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The Lineage Before Time: Circadian and Nonclassical Clock Influences on Development

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Abstract

Diverse factors including metabolism, chromatin remodeling, and mitotic kinetics influence development at the cellular level. These factors are well known to interact with the circadian transcriptional-translational feedback loop (TTFL) after its emergence. What is only recently becoming clear, however, is how metabolism, mitosis, and epigenetics may become organized in a coordinated cyclical precursor signaling module in pluripotent cells prior to the onset of TTFL cycling. We propose that both the precursor module and the TTFL module constrain cellular identity when they are active during development, and that the emergence of these modules themselves is a key lineage marker. Here we review the component pathways underlying these ideas; how proliferation, specification, and differentiation decisions in both developmental and adult stem cell populations are or are not regulated by the classical TTFL; and emerging evidence that we propose implies a primordial clock that precedes the classical TTFL and influences early developmental decisions.

Keywords

circadian; cell cycle; epigenetics; metabolism; stem cell; development

1. COUPLING OF THE CIRCADIAN CLOCK TO METABOLISM, EPIGENETIC STATE, AND CELL DIVISION

1.1. The Molecular Circadian Clock in Mammals

The mammalian circadian clock drives an oscillation of 24-hour period in diverse physiological functions, including epigenetic modification, metabolism, and cell division, in the whole body. Each cell possesses an intrinsic circadian clock driven by transcriptional-translational feedback loops (TTFLs) composed of circadian clock genes (Balsalobre

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et al. 1998, Yamazaki 2000). At the center of the TTFLs are two basic helix-loop-helix transcription factors: CLOCK and BMAL1 (ARNTL) (Figure 1a). These proteins heterodimerize, then bind to E-box enhancer elements and transactivate associated genes, driving gene expression that includes their negative regulators Period (PER) and Cryptochrome (CRY). Once the CLOCK:BMAL1 heterodimer forms a complex with PER and CRY proteins, the CLOCK:BMAL1 heterodimers dissociate from DNA, ending transactivation (Chiou et al. 2016). A series of phosphorylation and ubiquitination events as well as other checkpoints regulates the kinetics of PER and CRY nuclear reentry and competence to associate with CLOCK:BMAL1 (Patke et al. 2020). The latency of interactions between the positive CLOCK:BMAL1 and the negative CRY-PER arms contributes to the 24-hour period of the core TTFL loop.

The cell-autonomous circadian clock also contains secondary loops that serve to stabilize 24-hour transcriptional rhythms. A prominent example, particularly in development, is modulation by NR1 family transcription factors, which include transactivating RORα/β and repressive REVERBα/β family members (Ueda et al. 2005). These proteins compete for binding to RORE *cis* elements that regulate expression of clock genes, including *Bmal1* and the *Reverbs*, making these family members' interactions a self-contained regulatory loop that directly modulates BMAL1 expression to influence the core TTFL.

1.2. Circadian Clock Interaction with Metabolism, Chromatin Remodeling, and the Cell Cycle

While the composition of the circadian TTFL is relatively consistent, its coupling to inputs and outputs from other cellular signaling pathways varies substantially across cell types. Major pathways commonly integrated with the circadian TTFL, with functions that vary throughout and exert important influences upon developmental lineage restriction, include cellular metabolism, epigenetics, and the cell cycle (Figure 1).

1.2.1. Metabolism.—The primary function of circadian rhythms is to synchronize behavior and physiology with the diurnal rhythm of Earth rotating around its own axis as it revolves around the Sun. Perhaps the core function of this arrangement is to align the sleep/wake cycle of each organism with the solar cycle to maximize the opportunity to acquire available nutrients from its environment. It is therefore no surprise that extensive coupling exists between circadian rhythms and cellular metabolism throughout both development and adult function. For example, the abundance of ~50% of metabolites produced within the mouse liver is regulated by circadian clocks (Krishnaiah et al. 2017). These cyclically regulated metabolites include cofactors for chromatin-modifying enzymes such as nicotinamide adenine dinucleotide (NAD^+) and S-adenosyl methionine (SAM), suggesting that chromatin structure is dynamically regulated in tune with circadian rhythms through cellular metabolism (Krishnaiah et al. 2017).

Cellular metabolism includes the set of pathways through which molecules are broken down into smaller units to generate energy (catabolism) and through which biomolecules such as fatty acids, nucleotides, and amino acids are synthesized from smaller units (anabolism). The primary catabolic pathways present within all living cells include glycolysis, the

tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (OXPHOS). Together these pathways combine to break down glucose, generating crucial metabolites including most of the adenosine triphosphate (ATP) used by the cell to provide energy for biochemical reactions. Glycolysis, the TCA cycle, and OXPHOS each exhibit reciprocal interaction with circadian rhythms by requiring TTFL-expressed enzymes and generating metabolites involved in the modification of both chromatin and core clock proteins (Figure 1b, Table 1).

1.2.1.1. Interaction of glycolysis with circadian rhythms through NAD^+ and glucose catabolism.: Glycolysis is a sequence of cytosolic oxidation-reduction (redox) reactions that converts glucose into two pyruvate molecules, while also generating two net ATP and reducing two NAD^+ molecules to NADH. While glycolysis generates fewer net ATPs than OXPHOS, this pathway can function in a low-oxygen environment such as the postimplantation uterus and certain adult stem cell niches (Fischer & Bavister 1993, Simsek et al. 2010).

In particular, NAD^+ is a key metabolite for interactions among metabolism, chromatin remodeling, and the circadian clock (Figure 1a). NAD^+ shows robust diurnal rhythms in vitro and in vivo (Bellet et al. 2013, Nakahata et al. 2009, Ramsey et al. 2009), and operates as a cofactor for the sirtuins, a family of class III histone deacetylases (HDACs). NAD+ itself influences the circadian TTFL through the SIRT1-dependent deacetylation of PER2, a CLOCK:BMAL1 inhibitor (Asher et al. 2008). In turn, the circadian TTFL controls NAD^+ levels through the NAD^+ biosynthetic salvage pathway, in which nicotinamide is converted into the NAD^+ precursor β-nicotinamide mononucleotide. This reaction is catalyzed by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT), whose expression is driven by the direct binding of CLOCK:BMAL1 to E-boxes in the Nampt promoter (Nakahata et al. 2009, Ramsey et al. 2009). While formation of fructose-1,6-bisphosphate upstream of NAD^+ reduction is the rate-limiting step of glycolysis, increasing the concentration of NAD^+ is sufficient to increase ATP production in the cytosol (Pitelli et al. 2011). Thus, a transcriptional-enzymatic feedback loop controls NAD^+ biosynthesis and links circadian rhythms to the glycolytic catabolism of glucose.

Additionally, the availability of glucose itself contributes to circadian TTFL function by providing derivatives that serve as substrates for posttranslational modifications to core clock machinery. For example, CLOCK, BMAL1, and PER2 can be posttranslationally modified through the addition of O-linked N-acetylglucosamine (GlcNAc).This modification, catalyzed by the enzyme O-GlcNAc transferase (OGT), changes the activity of each clock protein (Kaasik et al. 2013, Li et al. 2013). Liver-specific OGT ablation dampens *Bmal1* oscillation.

1.2.1.2. Interaction of the TCA cycle with circadian rhythms through acetyl-CoA and α-ketoglutarate.: When oxygen is available, pyruvate (primarily generated by glycolysis) is transported to the inner mitochondrial matrix and converted into acetyl-CoA to fuel the TCA cycle. In this pathway, acetyl-CoA produced from pyruvate or through fatty acid oxidation is further oxidized to generate NADH and $FADH_2$. In addition to its catabolic function, several partially oxidized TCA cycle intermediates can be extracted to serve as building blocks for anabolic processes, including lipid, amino acid, and nucleotide

biosynthesis as well as posttranslational protein modifications (reviewed by Boroughs & DeBerardinis 2015; see also Wellen et al. 2009). In this way the TCA cycle represents a central hub of energy metabolism, in which many pathways involved in central carbon metabolism, chromatin remodeling, and circadian rhythms intersect. For example, TCA cycle intermediate α-ketoglutarate inhibits JumonjiC domain–histone demethylase (JARID), which associates with CLOCK:BMAL1 to facilitate *Per2* transcription (DiTacchio et al. 2011).

