-old mouse to a full-term newborn, and a 3-week-old mouse to an approximately 3-year-old child (Figure 2) (53). Thus, late-embryonic and neonatal mice offer the opportunity to explore perinatal insults in the context of premature birth, whereas post-weaning mice enable exploration of the effects of initial insults on subsequent lung structure and function in the context of pediatric disease.

**Clocks and the embryo**

The contribution of clocks to development is largely unknown, but characterizing the emergence of clocks throughout gestation may help us understand potential functional patterns. *Arntl* transcripts were initially considered to be present in unfertilized mouse eggs and the 2- and 16-cell stages (67). A more comprehensive study showed a peculiar pattern of the clock genes *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Clock*, and *Arntl*: while these genes were all expressed in the unfertilized egg and zygote (albeit at different levels), some transcripts disappeared at the 2-cell, 8-cell, and 16-cell stages, with complete restoration at the blastocyst stage (68). We can only speculate on the teleological rationale for this pattern of expression and disappearance. In vitro studies indicate that the pattern of emergence and disappearance continues throughout fetal development. Mouse embryonic stem cells from the late blastocyst stage lack a functional oscillatory clock. Differentiation of embryonic stem cells was sufficient to establish a clock rhythm, and strikingly enough, reprogramming differentiated cells to induced pluripotent embryonic stem cells triggered disappearance of the clock (69–71).

It is important to consider that absence of clock oscillation or synchrony does not imply absence of clock gene expression or functional relevance. Lack of synchrony during early stages of development and appearance upon differentiation strongly highlight a potential role of the clock in cell type specificity and differentiation. It is plausible that synchrony in the early embryo would inhibit differentiation by preventing various signals from targeting multiple differentiation pathways from progressing.

Evidence for clocks in later embryonic development derives from somitogenesis, a systematic process that establishes bilateral symmetry through sequential addition of somite pairs (mesodermal cells) on either side of the notochord along an anterior-posterior axis (beginning E8 in mice, 3 pcw in humans). This body axis segmentation lays the foundation for dermatomes, myotomes, and sclerotomes (72–74). Somitogenesis is under precise temporal control, orchestrated by rhythms of developmental signaling pathways (e.g., Notch, Wnt, and FGF). Before discovery of molecular clocks, the “clock and wavefront” model (75) hypothesized that an oscillator in presomitic mesodermal cells was halted by a wavefront moving posteriorly along the body axis, with wavefront timing corresponding to somite size and number (75). Somitogenesis can be viewed differently in the context of molecular clocks and oscillations. Notch transcription factors oscillate in concordance with segmentation periodicity (76, 77), as noted
increased by glucocorticoids and cAMP in human fetal alveolar type II cells (88), and by TGF-β in E10 mouse epithelial cells (89), while proinflammatory TNF-α inhibits NKX2.1 expression in human adenocarcinoma cells (90, 91). While it is unknown whether fetal TTF1 is under control of the clock, reports in other tissues have linked the two. Nkx2.1 in the rat brain pre- optic area is modulated by the light-dark cycle, while in GT1-7 cells (a neuronal cell line derived from murine hypothalamus), BMAL1-CLOCK represses Nkx2.1 promoter activity and CRY1 activates Nkx2.1 transcription (92). BMAL1-CLOCK–mediated suppression of Nkx2.1 is also reported in rat C6 glioma cells (93). Furthermore, TTF1 inhibits Nr1d1 transcription in GT1-7 cells (92). A genome-wide analysis found a differential role for TTF1 gene targets in early versus late mouse lung development with two clock pathways of note: at E11.5, Cry2 expression positively correlates with TTF1, while at E19.5, Clock expression positively correlates with TTF1 (94).