Acetyl-CoA, which interacts with circadian rhythms in part by providing acetyl groups as substrates for chromatin remodeling, exists in two separate pools in the cell: a mitochondrial pool that fuels the TCA cycle and a nuclear/cytosolic pool (reviewed by Albaugh et al. 2011). The nuclear/cytosolic pool, which is responsible for protein acetylation and fatty acid synthesis, is produced by two enzymes: ATP-citrate lyase (ACLY) and acetyl-CoA synthetase 1 (AceCS1). ACLY uses citrate produced during the TCA cycle as a substrate for the production of acetyl-CoA and is subject to circadian regulation. ACLY protein levels are cyclic in the liver (Mauvoisin et al. 2014), and ACLY activity controls global histone acetylation depending on glucose availability (Wellen et al. 2009).

Notably, AceCS1 is also subject to circadian regulation through NAD^+. To produce acetyl-CoA, AceCS1 uses acetate produced physiologically by alcohol metabolism and histone deacetylation as a substrate (reviewed by Shimazu et al. 2010). The deacetylation of Lys-661 on AceCS1 by SIRT1 leads to the activation of AceCS1 (Hallows et al. 2006). The deacetylation of AceCS1 is cyclic, and its rhythmicity requires both a functional circadian clock and the NAD^+-dependent deacetylase SIRT1 (Sahar et al. 2014). Thus, circadian rhythms directly link interconnected metabolic pathways to chromatin modification by dynamically regulating levels of acetyl-CoA produced through both ALCY and AceCS1 activity. In turn, intermediate metabolites produced by the TCA cycle such as α-ketoglutarate act as signaling molecules that reinforce the core circadian TTFL.

1.2.1.3. Interaction of OXPHOS with circadian rhythms through the NAD^+-dependent activation of SIRT1/3.: OXPHOS is the primary catabolic pathway in mature eukaryotic cells for generating ATP, and this pathway plays a critical role in maintaining bioenergetic homeostasis by linking glycolysis, the TCA cycle, and fatty acid oxidation with ATP synthesis. NADH and $FADH_2$, often from the TCA cycle, shuttle electrons to transmembrane protein complexes along the inner mitochondrial membrane that comprise the electron transport chain (ETC). The flow of electrons through these complexes pumps protons from the inner mitochondrial matrix to the outer matrix, creating a high electrochemical gradient. ATP is synthesized through ATP synthase, which uses the energy produced from transporting protons back to the inner mitochondrial matrix to catalyze adding a phosphate group to adenosine diphosphate (ADP).

OXPHOS is indirectly regulated by circadian rhythms, in part through NAD synthesis and thus the diurnal deacetylation of ETC Complex I through the activity of NAD^+-dependent HDACs. The reversible acetylation of Complex I inhibits its function, driving its rhythmic activity in a human hepatocyte cell line (HepG2 cells) (Cela et al. 2016). Complex I function peaks in phase with NAMPT and SIRT1/3 expression, implicating the circadian

NAD^+-salvage pathway as a key mediator of OXPHOS (Cela et al. 2016). Additionally, approximately 38% of mitochondrial proteins oscillate in abundance throughout the day in a PER1/2-dependent manner, including several rate-limiting enzymes contributing to the TCA cycle and ETC (Neufeld-Cohen et al. 2016). In turn, OXPHOS appears to directly regulate the core circadian clock by influencing BMAL1 expression. Disrupting OXPHOS either through pharmacological inhibition or the depletion of mitochondrial DNA dysregulates BMAL1 expression in HepG2 cells (Scrima et al. 2016). Together, these observations suggest that OXPHOS and circadian rhythms reciprocally interact through the production of metabolites that act as substrates for posttranslational modifications of metabolic enzymes and core circadian machinery alike.

1.2.1.4. Anabolism: pentose phosphate pathway (NADPH) and one-carbon metabolism (SAM).: Among anabolic pathways responsible for synthesizing biomolecules, the pentose phosphate pathway (PPP) and one-carbon metabolism each exhibit reciprocal regulation with the circadian clock through metabolite signaling pathways. The PPP uses the glycolysis intermediate glucose 6-phosphate to generate NADPH and ribose 5-phosphate, a precursor for nucleotide synthesis. Signaling from the PPP through production of the redox cofactor NADPH is an important regulator of transcriptional oscillations (Rey et al. 2016). Inhibiting NADPH production by the PPP remodels circadian gene expression in human osteosarcoma (U2OS) cells and is sufficient to alter rhythmic behavior and tissue clocks in *Drosophila* (Rey et al. 2016). However, whether metabolic oscillations of NADPH persist without a functional circadian TTFL is an area of future study that will be crucial to determining the full extent of interaction between circadian rhythms and the PPP.

Circadian rhythms are also influenced by cyclical methionine production, which is part of a broader set of transformations known as one-carbon metabolism. Methionine production generates S-adenosylmethionine (SAM) as an intermediate metabolite that interacts with circadian rhythms through its function as a methyl-donor cofactor. During transmethylation, SAM donates its methyl group to acceptor molecules to generate S-adenosylhomocysteine (SAH).SAM exhibits diurnal cycling in mouse liver, and the accumulation of SAH elongates the circadian period by inhibiting the m^6A-RNA methylation of core clock transcripts including *Clock*, *Bmal1*, and *Per1/2* (Fustin et al. 2013, Krishnaiah et al. 2017). Because SAH acts as a competitive inhibitor of SAM-dependent-transmethylation, the SAM/SAH ratio within the cell (known as the methylation potential) therefore dynamically regulates the circadian period by titrating the rate of m^6A-dependent mRNA degradation (reviewed by Carmel & Jacobsen 2011; see also Fustin et al. 2013).

1.2.2. Chromatin remodeling.—In eukaryotes, the storage and accessibility of genomic DNA is regulated at the level of chromatin, the DNA–protein complex that packages nuclear DNA. Chromatin is predominantly arranged into repeated arrays of nucleosomes composed of histone octamers. Due to their positive charge and the accessibility of their flexible N-terminal tails, histones are ideally suited to package DNA while acting as a platform for reversible, chemical posttranslational modifications that alter chromatin structure and function. These post-translational modifications include combinations of acetylation, methylation, phosphorylation, and ubiquitination events that

durably encode physiological and environmental information and in turn modulate the extent to which genome elements are either accessible or compacted depending on cellular needs (reviewed by Bannister & Kouzarides 2011, Smith & Shilatifard 2010). Specific cyclic chromatin transitions associated with circadian TTFL transcription events occur on a genome-wide scale (Masri & Sassone-Corsi 2010). For example, CLOCK:BMAL1 acts as a pioneer transcription factor capable of directly initiating chromatin remodeling, and rhythmic CLOCK:BMAL1 binding to E-box motifs drives circadian chromatin transitions through transcription of the ancillary clock protein DBP (Menet et al. 2014, Ripperger & Schibler 2006). In addition, the circadian TTFL indirectly contributes to chromatin remodeling through interaction with metabolic pathways that generate intermediate products capable of serving as substrates for chromatin modifications (Figure 1a,b).

1.2.2.1. Histone acetylation and deacetylation.: The acetylation of histone lysines is catalyzed by histone acetyltransferases (HATs) and requires acetyl-CoA as the acetyl-donating cofactor (reviewed by Sabari et al. 2017). Several proteins involved in the addition and removal of acetyl groups to histones are associated with the core circadian TTFL. CLOCK acts in concert with HATs such as p300, CBP (CREB-binding protein), and the CBP-associated factor PCAF (Curtis et al. 2004, Etchegaray et al. 2003, Lee et al. 2010, Takahata et al. 2000). CLOCK:BMAL1 inhibitors balance these modifications by recruiting HDACs: PER recruits SIN3A-HDAC1, and CRY1 associates with the complex SIN3B-HDAC1/2 (Duong et al. 2011, Naruse et al. 2004).

In particular, the NAD^+-dependent class III HDACs (sirtuins) represent a central node linking metabolism, chromatin remodeling, and circadian rhythms. SIRT1, SIRT3, and SIRT6 have each been functionally linked to circadian control, and they modulate cyclic outputs in response to metabolic cues across different subcellular compartments (reviewed by Sassone-Corsi 2016). While SIRT3 is localized to mitochondria and controls fatty acid oxidation and intermediary metabolism, SIRT1 and SIRT6 directly contribute to circadian patterns of histone deacetylation (Masri et al. 2013, 2014; Peek et al. 2013). SIRT6, which is the only sirtuin localized exclusively to chromatin, interacts directly with CLOCK:BMAL1 (Masri et al. 2014, Tennen et al. 2010). SIRT6 controls circadian chromatin recruitment of SREBP-1, cyclically regulating genes implicated in fatty acid and cholesterol metabolism (Masri et al. 2014). SIRT1 is localized to both the nucleus and cytoplasm and deacetylates histones and nonhistone proteins, including the clock proteins BMAL1 and PER2 (Asher et al. 2008, Hirayama et al. 2007). Through deacetylation of other histone-modifying enzymes such as methyltransferase MLL1 and metabolic enzymes such as AceCS1, SIRT1 positions circadian control of NAD^+ production as a principal regulator of epigenetic and metabolic function (Aguilar-Arnal et al. 2013, Sahar et al. 2014).

1.2.2.2. Histone methylation and demethylation.: Histone methylation reactions, which are catalyzed mostly on lysine residues by histone methyltransferases, are also capable of influencing circadian TTFL function. For example, the histone H3 lysine K4 methyltransferase MLL1 (mixed-lineage leukemia 1) interacts with CLOCK:BMAL1 and is needed for the proper regulation of circadian transcription (Katada & Sassone-Corsi 2010). The activating trimethylation of H3K4 has been linked to clock control, and it seems to

be essential to permit circadian chromatin transitions that promote clock gene expression (Ripperger & Schibler 2006). Conversely, the repressive trimethylation of H3K27 is clock controlled at the *Per1* promoter through a mechanism that involves the methyltransferase EZH2 (Etchegaray et al. 2006). All histone methyltransferases require SAM generated from methionine metabolism as the methyl-donor cofactor. Thus, in addition to its influence on m^6A-RNA methylation pathways, methionine metabolism interacts with circadian TTFL function through the SAM-dependent histone methylation.

The removal of methyl modifications is also critical for cyclic chromatin remodeling. However, while several histone demethylases associate with CLOCK:BMAL1, the mechanism through which histones are catalytically demethylated at clock genes remains unclear and is further complicated by the interactions among other chromatin-modifying enzymes. For example, lysine-specific demethylase 1a (LSD1) is an FAD (flavin adenine dinucleotide)-dependent histone demethylase that targets H3K4 and H3K9 and undergoes circadian phosphorylation when complexed with CLOCK:BMAL1 to promote the activation of *Per2* (Nam et al. 2014, Shi et al. 2004). However, the loss of circadian phosphorylation on LSD1 does not appear to affect H3K4 or H3K9 methylation but instead inhibits the acetylation of H3K9 (Nam et al. 2014). The mechanism through which LSD1 affects histone acetylation may be related to its association with SIRT1, but how H3K4 and H3K9 undergo circadian demethylation remains unclear (Mulligan et al. 2011). Another histone demethylase implicated in clock function is JARID1a. JARID1a also associates with CLOCK:BMAL1 to facilitate *Per2* transcription, but similarly to LSD1 its activity increases histone acetylation by inhibiting HDAC1 independent of demethylation (DiTacchio et al. 2011). Thus, understanding how circadian histone demethylation is achieved will likely require further study of the interactions among chromatin-modifying enzymes.

1.2.3. The cell cycle.—The cell cycle consists of the cell growth (G_1), DNA replication (S), and cell division (G_2/M) phases. Often the G_1/S transition, when signals ranging from growth factors to DNA damage are integrated to determine entry into S phase or exit to a noncycling quiescent phase (G_0), is rate determining for cell division. However, the G_2/M transition can also become rate determining under conditions of rapid proliferation in which the G_1 duration is shortened early in embryonic development (Figure 2) (Van Oudenhove et al. 2016). The circadian TTFL can exert control over both of these major checkpoints. Increasing the flexibility of this relationship, modeling approaches suggest that circadian TTFL coupling can drive ultradian (<24-hour period) or infradian (>24-hour period) harmonics of the circadian period in cell division in addition to the classical 24-hour rhythm, depending upon what intracellular coupling mechanisms and extracellular signaling are present (Almeida et al. 2020, El Cheikh et al. 2014, Feillet et al. 2014, Gupta et al. 2016, Kang et al. 2008, Traynard et al. 2016). Conversely, the cell cycle has also been proposed to entrain the circadian TTFL, and cyclin-dependent kinase signaling can regulate the circadian clock, suggesting that TTFL/cell cycle interactions are reciprocal (Bieler et al. 2014, Ou et al. 2019, Paijmans et al. 2016, Yan & Goldbeter 2019).

Perhaps the most prominent TTFL influence on the cell cycle is the BMAL1-driven, REVERBα suppression of *p53* and *p21* expression. This in turn gates the cyclin-dependent kinase (CDK) activity necessary for G_1-to-S progression, among other cell cycle

checkpoints (Figure 1c) (Fu & Kettner 2013, Gérard & Goldbeter 2012, Gréchez-Cassiau et al. 2008, Karimian et al. 2016). More recently, the TTFL has also been shown to exert control on the cell cycle downstream of the CDKs through posttranslational modification of the retinoblastoma protein (Lee et al. 2019).

Another salient example is c-MYC, which accelerates the cell cycle by promoting the G_1/S transition in spite of p27-dependent CDK inhibition and promotes the cyclin expression needed for progression through the S phase (Figure 1c) (Pérez-Roger et al. 1997). The CLOCK:BMAL1 complex and c-MYC inhibit one another's activity by a startling array of mechanisms, and this balance is disrupted by jet lag (Altman et al. 2015; Fu et al. 2002, 2005; Lee et al. 2019; Repouskou & Prombona 2016; Shostak et al. 2016). The TTFL/c-MYC balance is of particular interest in stem cells, given the role of c-MYC in pluripotency (Cartwright 2005, Takahashi & Yamanaka 2006). Other mechanisms coupling the circadian TTFL to the G_1/S transition include the clock-regulated expression of cyclins and Wnt signaling components, among other factors (Fu & Kettner 2013).

The classical mechanism coupling the circadian TTFL to the G_2/M checkpoint is CLOCK:BMAL1 control of *Wee1* expression, which inhibits the initiation of mitosis by the cyclin B1/CDK1 complex (Figure 1c) (Matsuo 2003). More recently, the TTFL regulation of cyclin B1 expression itself has been shown to be relevant (Farshadi et al. 2019). Meanwhile the Timeless protein, while arguably nonessential for TTFL function in mammals, mediates the reciprocal regulation of the TTFL and the cell cycle G_2/M checkpoint via Chk signaling (Engelen et al. 2013, Neilsen et al. 2019, Ünsal-Kaçmaz et al. 2005, Yang et al. 2010).

2. CIRCADIAN CLOCKS IN DEVELOPMENT

Embryonic development is a complex process with numerous decision points that any given cell must make to produce differentiated tissues, all of which first derive from pluripotent source material. Although most somatic cells are equipped with an intrinsic circadian clock, the TTFL arises gradually during embryonic development. Most data suggest that the core circadian feedback loops are completely absent in gametes and very early fertilized zygotes, with the TTFL being induced and progressively strengthened as lineage choice becomes more defined (Alvarez et al. 2003, Amano et al. 2009, Morse et al. 2003, Umemura et al. 2017). Thus, the circadian TTFL is completely absent in pluripotent stem cells (PSCs), and these are often used as a model for clock suppression and ontogeny in the context of development (reviewed by Umemura & Yagita 2020; see also Dolatshad et al. 2010, Yagita et al. 2010). At what point the TTFL is initially induced as pluripotency is lost; how metabolic, epigenetic, and cell cycle factors contribute to this shift; and the possible role of the TTFL in early lineage commitment remain unclear.

2.1. Circadian Clocks in Gametes

In both sexes, gametes lack an intrinsic circadian TTFL, and circadian regulation of their biology appears to occur non–cell autonomously. In male mammals, the protein expression of clock genes in testis germ cells is largely stable across the day; this may arise from a loss of transcriptional rhythms, as some studies find stable transcript expression (Alvarez et al. 2003, Morse et al. 2003), or from a lack of posttranscriptional circadian control, as

others report circadian or ultradian rhythms in transcript levels despite unchanging protein expression (Bebas et al. 2009, Liu et al. 2007). Histological approaches show stable PER1 expression in developing sperm cells that is controlled by the CREM transcription factor rather than by classical clock control (Alvarez et al. 2003, Morse et al. 2003). Most rhythmic clock gene expression in the male reproductive system is confined to extragametic structures, and poor sperm production in *Bmal1*$^{-/-}$ mice is attributable to defects in rhythmic testosterone production by Leydig cells of the testes rather than to cell-autonomous defects in sperm (Alvarez et al. 2008, Bebas et al. 2009). While spermatogenesis is circadian regulated, this regulation thus seems to be entirely mediated by extracellular signaling from surrounding clock-competent tissues (Rienstein et al. 1998).