cAMP may be an important aspect of embryonic clock development. cAMP modulates clock properties via CREs in Per1 and Per2 promoters (16), regulates NKKX2.1, and regulates TTF1’s interaction with CREB to subsequently increase transcription of its downstream targets (95). In the developing fetal lung, cAMP signaling regulates surfactant protein A gene expression in type II cells (95). These loosely linked data suggest relationships between clock, cAMP signaling, NKX2.1/TTF1, and developing lung that need to be better delineated in the context of actual changes in lung structure and function, as well as cellular and temporal patterns.
While a myriad of signaling pathways have been implicated in different aspects of embryonic lung development, four key pathways may be relevant in terms of clocks: FGF, BMP, Wnt, and SHH. In nonpulmonary tissues, the clock has been implicated in Wnt and BMP signaling pathways. For example, Wnt regulates clocks in the Drosophila intestinal stem cell niche (96), and CRY1 regulates adipogenic differentiation in mouse 3T3-L1 embryonic fibroblasts (97). In preadipocyte 3T3-L1 cells in vitro, proliferation and components of the Wnt signaling pathway are under transcriptional control of CLOCK (98). Additionally, CRY1 can regulate osteoblast differentiation in human osteosarcoma cells via Wnt signaling (99). In human aortic endothelial cells, loss of BMAL1 drives endothelial-mesenchymal transition through increases in BMP signaling and ROS accumulation (100). Furthermore, promoters of Bmp genes have E-box elements for clock control, and in uterine endometrial stromal cells, REV-ERBα transcriptionally represses Bmp expression while dampening of clock upregulates Bmp-encoding genes (101). These many disparate data regarding clock elements in other systems may provide important insights into lung development, given known roles of both Wnt pathways (102–104) and BMP signaling (105–108) in this process. Relationships between clock and other critical elements such as FGF or SHH remain to be established in any organ system, but are exciting areas to investigate in the context of understanding clock regulation of lung development.

Another important factor to consider is the link between clocks in the fetal lung and hypoxia. Fetal development occurs in a relatively hypoxic environment in utero, which is critical during pseudoglandular and canalicular stages (109). In low-oxygen environments, HIFs are essential transcription factors for embryonic development, as evidenced by embryonic lethality of Hif1a-knockout mice by E11 (110). HIFs regulate downstream pathways involved in energy metabolism, proliferation, angiogenesis, extracellular matrix formation, and apoptosis (111, 112). Recent studies have identified yet another bidirectional relationship between HIF-1α and the clock that suggests a potential link in utero: In mice treated with DMOG, a drug that stabilizes HIF-1α, BMAL1 drives Hif1a gene expression (113). Additionally, in U2OS human bone osteosarcoma epithelial cells, HIF-1α is bound to the PER2 promoter, which regulates its expression (113), while in mouse fibroblast cells, Nrd1, Per1, and Per2 are induced by HIF-1α stabilization (113). Furthermore, HIF-1α dimerizes with BMAL1 with substantial overlap of downstream target genes (114). Conversely, mouse skeletal myotubes lacking BMAL1 exhibit reduced Hif1a expression and increased HIF-1α turnover, while Per2lac oscillatory activity is dependent on HIF-1α stability (115). Lastly, an elegant study found diurnal variation in oxygenation in blood, brain, and kidney in adult mice, with kidneys displaying a different time-of-day peak of HIF-1α nuclear localization compared with brain. In vitro experiments using Hepa-1c1c7 (murine hepatoma) and NIH 3T3 (murine fibroblast) cells and rhythmic O2 exposures (12 hours 5% O2/12 hours 8% O2) “reset” clock oscillation, a phenomenon dependent on HIF-1α (116). While these studies were primarily done in nonfetal models or in cell lines, one study did use SCN slices from postnatal mice at P3–P6 carrying a Per2:Per2:luc reporter; when anoxia was mimicked ex vivo, the period of clock oscillation was lengthened and amplitude diminished (113). Taken together, these studies suggest that O2 is a signal to the fetal clock during development, and oscillations in fetal O2 may prime the fetus for postnatal life. The precise role of hypoxia and the clock during lung development can only be speculated, but previous work suggests that oxygen exposure may regulate the pattern of clock emergence. On the other hand, rat pups born to mothers exposed to hypoxic gas (10% O2, 90% N2) when their fetuses were at E5 have profound behavioral and locomotor abnormalities, phase advancement, and failed entrainment to new light-dark cycles (117). Conversely, clock oscillations are altered by hyperoxia exposure in neonatal mice through REV-ERBa (118). Thus, the timing and extent of oxygen exposure may also be a critical driver of lung development.