A similar situation prevails in the ovary in female mammals (reviewed in Sellix & Menaker 2010). Unfertilized oocytes have static clock gene expression, with especially high levels of *Bmal1*, *Clock*, and *Cry1* transcripts that are likely maternally derived and may play a role in maintaining oocyte transcriptional repression (Amano et al. 2009, Ko et al. 2000). The ovary as a whole has inducible circadian TTFL function coupled to luteinizing and gonadotrophin hormone release, but this rhythm is localized in various support cell populations of the ovary and is excluded from quiescent and developing ova (Fahrenkrug et al. 2006, He et al. 2007, Karman & Tischkau 2006, Yoshikawa et al. 2009). A subset of these support cells' clocks gates the circadian timing of ovulation. Deletion of *Bmal1* in theca cells, but not in granulocytes, abolishes rhythmic sensitivity to luteinizing hormone exposure, impairing the normal circadian control of ovulation timing via hormone release (Mereness et al. 2016).

2.2. Circadian Clocks in Embryonic and Induced Pluripotent Stem Cells

While some core clock proteins such as BMAL1 are constitutively expressed early in embryonic development, circadian expression patterns only begin following the metabolic shift from glycolysis to OXPHOS in PSCs transitioning out of pluripotency (Umemura et al. 2019). During differentiation, CLOCK protein expression appears to be a rate-limiting factor for circadian TTFL network maturation (Umemura et al. 2017). CLOCK expression is suppressed posttranscriptionally in PSCs, and this suppression gradually subsides during embryonic development concomitant with an increased amplitude of circadian molecular oscillation (Figure 2) (Umemura et al. 2017). The primary mechanism of posttranscriptional repression of Clock mRNA appears to be Dicer/Dgcr8-dependent microRNA (miRNA)-mediated repression, as knockout of either *Dicer*or *Dgcr8* led to early-onset CLOCK expression in mouse embryonic stem cells (mESCs) (Umemura et al. 2017). Despite early CLOCK expression, however, *Dicer*$^{-/-}$ and *Dgcr8*$^{-/-}$ mESCs failed to demonstrate circadian rhythms even after DIV28, far beyond the previously observed milestone of their development (Umemura et al. 2017). In this study the lack of circadian oscillation in these cells was ascribed to their failure to properly differentiate, highlighting the crucial role of the transition from pluripotency in the development of the circadian clock. In line with this idea, the disruption of cellular differentiation by genetic and epigenetic perturbations [such as DNA methyltransferase (*Dnmt1*, *Dnmt3a*, and *Dnmt3b*) knockouts or c-MYC overexpression] prevents the development of circadian rhythms in mESCs (Figure 1) (Umemura et al. 2014, 2017).

In fact, the reprogramming of somatic cells using Yamanaka factors [OCT3/4, KLF4, SOX2, and c-MYC (Takahashi & Yamanaka 2006)] to produce human induced PSCs (hiPSCs) abolishes the established circadian rhythm, further demonstrating that circadian clock development is tightly coupled with cellular differentiation (Yagita et al. 2010). In the early stages of reprogramming, Yamanaka factors induce cell proliferation, which increases mitochondrial respiration and shuttles glucose to the PPP to meet the demand for increased nucleotide synthesis (Hawkins et al. 2016). Peaks in cell proliferation and OXPHOS contribute to reactive oxygen species (ROS) accumulation and hypoxia-inducible factor 1α activation, which drives the metabolic switch toward glycolytic energy production (Hawkins et al. 2016). Together these observations lay the foundation for a paradigm through which the dynamic changes in cellular metabolism, chromatin remodeling, and the cell cycle that occur during cell fate transition are intertwined with ontogeny of the circadian clock.

In the mouse embryo, tissues such as the heart and liver begin to demonstrate synchronous circadian rhythms at E13 (Dolatshad et al. 2010, Inada et al. 2014, Landgraf et al. 2015). By E14.5, synchronous circadian rhythms are observable in the mouse suprachiasmatic nucleus (SCN), the region of the hypothalamus responsible for aligning circadian rhythms across peripheral tissues (Carmona-Alcocer et al. 2018). The whole-body circadian clock also emerges gradually during human development. Circadian rhythms in body temperature are present in newborn infants, but circadian rhythms in the sleep/wake cycle only stabilize after about six weeks of life (Kleitman & Engelmann 1953, Rivkees 2003). The gradual development of the mouse and human circadian clocks is recapitulated in vitro: The differentiation of PSCs drives the cell-autonomous circadian oscillation of clock gene expression only after 14 days in vitro (DIV14) in mouse PSCs and after DIV42 in human PSCs (with robust circadian rhythms only observable in human PSCs after DIV90) (reviewed by Umemura & Yagita 2020; see also Umemura et al. 2019, Yagita et al. 2010).

2.3. Differentiation of Pluripotent Stem Cells by Changes to Metabolites That Regulate Chromatin Structure and Eventually Interact with Circadian Rhythms

Pluripotency is the ability of a stem cell to differentiate into any of the three primary germ layers: ectoderm, mesoderm, and endoderm. These three layers can give rise to all cell types of the adult body. Rather than a single fixed stage, pluripotency encompasses a gradient of cellular phenotypes from naïve PSCs of the inner cell mass in early blastocysts to primed PSCs of early postimplantation epiblasts (Figure 2) (reviewed by Weinberger et al. 2016). Although pluripotency exists transiently in vivo, several stages can be stably recapitulated in vitro (Evans & Kaufman 1981).In addition to the ability to give rise to all somatic lineages, naïve PSCs are able to incorporate into a developing blastocyst to create chimeric embryos and use both glycolysis and OXPHOS to generate ATP. In contrast, primed PSCs retain pluripotency, but unlike naïve PSCs they are incapable of integrating into a developing blastocyst to form chimeric embryos and are predominantly glycolytic. Although the mouse naïve-to-primed PSC transition is accompanied by increased glycolytic activity, in human PSCs this increase is not observed (Gu et al. 2016, Sperber et al. 2015, Zhou et al. 2012). However, during differentiation both mouse and human primed PSCs undergo a metabolic

shift toward using OXPHOS as the primary source of ATP generation (Diano & Horvath 2012, Zhang et al. 2011).

Metabolites appear to play a significant role in regulating cell fate and development, including PSC differentiation. This regulation occurs primarily through the modification of chromatin that contributes to gene expression (reviewed by Mathieu & Ruohola-Baker 2017, Tsogtbaatar et al. 2020). Interestingly, many of the metabolites that contribute to the maintenance of stem cell pluripotency also influence the function of core circadian machinery following the development of circadian rhythms in differentiating cells (Table 1). This crucial link between cell fate acquisition and circadian TTFL function underlies the potential of metabolic chromatin remodeling pathways to drive the overall trajectory of development with progressive circadian entrainment as a central conductor. For example, entrainment to circadian feeding/fasting cycles triggers islet maturation in human ESCs (hESCs) by inducing the cyclic synthesis of energy metabolism and insulin secretion effectors (Alvarez-Dominguez et al. 2020). After entrainment, hESC-derived islets gain persistent chromatin changes and rhythmic insulin responses with a raised glucose threshold, a hallmark of functional maturity (Alvarez-Dominguez et al. 2020). As such, exploring how metabolites influence cell fate acquisition will provide crucial insights into how cellular differentiation might drive the initiation of circadian rhythms.

2.3.1. The TCA cycle (acetyl-CoA and α-ketoglutarate).—As a central pathway linking chromatin remodeling, glycolysis, and OXPHOS, the TCA cycle is well positioned to influence the metabolic shifts that drive the transition from naïve to primed pluripotency and ultimately the differentiation of primed PSCs to multipotent primordial germ cells. For example, the TCA cycle intermediate α-ketoglutarate promotes naïve stem cell pluripotency through the elevated demethylation of histones and DNA (Carey et al. 2015, Tischler et al. 2019). However, following the metabolic shift toward glycolytic dependence in the transition to primed pluripotency, α-ketoglutarate functions to promote primordial germ cell differentiation by supporting oxidative metabolism (TeSlaa et al. 2016, Tischler et al. 2019). Blocking acetyl-CoA production by the TCA cycle causes a loss of pluripotency in hESCs, while preventing acetyl-CoA consumption delays differentiation (Moussaieff et al. 2015). During the first 24hours of mESC differentiation, a metabolic switch takes place in which pyruvate becomes fully oxidized in mitochondria, leading to acetyl-CoA deprivation, the loss of histone acetylation, and the subsequent loss of pluripotency marker expression (Moussaieff et al. 2015). Together these observations demonstrate that crucial phases of cell fate transition are driven by metabolic shifts that alter substrate availability for chromatin remodeling.

2.3.2. Glycolysis (NAD^+).—The connection between acetyl-CoA deprivation and histone acetylation in the maintenance of pluripotency implicates the NAD^+-dependent HDAC SIRT1, which promotes acetyl-CoA production through the deacetylation of AceCS1 and directly participates in histone deacetylation. Indeed, the activity of SIRT1, which has been shown to be crucial for reprogramming, regulates mESC pluripotency (Calvanese et al. 2010, Y. Lee et al. 2012, Tang et al. 2014). Interestingly, the downregulation of SIRT2 and the upregulation of SIRT1 are together a molecular signature of primed human PSCs; these

proteins regulate primed pluripotency through distinct mechanisms (Cha et al. 2017). NAD^+ also promotes the cell fate transition from primed to naïve pluripotency in hESCs through the acquisition and maintenance of a bivalent metabolism (Lees et al. 2020). Increasing intracellular NAD^+ levels in hESCs stimulates mitochondrial metabolism while suppressing glycolytic metabolism. This bivalent metabolism has been shown to coincide with increased pluripotency marker expression and decreased global H3K27 trimethylation, suggesting a potential link to SAM metabolism and methylation potential (Lees et al. 2020).

2.3.3. The methionine cycle (SAM).—SAM levels are crucial during the naïve-to-primed hESC transition, in which the epigenetic landscape changes through increased H3K27 trimethylation (Sperber et al. 2015). H3K27 trimethylation regulates key signaling pathways important for the metabolic changes necessary for early human development (Sperber et al. 2015, Z. Xu et al. 2016). These trimethyl modifications are generated by Polycomb repressive complex 2, which among other proteins includes the methyltransferase EZH2 (Vizán et al. 2015). Methionine and SAM are required for hESC self-renewal through the methylation of other histone targets, and the depletion of SAM leads to reduced H3K4 trimethylation and defective maintenance of the hESC state (Shiraki et al. 2014). In naïve stem cells, SAM is consumed by the metabolic enzyme nicotinamide N-methyltransferase, limiting its availability and thus maintaining low levels of histone methylation (Sperber et al. 2015). SAM, the universal donor of methyl groups for histone modifications, is therefore a key regulator both for promoting the primed pluripotent state of ESCs and for regulating their differentiation.

2.3.4. Core clock gene function prior to differentiation.—The observation that CLOCK expression is posttranscriptionally suppressed in mESCs prior to differentiation demonstrates that CLOCK function is not required for either PSC self-renewal or the transition from naïve to primed pluripotency (Umemura et al. 2017). Interestingly, however, *Clock* knockout in mESCs appears to decrease proliferation and increase apoptosis, potentially indicating a function of CLOCK in PSCs undergoing the first steps of cellular differentiation (Lu et al. 2016).

In contrast to CLOCK, BMAL1 is robustly expressed in both mouse and human PSCs, and several recent reports have demonstrated diverse functions of this transcription factor prior to the development of circadian rhythms. For example, loss of BMAL1 inhibits glycolytic metabolism in mouse PSCs and appears to disrupt the ability of these cells to exit pluripotency (Ameneiro et al. 2020). *Bmal1*-knockout mESCs express higher levels of Nanog and exhibit altered expression of pluripotency-associated signaling pathways (Ameneiro et al. 2020). Furthermore, *Bmal1*-knockout mESCs display deficient multilineage cell differentiation capacity during the formation of teratomas and gastrula-like organoids (Gallardo et al. 2020). Considering the clock-independent functions of BMAL1 that have been observed both prior to circadian clock development and in adult function, it will be interesting to explore the factors that dynamically regulate the extent to which BMAL1 functions independently of the core circadian TTFL within single cells (Lipton et al. 2015).

3. CIRCADIAN CLOCKS IN ADULT STEM CELLS

3.1. Neural Stem Cells

Most ectodermally derived neural stem cells (NSCs) that populate the developing nervous system, radiating neurons and glia outward from the ventricles, are transient. What NSCs remain in the adult nervous system are canonically localized to specific niches within the telencephalon that have relatively high baseline levels of neurogenesis: the subgranular zone and dentate gyrus of the hippocampus as well as the subventricular zones (SVZs) of the lateral ventricles. Most research on circadian control of neurogenesis has focused on these traditional niches. More recent studies also suggest broader circadian control of inducible adult NSCs in ventral forebrain-derived structures including the retina and hypothalamus (Figure 3).

3.1.1. Hippocampal neural stem cells.—Prolific neurogenesis in hippocampal NSC niches underpins the region's impressive plasticity, which is essential for its role in learning and memory formation. Proliferating hippocampal NSCs in vivo express BMAL1 and PER2 (Borgs et al. 2009, Bouchard-Cannon et al. 2013) and lack PER1 (Gilhooley et al. 2011). However, most circadian gene expression analyses purporting to show either rhythmic (Bouchard-Cannon et al. 2013) or constitutive (Borgs et al. 2009) clock gene expression in hippocampal NSCs only looked broadly at the hippocampal domains in question without colocalization with markers of NSC fate, rendering the experiments uninformative. In the only direct test (Malik et al. 2015a) of whether hippocampal NSCs have an autonomous clock to date, PER1::Luc rhythms were detected in hippocampal NSC neurosphere cultures shortly after, but not before, the induction of differentiation. While the possibility of a PER1-independent clock in undifferentiated hippocampal NSCs deserves further scrutiny, the preponderance of available evidence currently suggests that they lack an autonomous clock.

Most studies (reviewed in Draijer et al. 2019), particularly later ones with more refined methodologies, agree that hippocampal NSC proliferation is circadian across vertebrates, with peaks in the G_1/S transition in the morning, S phase early in the evening, and mitosis overnight. In young mice constitutively null for *Bmal1*, *Per2*, or *Reverbα*, hippocampal NSCs are arrhythmic and hyperproliferative, demonstrating that clock-controlled cues regulate their mitosis (Ali et al. 2015, Bouchard-Cannon et al. 2013, Schnell et al. 2014). In particular, multiple clock components, including BMAL1 and PER2, seem to gate entry of quiescent hippocampal NSCs into mitosis, while BMAL1 serves the additional role of regulating the number of divisions an active progenitor undergoes (Bouchard-Cannon et al. 2013). When clock control of these NSCs' cell cycles is compromised, hyperproliferation exacerbates the rundown of the finite proliferative capacity of hippocampal NSCs, leading to decreased hippocampal neurogenesis with age in *Bmal1*$^{-/-}$ mice in vivo (Ali et al. 2015, Encinas et al. 2011). Similar results have been reported in culture. Young adult *Per2*$^{-/-}$ hippocampal NSCs yield larger neurospheres with increased percentages of mature neurons at the expense of undifferentiated NSCs (Borgs et al. 2009). In contrast, older *Bmal1*$^{-/-}$ and *Cry1/2*$^{-/-}$ hippocampal NSCs, and *Reverbβ*$^{-/-}$ forebrain NSCs of unreported age, generate smaller, more differentiated neurospheres (Malik et al. 2015a, Shimozaki, 2018). In sum, the

niche circadian clock seems to antagonize hippocampal NSC proliferation and prevent their premature exhaustion.

Most mechanisms proposed to couple the clock to hippocampal NSC mitosis are cell autonomous. Modeling based on *Bmal1* and *Per2* loss-of-function phenotypes has suggested an inhibitor of cyclin-D/CDK4–6 as a key clock-regulated mediator of the cell cycle (Bouchard-Cannon et al. 2013). This model is consistent with rhythmic *p20* expression at the G_1/S transition and the successful delay of cell cycle progression by a selective CDK4–6 inhibitor in zebra fish NSCs (Figure 1c) (Akle et al. 2017). Separately, REVERBα is proposed to suppress hippocampal NSC proliferation by downregulating expression of the FABP7 fatty acid–binding protein (Schnell et al. 2014). Presumably, currently unidentified clock-controlled extracellular signaling couples the niche circadian TTFL to these cell-autonomous proximal regulators of NSC mitosis.