**Maternal cues to the fetal lung**

Some insights regarding maternal cues in fetal clock development are provided by behavioral differences in offspring of precocial animals (born at an advanced stage of independence, e.g., monkeys, sheep) versus altricial animals (born at an underdeveloped stage, e.g., rats, hamsters, mice). Humans are a unique blend of precocial in many aspects of bodily form but neurologically and behaviorally altricial. In recognized precocial mammals, there are distinct fetal physiological rhythms in heart rate, breathing, movement/activity, and plasma cortisol (119–122), suggesting that clock develops prenatally: In altricial mammals, physiological rhythms in terms of behavior, temperature, activity, and corticosterone may be more substantially impacted postnatally depending on the length of gestation: rhythms may be established in utero of humans with longer gestation times, whereas species with shorter gestation times are still establishing rhythms postnatally. Alternatively, emergence of rhythms may depend on SCN development: in precocial species, the...
fetal SCN is developed by midgestation, but it begins to form around E14 in altricial species, completing development around birth (123). However, in studies using Per1:Luc mouse models, luciferase activity appeared before the fetal SCN develops (87), suggesting that peripheral fetal clocks emerge independent of central clock signals. Additionally, the maternal SCN may be regulating the fetal clock before development of a functional fetal master clock. In fact, studies in which rat or hamster maternal SCN was ablated support the notion of an endogenous clock established by the fetus itself, synchronized to the maternal SCN (124–126). Additionally, these studies suggest that clock synchrony with maternal SCN during development can dictate postnatal physiological rhythms (124–126). More recent studies found that heterozygous mouse pups from mothers harboring double knockouts of either Per1 and Per2 or Per2 and Cry1 lacked activity rhythms compared with wild-type littermates (126). These data support the concept that the fetal clock develops endogenously, not through the mother, and that the maternal SCN serves to entrain/signal/synchronize the fetus.

How does the maternal SCN signal and entrain the fetal clock? Candidates include maternal feeding and endocrine signals such as melatonin, glucocorticoids, and other hormones. Melatonin is considered a synchronizing signal for the fetal clock, as demonstrated by exogenous melatonin rescuing rhythmicity in pups born to hamsters in which the SCN was ablated (127). Additionally, the fetal adrenal clock can be manipulated by maternal light exposure; suppression of maternal melatonin by light results in complete loss of fetal BMAL1 and PER2 adrenal oscillations, which are rescued by exogenous melatonin (128).

Glucocorticoids exhibit circadian rhythmicity, reaching peak levels in the morning in humans. This rhythmicity stems from the SCN and the hypothalamic-pituitary-adrenal axis, leading to a circadian pattern of glucocorticoid secretion in a clock-dependent manner (129, 130). Glucocorticoids serve as an entrainment signal to peripheral clocks, and circadian disruption can disrupt glucocorticoid oscillations and therefore downstream, peripheral cellular functions (131). Thus, glucocorticoids could be one mechanism by which the fetus entrains to the maternal SCN, and may additionally explain how the fetal lung begins receiving a clock-stimulating signal. The glucocorticoid receptor is expressed in fetal lung during early gestation and drives production of surfactant-associated proteins, cell maturation/differentiation, and lung morphology (132, 133). However, the placental glucocorticoids to entrain peripheral clocks (135), the timing/pattern of glucocorticoid signaling during lung development and growth becomes worthy of investigation.