Bmal1$^{-/-}$ hippocampal NSCs tend to overproduce astrocytes at the expense of neurons both in vivo and in neurosphere culture, suggesting that BMAL1 has a proneural effect on neuron/glial fate choice in differentiating progenitors that is independent of the clock regulation of mitosis (Ali et al. 2015, Malik et al. 2015a). However, *Per2*$^{-/-}$ neurospheres from P10 mouse pups yield a higher percentage of neurons with no change in glial yields, while *Cry1/2*$^{-/-}$ neurospheres from 5- to 8-month-old adult mice yield an increase in astrocyte production with no change in neuron yield (Borgs et al. 2009, Malik et al. 2015a). Given the age confound, *Per* and *Cry* mutants' apparent effects on the neuron/glia fate decisions of NSCs seem likely to simply reflect the overproliferation and depletion of NSCs, as hippocampal NSCs produce more glia at the end of their useful lives as progenitors (Encinas et al. 2011). Thus, BMAL1 has a bona fide proneural influence on the fate of individual hippocampal NSCs' daughter cells independent of the TTFL, while the circadian clockwork as a whole indirectly maintains the neurogenic capacity of the hippocampal niche by limiting the NSCs' rate of proliferation over developmental time.

3.1.2. Subventricular zone neural stem cells of the lateral ventricles.—

High baseline neurogenesis in the SVZ provides replacement cells to the olfactory bulb, where exposure to aerosolized environmental toxins necessitates continuous turnover of mature cells throughout the life span. Similarly to the hippocampus, SVZ NSCs express some clock genes in arrhythmic or very weakly rhythmic patterns that are rapidly induced into rhythmic expression upon exposure to differentiation cues (Kimiwada et al. 2009, Malik et al. 2015b). Of some concern, PER1::Luc is again the only sensor used to directly demonstrate this in live-imaged SVZ neurospheres. *Per1* has among the weakest and least rhythmic expression among clock genes in undifferentiated SVZ NSCs in the Kimiwada et al. (2009) study, while some other clock genes including *Per2* appear to be potentially circadian in their expression, albeit with very low amplitude. Thus, while available evidence argues against the presence of a cell-autonomous TTFL in SVZ NSCs, this case further emphasizes the need for an attempt to discern whether PER1-independent rhythms are present in undifferentiated NSCs of both major telencephalic neurogenic niches.

Unlike in the hippocampus, the timing of SVZ NSC proliferation may be uncoupled from the circadian clock, with the G_2/M phase transition occurring equally across the day and

no data currently available for other stages of the cell cycle (Tamai et al. 2008). However, *Bmal1*$^{-/-}$ SVZ NSCs in 3-month-old mice show reduced proliferation in vivo (Ali et al. 2019), while *Bmal1* and *Clock* RNAi reduce the production of mature neurons but not astrocytes from 6-week-old SVZ-derived neurospheres (Kimiwada et al. 2009). These data are consistent with the rundown of the SVZ NSCs with age in the absence of *Bmal1*, as well as a role of BMAL1 in promoting neural fate in SVZ daughter cells. Thus, SVZ NSCs are probably regulated by the circadian clock more similarly to hippocampal NSCs than is suggested by the currently available data directly examining this question.

3.1.3. Ventral forebrain neural stem cells.—The retina and hypothalamus of the mammalian brain develop from a common compartment that segregates prior to developmental neurogenesis, producing distinct transient NSC pools that yield neurons and glia with dramatically different morphology and function to serve their respective visual and organism-wide homeostatic functions (reviewed in Bedont et al. 2015, Byerly & Blackshaw 2009, Cepko 2014). Among the more obvious legacies of this shared heritage are Müller glia of the retina and tanycytes of the tuberal hypothalamic third ventricle, which are distinct populations of Rax(+) radial glia-like NSCs. While to our knowledge circadian regulation of these niches' neurogenesis has not been studied directly, the robust roles of the retina and hypothalamus in organismal circadian rhythmicity suggest that such regulation is extremely likely, and considerable circumstantial evidence supports this viewpoint.

Tanycytes are traditionally divided into lateral α1/2 and ventral β1/2 subpopulations by morphology (Rodriguez et al. 2005). Tanycytes regulate such aspects of homeostasis as feeding and metabolism, seasonality, and hormone release, both directly and by generating daughter cells that integrate into the relevant hypothalamic nuclei. While the sources of hypothalamic neurogenesis are hotly debated, studies (reviewed in Yoo & Blackshaw 2018) have characterized two distinct tanycytic neurogenic niches that contribute at least some of these cells in mammals. β2-Tanycytes of the hypothalamic proliferative zone (HPZ) form a diet-responsive niche that is primarily neurogenic in adolescent and young adult mice (Haan et al. 2013, D. Lee et al. 2012). In contrast, α-tanycytes are robustly proliferative in a growth factor–regulated manner as late as 16 months of age in mice (Chaker et al. 2016, Pérez-Martín et al. 2010, Robins et al. 2013). Likely as a consequence of their extended proliferation, α- but not β-tanycyte pools run down with aging in mice in a manner reminiscent of hippocampal NSCs (Chaker et al. 2016, Encinas et al. 2011). In contrast to the telencephalic neurogenic niches, the tanycytes of the third ventricle have robustly rhythmic clock gene expression. The transcript-level expression of clock genes tends to be either across the entire tanycyte population or enriched in the HPZ (Lein et al. 2007, Yasuo et al. 2008). However, close visual inspection of in-slice sample images from the only protein-level data available suggests that the PER2::Luc signal is rhythmic in both the nonproliferative β1 and adult-quiescent β2 populations, with much stronger amplitude and overall higher expression in the former than in the latter. The relative strength of the α-tanycyte rhythm in these images is difficult to make out due to low resolution and strong signal in the adjoining parenchyma, but these cells appear to less robustly express PER2::Luc than the adjacent hypothalamic nuclei and may not have active autonomous clocks (Guilding et al. 2009). While further study is warranted, it is tempting to speculate

that, at least for the HPZ, the observed intermediate rhythms in clock gene expression contribute to their progressive quiescence as mice mature into adulthood.

Such a role would be consistent with the developmentally related Müller glia of the retina. While these cells are constitutively proliferative throughout the life span in nonmammalian vertebrates, including zebra fish, they are generally minimally proliferative or nonproliferative in adult mammals (Bernardos et al. 2007, Das et al. 2006). What is less clear is whether Müller glia retain any residual neurogenic capacity in mammals in response to injury. While bipolar cell and rod production sourced from Müller glia has been reported after injury in rat retina, it is unclear whether mammalian Müller glia are capable of acting as NSCs supporting full retinal regeneration, which is seen after injury in less complex vertebrates (Ooto et al. 2004, Thummel et al. 2008). Adult Müller glia have a robust autonomous circadian clock (L. Xu et al. 2016). In addition, intriguingly, transcript expression of *Per*, *Cry*, and *Reverb* inhibitors of Bmal1 is higher, while positive regulators of *Bmal1* are generally lower in Müller glia compared to those in retinal progenitors, and retinal progenitor–like changes in many of these genes' expression occur in Müller glia injured with N-methyl-d-aspartate (NMDA) (Lin et al. 2019). Phototoxic insult has somewhat similar effects on Müller glia, suppressing the expression of clock genes, including *Bmal1* and *Per1*, which are central to their clockwork (Sifuentes et al. 2016, L. Xu et al. 2016). Much as with hypothalamic tanycytes, this hints that the circadian clock may hinder dedifferentiation and neurogenesis of Müller glia in the mammalian retina. The potential for the application of latent Müller glia neurogenic capacity to regenerative medicine is a topic of considerable translational interest (reviewed in Lahne et al. 2020), making circadian manipulation as a possible tool for modifying this capacity a topic of particular interest.

3.2. Epithelial Stem Cells

The hair follicle is a useful case study of a relatively well-characterized, mixed population of stem cell populations hailing from two distinct developmental lineages: hair follicle stem cells (HFSCs) derived from the ectoderm proper and melanocyte stem cells (MnSCs) derived from the neural crest (Figure 3). These stem cells provide the fuel for a lifelong cycle of anagen (growth), catagen (degeneration), and telogen (quiescent) stages in the hair follicle as a whole. Commingled HFSCs and MnSCs are housed in a relatively quiescent state in a bulge adjoining each hair follicle and, at some stages of the growth cycle, in a more proliferative primed state in secondary hair germ; the hair germ in turn produces more-differentiated transient amplifying cells (TACs) that actually generate most of the terminal cell types incorporated into the hair follicle. In physiological conditions, the interfollicular skin is replenished by a physically separate population of epidermal keratinocyte stem cells (EKSCs); these cells proliferate at the basal layers of the skin and migrate upward as they first differentiate into TACs and then give rise to terminally differentiated cells, similar to the process in the hair follicle (reviewed in Hsu et al. 2014) (Figure 3).

3.2.1. Hair follicle stem cells.—HFSCs are impressively multipotent, with the capacity to contribute TACs to supplement epidermal keratocyte replenishment of the interfollicular skin after wounding (Ito et al. 2005) and produce numerous ectodermal and mesodermal

lineages under laboratory conditions (reviewed in Najafzadeh 2015). Despite their malleable fate, the overall population of HFSCs has a robust intrinsic circadian clock even during the quiescent telogen phase (Janich et al. 2011, Lin et al. 2009, Plikus et al. 2013). The expression of HFSC clock genes tracks with the expression of key regulators of their proliferation and differentiation; similarly, HFSC subpopulations collected simultaneously and parsed by clock amplitude unequally express differentiation factors (Janich et al. 2011, Lin et al. 2009). Meanwhile, across time, higher HFSC clock amplitude as anaphase begins correlates with self-renewal and outer root sheath fates in daughter cells, while primed germ progenitors are produced by HFSCs oscillating at lower amplitude in late catagen (Ito et al. 2004, Janich et al. 2011, Rompolas et al. 2013). More lineage-committed TAC progeny have even higher amplitude rhythms than HFSCs during anaphase, demonstrating a progressive increase in clock amplitude during differentiation within the hair follicle niche (Plikus et al. 2013). Together, this suggests a role for the HFSC and TAC clocks in setting the terminal fates of their progeny.

The circadian clock plays a well-established role in driving the progression of HFSCs through the proliferative, refractory, and competent cell cycle states necessary for cyclical hair growth (Janich et al. 2011, Plikus et al. 2011). Clock amplitude in individual HFSCs positively correlates with the expression of pro-proliferative markers, including the Wnt and BMP-inhibitor pathways (Janich et al. 2011). Many of these signaling genes have Bmal1-competent E-boxes in their enhancers, and K14Cre-mediated *Bmal1* deletion in HFSCs reduces these pro-proliferative genes' expression and impedes cells' proliferative capacity (Janich et al. 2011). This is consistent with gene expression changes in global *Bmal1*-null skin that specifically upregulate the inhibitory cell cycle regulator *Cdkn1a*, likely secondary to a dysregulated ROR/REVERBα balance (Lin et al. 2009). Global *Period1/2* deletion enhances the HFSC proliferative capacity, supporting an interpretation of the *Bmal1* proliferation phenotypes as clock effects (Janich et al. 2011).

Clock-null HFSC proliferation defects would, at first blush, then be expected to hinder hair growth. Indeed, a stalled first anagen delays initial hair growth in global $Bmal1^{-/-}$ and $Clock^{-/-}$ mice (Lin et al. 2009). However, K14Cre-mediated *Bmal1* deletion in HFSCs fails to phenocopy first anagen delay (Geyfman et al. 2012, Janich et al. 2011), suggesting that Bmal1 dependence of the first anagen is a local niche effect on the HFSCs. Alternatively, the pacing of the first anagen may be set not by the HFSCs but by the more-differentiated TAC progenitor population. TACs contribute most cells directly incorporated into the hair follicle; these cells have the timing but not the overall throughput of their mitosis disrupted by K14Cre-driven *Bmal1* or global *Cry1/2* deletion (Geyfman et al. 2012, Plikus et al. 2013, Rompolas et al. 2013).

Surprisingly, tamoxifen-gated *Bmal1* deletion in adulthood accelerates hair regrowth after depilation, suggesting that the clock inhibits hair growth later in life (Yang et al. 2016). Intriguingly, these results could reflect gating of the clock by pro–hair growth effects of light. Light signaling conveyed through a retina → SCN → sympathetic relay promotes hair regrowth after manual depilation via accelerated anagen entry (Fan et al. 2018). While the authors did not demonstrate a relationship between this light promotion of hair regrowth and gating by either the HFSC or TAC clocks, a relationship seems plausible.

3.2.2. Melanocyte stem cells.—MnSCs were recently demonstrated to have a cell-autonomous clock (Buhr et al. 2019). Human melanocyte phenotypes including dendritic outgrowth are modified by *Bmal1* and *Per2* short hairpin RNA (shRNA) injected into the intact hair follicle but not into differentiated melanocytes in culture, possibly reflecting a clock role in MnSC differentiation (Hardman et al. 2015). MnSCs also express neuropsin, allowing local entrainment of their rhythms by light (Buhr et al. 2019). One intriguing possibility is that local clock entrainment in MnSCs tweaks melanocyte proliferation and differentiation to help implement pigmentation changes dependent upon light dosage. Indeed, transcriptomics in snowshoe and mountain hare skin demonstrates seasonal changes not only in pigmentation gene expression but also in Wnts, other HFSC/MnSC proliferation signals, and visual transduction genes (Ferreira et al. 2020). However, the sympathetic signaling that mediates the SCN-dependent light responsiveness of HSFCs also appears to regulate age-related graying through MnSC β-adrenergic receptors (Fan et al. 2018, Zhang et al. 2020). While the MnSC clock itself is insensitive to SCN light cues (Buhr et al. 2019), whether internal and/or external light sensitivity guides other aspects of MnSC adaptation to light is unclear.

3.2.3. Epidermal keratinocyte stem cells.—Cultured human EKSCs have robust cell-autonomous clocks that drive five daily peaks of EKSC gene expression: the first three peaks of (*a*) *Reverbα/β*, (*b*) *Per1/3*, and (*c*) *Per2*+*Cry2* correlate with distinct modules of genes required for EKSC differentiation (Janich et al. 2013). Administering Ca^{2}+ or BMP to EKSCs during these peaks exerts a stronger differentiation response than at other times, and forcibly overexpressing *Per1* or *Per2* forces differentiation of EKSCs (Janich et al. 2013). Both $Clock^{-/-}$ and $Per2^{-/-}$ mice exhibit skewing of luminal and epithelial cell fates present in mammary tissue, supporting a role for the clock in guiding EKSC differentiation (Casey et al. 2016, McQueen et al. 2018). EKSC clock expression peaks of (*d*) *Cry1* and (*e*) *Bmal1* associate with modules controlling DNA repair and cell division (Janich et al. 2013). Constitutive $Bmal1^{-/-}$ and $Clock^{-/-}$ mutants, as well as K14Cretargeted Bmal1 deletion, result in the hyperproliferation of murine EKSCs, suggesting that the clock's effect on mitosis in this lineage is inhibitory (Casey et al. 2016, Geyfman et al. 2012).

3.3. Bone Marrow Stem Cells

Much like the hair follicle, the bone marrow presents another useful case of distinct adult stem cell lineages colocalized within a common niche: mesoderm-derived hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) derived from the neural crest (Isern et al. 2014) (Figure 3). HSCs give rise to the multipotent progenitors and common lymphocyte progenitors required for diverse white and red blood cell production. In service of this function, HSCs are unusual in being able to leave their niche, trafficking out into the blood and sites important for immune function such as the spleen and thymus, all while retaining the ability to home back into the bone marrow for maintenance and self-renewal (Kumar et al. 2019). Within the bone marrow, HSCs occupy a heavily vascularized stromal niche in close proximity to the MSCs that is distinct from the endosteal compartment housing more lineage-restricted progenitor cells (Ding & Morrison 2013). The MSCs maintain conditions suitable for HSC self-renewal and help to regulate HSC trafficking in addition to their own role as stem cells for adipogenesis (fat), osteogenesis (bone), and

chondrogenesis (cartilage) (reviewed in Murray et al. 2014; see also Méndez-Ferrer et al. 2010).