**Fetal origins hypothesis meets the clock**

In the 1990s, a “fetal origins hypothesis” postulated that in utero development programs the fetus for lifelong health versus future disease, regardless of health status at birth (136, 137). This notion implies that external factors experienced as an adult (e.g., diet, exercise, cigarette smoke, sleeping habits) may not be the sole determinants of disease. Thus, inadequate priming during fetal development can suppress latent effects on disease progression. Numerous epidemiological studies have linked factors such as low birth weight, nutrition, or stress with coronary artery disease, hypertension, obesity, insulin resistance, cancer, and other chronic diseases (138). The role of the clock in fetal development initially did not receive much attention because animal models lacking clock genes are not embryonic lethal and do not show striking morphological changes. However, the effects of clock knockouts are evident later in life. For example, BMAL1-knockout mice are visually similar to their littermates at birth but show impaired growth and weight gain around 16–18 weeks of age (139), while adults are infertile (140). BMAL1-deficient mice exhibit multiple symptoms of premature aging and increased ROS in several tissues (139), which can be reversed with N-acetyl-l-cysteine (141), suggesting that BMAL1 modulates ROS homeostasis. While these studies used whole-body conventional knockout mice, a pivotal study dissociated the role of BMAL1 during embryogenesis from BMAL1 disruption later in life with rather surprising findings: mice with intact BMAL1 during fetal development but lacking BMAL1 in adulthood did not display the same premature aging, indicating that the timing/pattern of BMAL1 expression is important to organismal health. Indeed, mice lacking BMAL1 in both embryogenesis and adulthood exhibited altered lifespan, fertility, body weight, and blood glucose levels as well as age-dependent arthropathy. However, the presence of BMAL1 during fetal development (deficient only in adulthood) rescued these phenotypes (142). These data strongly suggest that BMAL1’s function during fetal development has effects later in life. Other clock genes may also have a role in embryogenesis, priming the fetus through development and setting the stage for health versus disease throughout life (Figure 1).

**Clocks and the neonatal lung**

Immediate and efficient transition of the lung to extraterine life is critical for postnatal survival. This involves coordinating clearance of fetal lung fluid with increased pulmonary blood flow and decreased pulmonary vascular resistance, secretion of surfactant, breathing mechanics, and metabolic adaptation to increased oxygen exposure (143). Maladaptation (as occurs in early birth) runs a high risk of inflammation and oxidative stress in lungs prematurely exposed to normoxia and the extraterine environment. Data in other cell systems suggest that the clock is bidirectionally linked to immune responses and ROS regulation, raising the question of whether the clock is also involved in the fetal-to-neonatal transition, and additionally whether lack of a functional clock plays a role in responses of the premature lung.

REV-ERBa may be a key connection between the clock and neonatal lung. Abundantly expressed in adult lung (144), REV-ERBa may be regulated by oxidative stress, as evidenced by downregulation of lung Nr1d1 in adult mice exposed to cigarette smoke (145). Multiple studies show the intersection between oxidative stress, inflammation, and REV-ERBa in neonatal lung. Initial reports identified hyperoxia-induced NF-κB activation in neonatal mouse lungs (but not in adult) that protected against hyperoxia-induced
lungs injury via inhibition of apoptotic pathways (146). Additional
in vitro studies in neonatal mouse lung-derived fibroblasts and in
vivo neonatal lung show a connection between NF-κB and Nr1d1
(147). Mouse lung Nr1d1 mRNA expression increases from P1 to P21
and remains elevated in adults. Additionally, hyperoxia-induced
increases in neonatal lung Nr1d1 levels are exaggerated in the
absence of p50 NF-κB subunit (147). The Nr1d1 promoter contains
not only an NF-κB–binding site, but also an Nrf2-binding site.
Inflammatory stimulus via TNF-α results in NF-κB-mediated reduc-
tion in Nr1d1 expression, while hyperoxia-induced oxidative stress
upregulates Nr1d1 in an Nrf2-dependent manner (147). Importantly,
in neonatal mouse lung fibroblasts, hyperoxia-mediated oxidative
stress and NF-κB disruption dampen Nr1d1 oscillation induced by
serum shock (147). Furthermore, hyperoxia increases expression
of the transcription factor C/EBPα in neonatal mouse lung (148),
esential for fetal maturation of lung epithelium and surfactant pro-
duction (149). C/EBPα was also identified as a regulator of postnatal
alveolar epithelial cell proliferation and differentiation in hyperoxia
(148), with studies in other cell types showing Per2 and Nrlid1 as
its transcriptional targets (150, 151). However, the link between C/
EBPαs and the clock in the neonatal lung is not yet established.

Overall, emerging studies strongly suggest that oxidative
stress and inflammation regulate oscillation of at least REV-ERBα,
but the relationships (likely bidirectional) between lung responses
to perinatal insults such as hyperoxia or inflammation and
other key clock drivers such as BMAL1, PERs, and CRYs are unknown.
Indeed, if REV-ERBα is modulated by oxygen or inflammation
and drives lung cell phenotype, then it is likely that other clock
genes are both mediators and modulators of early postnatal lung
growth or, at the least, become important in the context of insults.
Notably, these relationships are being considered in the broad
context of “the lung,” but cellular heterogeneity in clock gene
expression, oscillatory patterns, and functional roles likely exists
and needs investigation.

Harnessing LungMAP to explore developmental
clock patterns
Given the many technical and interpretive limitations in dis-
secting out the when and how of clocks in the developing lung,
insights can be gained from pilot studies mapping spatiotempo-
ral patterns of lung development and growth. Here, the National
Heart, Lung, and Blood Institute’s LungMAP consortium (http://
www.lungmap.net) (152) is particularly appealing, given its focus
on both prenatal and postnatal time points. For example, LungMAP
data sets from mouse and human RNA sequencing after laser cap-
ture microdissection of alveolar parenchyma, or data from cell
sorting for endothelial, epithelial, mesenchymal, and immune
cells, allow visual assessment of spatiotemporal patterns in
expression of core clock genes. Such assessments show that Per1,
Cry2, Arntl, and Clock expression does occur at key prena-
tal and postnatal developmental points (with the understanding
that additional genes and regulators may also be dynamically
involved) (Figure 3). While more in-depth analyses are necessary,
this initial evidence leads to intriguing questions:

A. Are these genes oscillating in expression even within the
fetal lung, and if so, does the timing of acquisition of tissues (and
subsequent gene analysis) matter?

B. If there are temporal variations, are they intrinsically syn-
chronized, or obtaining cues from maternal patterns?

C. Do clock genes or their patterns matter to lung growth, i.e.,
are clock genes functional?

D. Are there species differences in clock gene expression, par-

ticularly in perinatal temporal patterns and functionality in altrici-

cal versus precocial species?

The LungMAP data provide glimpses into the spatial and tem-
poral dynamism of clock gene expression in the postnatal lung,
perhaps a more relevant time period in the context of healthy
growth and perinatal/pediatric disease. Clearly, some clock
genes, such as Per1, appear to be important in the early postnatal
period, and could be appealing to explore in the context of lung
growth, responses to insults such as oxygen or inflammation in
prematurity, and initiation of chronic lung diseases. Here, it may
also be important to consider whether differential expression in
epithelial versus mesenchymal cells is relevant to specific disease
progression. Conversely, the relative stability (or at least lack of
reduction) of Clock or Cry2 suggests that these genes permit
upstream and downstream modulation of clock pathways as well
as growth and inflammation.

Clinical significance of clocks in the developing lung
Introducing the clock to the fetal origins hypothesis presents the
notion of fetal programming. Do adult diseases replicate inade-
quate fetal programming? The Dutch Hunger Winter Study found
a significant link between gestational malnutrition and adult car-
diovascular and metabolic diseases (153). Many factors can influ-
ence fetal development and thereby “program” disease, including
uteroplacental blood flow, hypoxia, oxidative stress, malnutrition,
and maternal hormones (154). We can speculate that the placent-
ta serves as a gatekeeper to mediate when (in terms of gestational
time and/or time of day) and how much of maternal signals (what-
ever they are) reach the fetus. This may also depend on develop-
mental cues necessary for fetal development. Clock biology is
inherently adaptive to its environment, so reasonable speculations
can be discussed. The placenta may create a maternal-fetal sig-
aling gradient over gestational time to wean the fetus off mater-
nal cues toward parturition, an effort to prepare the fetus’s own
adaptive mechanisms for postnatal life. Increased susceptibility
to chronic airway diseases in premature infants exposed to insults
may reflect inadequate clock establishment or lack of clock pro-
gramming before birth. Additionally, premature infants in the neo-
natal ICU experience circadian disruption (155, 156), while entrain-
ment strategies improve outcomes (157). If the effect of circadian
disruption on the fetal lung is as severe as in the adult, mindfulness
of a newborn’s developing circadian system may prove beneficial.

Targeting the clock may provide solutions to prevent or treat
airway disease progression in premature infants and in those
with airway diseases. Multiple mechanisms relevant to airway
disease are already associated with clock biology, even if in
nonpulmonary tissues (e.g., immune responses, contractility,
mitochondrial dynamics, metabolism, senescence pathways). Addition-
ally, the clock is not a two-way street. Many cellular pathways feed into the clock feedback loop, which provides sens-
ing of environmental changes, while core clock components reg-
ulate downstream pathways to provide an adaptive advantage.
This bidirectional relationship highlights the potential benefit of therapeutically targeting the clock (chronotherapeutics), albeit after a more complete understanding of functional roles of the clock in the developing and perinatal lung.

Conclusions
Understanding of clock biology during fetal and neonatal lung development is currently limited, but holds promise for assessment of mechanisms and potential targets for chronic lung diseases. Substantial insights can be gained from data in other organ systems, and emerging data in adult peripheral lung clocks and lung diseases. Fetal lung clock emergence, synchrony, and function during development add a level of complexity to the circadian field and are a relatively unexplored niche.

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9. Ewer J, Rosbash M, Hall JC. An inducible promot-
15. Cuesta M, Cermakian N, Boivin DB. Gluco-
16. O’Neill JS, Reddy AB. The essential role of CAMP/ Ca2+ signaling in mammalian circadian time-
17. Oishi K, Sakamoto K, Okada T, Nagase T, Ishida N. Dual origins of the intracellular circadian calci-
21. Ewer J, Rosbash M, Hall JC. An inducible promot-
29. Ewer J, Rosbash M, Hall JC. An inducible promot-
35. Chaix A, Zarrinpar A, Panda S. The circadian clock for imaging of intracellular calcium in single suprachiasmatic nucle-
44. Hwang JW, Sundar IK, Yao H, Sellitt MT, Rahman I. Circadian clock function is disrupted by environmental tobacco/cigarette smoke, leading to circadian field and are a relatively unexplored niche.


ing down clock control gene CRV1 decreases adipogenesis via canonical Wnt/β-catenin sig-
100. Zhu M, Tang H, Xu A, Guo D, Chen F. BMAL1 suppresses ROS-induced endotheli-
103. Cohen JC, Larson JE, Killeen E, Love D, Takema-
104. Shu W, Jiang YQ, Lu SM, Morrissey EE. Wnt7b regulates mesenchymal proliferation and vas-
105. Yun EJ, Vu TH. mSmile is necessary for bronchial smooth muscle and alveolar myofibroblast develop-
106. Zhang XQ, et al. Regulation of pulmonary sur-
107. Southwood M, et al. Regulation of bone morpho-
108. Shi W, Zhao J, Anderson KD, Warburton D. Gremlin negatively modulates BMP-4 induction of embryonic mouse lung branching morpho-
111. Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC. Differential roles of hypoxia-inducible fac-
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