3.3.1. Hematopoietic stem cells.—An HSC-enriched fraction of blood and tissues is obtainable by flow cytometry. Furthermore, host bone marrow HSCs can be readily ablated and replaced with donor HSCs, allowing researchers to easily distinguish cell-autonomous and non-cell-autonomous effects. These tools were leveraged to show that HSCs have a functional circadian TTFL (Garrett & Gasiewicz 2006, Puram et al. 2016, Tsinkalovsky et al. 2006). Indeed, Clock1a expression is an instructive positive regulator of hematopoietic system formation in zebra fish development via control of rhythmic Smad3 expression involved in the nodal induction of mesoderm (Bian et al. 2017). Clock similarly regulates Smad3 expression in 3T3 cells, suggesting that an already-functional circadian TTFL may be essential for HSC production in mammalian development (Bian et al. 2017).

Circadian rhythms in HSC number and associated colony-forming activity have generally been reported in the blood and spleen (García-García et al. 2019, Golan et al. 2018, Kollet et al. 2013, Lucas et al. 2008, Méndez-Ferrer et al. 2008, Stenzinger et al. 2019), as well as by most studies in bone marrow (Garrett & Gasiewicz 2006, Kollet et al. 2013, Singh et al. 2011; but see also Stenzinger et al. 2019). Both rhythms and overall levels of HSCs are perturbed by circadian disruptions, including constant light, jet lag, and most clock gene deletions examined, often but not always in a non-cell-autonomous manner (Bhatwadekar et al. 2013, Kollet et al. 2013, Méndez-Ferrer et al. 2008, Singh et al. 2011, Stenzinger et al. 2019; but see also Bhatwadekar et al. 2017, Cao et al. 2017, Lucas et al. 2008, Wang et al. 2016). Contradictory reports in the literature likely stem from multiple sources, including a tendency to infer circadian rhythmicity from two daily time points; HSC numbers reflecting the integration of HSC cell division and specification dynamics with HSC migration dynamics; and the modulation of all of these factors by aging. We explore these interactions at length below.

Cell division in whole bone marrow of mammals, ranging from mice to humans, definitively occurs on a circadian rhythm (Aardal & Laerum 1983, Bourin et al. 2002, Clark & Korst 1969, Scheving et al. 1978, Smaaland et al. 1992). However, multipotent and lineage-committed progenitors contribute to this, and the rhythmic nature of HSC cell division is unclear. A human $CD34^+$ population enriched for HSCs reportedly displays circadian DNA replication (Abrahamsen et al. 1998). Meanwhile, the percentage of murine LSK HSCs in quiescence varies in more of a 12-hour ultradian pattern, with peaks in HSC differentiation/egress at ZT5 and self-renewal at ZT17 reported to be times of low HSC quiescence (Garrett & Gasiewicz 2006, Golan et al. 2018). Theonly study we found reporting no time-of-day difference in DNA replication of LSK HSCs in blood, spleen, and bone marrow looked only at ZT2 and ZT14 (Stenzinger et al. 2019), time points with similar HSC quiescence rates that predictably generated a false negative result (Garrett & Gasiewicz 2006). Thus, time of day does seem to affect HSC cell division, although more direct studies examining LSK HSC cell division with finer circadian time courses are warranted. *Bmal1*$^{-/-}$ HSCs show no defects either in colony formation in vitro or in bone marrow renewal in competition with wild-type HSCs in vivo (Ieyasu et al. 2014), suggesting that the cell-autonomous TTFL is not directly coupled to the cell cycle in HSCs. However, perturbing systemic circadian

rhythms by administering an aryl hydrocarbon receptor agonist appears to shorten the HSC cell cycle (Garrett & Gasiewicz 2006), while clock-controlled extracellular cues such as the release of the chemokine CXCL12 and the cytokine TNF-α regulate HSC proliferation (Golan et al. 2018, Méndez-Ferrer et al. 2008, Nie et al. 2008, Stenzinger et al. 2019). Thus, circadian control seems to be primarily exerted non–cell autonomously on the HSC cell cycle. That said, the chemokine receptor CXCR4 is probably expressed in a temporally regulated manner in HSCs (ZT5 > ZT13 but ZT2 = ZT14), and adult CXCR4 deletion with ROSACreER increases HSC proliferation in a cell-autonomous manner that is likely dependent on cyclin D1 (Lucas et al. 2008, Nie et al. 2008, Stenzinger et al. 2019). This suggests that HSCs gate their responsiveness to external cell cycle control through the temporal regulation of CXCR4 expression.

HSC migration closely cooperates with HSC cell cycle kinetics to set the hematopoietic proliferative capacity of various tissues. The circadian control of HSC egress from and homing back to bone marrow is primarily mediated non–cell autonomously by clock-controlled sympathetic/parasympathetic release of the neurotransmitters norepinephrine and acetylcholine, as well as by rhythmic corticosteroid and melatonin release (García-García et al. 2019, Golan et al. 2018, Méndez-Ferrer et al. 2008, Stenzinger et al. 2019). These long-range cues converge on other cells present in the bone marrow niche that regulate HSC trafficking through local release of additional signals, including rhythmic expression of the cytokine CXCL12 by MSCs and TNF-α by leukocytes (Golan et al. 2018; Kollet et al. 2013; Méndez-Ferrer et al. 2008, 2010; Stenzinger et al. 2019). This extrinsic circadian control of HSC trafficking is again likely gated by temporal variations in the HSC expression of CXCR4 (Lucas et al. 2008, Stenzinger et al. 2019).

Interestingly, considerable evidence suggests an age-related transition from extracellular circadian control to cell-autonomous HSC TTFL control in setting daughter cells' specification and differentiation, perhaps related to a withdrawal of norepinephrinergic innervation and the associated displacement of HSCs from their normal niche in aged mice (Maryanovich et al. 2018). Younger constitutive *Bmal1*$^{-/-}$ mice have lower HSC cell counts in bone marrow, defects in lymphocyte cell numbers, and inefficient differentiation in lymphoid B-cell lineages, all of which appear to be mediated by non-cell-autonomous cues (Puram et al. 2016, Sun et al. 2006). Complementing these findings, young adult constitutive *Cry1/2*$^{-/-}$ mice have more differentiated B cells, while 4-month-old constitutive *Per2*$^{-/-}$ mice have an increase in HSCs in younger adult marrow that is depleted by 12 months of age, suggesting unsustainable hyperproliferation with inadequate self-renewal in young mouse HSCs (Bhatwadekar et al. 2013, Cao et al. 2017). While *Bmal1*$^{-/-}$ HSCs show normal colony formation in vitro and MxCre>Bmal1-deletion in HSCs has no impact on the HSC content of the bone marrow of younger mice, TekCre>Bmal1-deletion in HSCs results in a loss of bone marrow HSCs and colony-forming capacity by 8–10 months of age, suggesting a failure of self-renewal (Bhatwadekar et al. 2017, Ieyasu et al. 2014, Puram et al. 2016). Conversely, very old 20-month *Per2*$^{-/-}$ HSCs of the lymphoid-biased lineage have cell autonomously enhanced self-renewal, likely due to a role for PER2 in driving specific DNA repair pathways that inhibit proliferation (Wang et al. 2016). Interestingly, the contrast of Wang et al. (2016) with the Bhatwadekar et al. (2013) results in *Per2*$^{-/-}$ mice implies a reversal of PER2's effects on HSC self-renewal at a very advanced age in mice. This may be

precipitated by the withdrawal of circadian sympathetic signaling and other changes in the niche that have occurred by 20 months of age (Maryanovich et al. 2018).

3.3.2. Neural crest mesenchymal stem cells.—MSCs have a cell-autonomous circadian TTFL (Boucher et al. 2016, Huang et al. 2009,Wu et al. 2008).The TTFL's effects on the MSC cell cycle have not been heavily studied.While *Clock* or *Per2* RNA interference (RNAi) has surprisingly been reported to increase the proportion of quiescent MSCs in culture (Boucher et al. 2016), *Reverb*α overexpression has a similar effect (He et al. 2015). At present, there are insufficient reports to confidently determine the relationship between the TTFL and the cell cycle in MSCs.

In contrast, numerous studies have examined the effects of circadian clock components in the specification and differentiation of MSC daughter cells into the fat and bone lineages, motivated in no small part by mutant mouse phenotypes. *Bmal1*$^{-/-}$, *Clock*$^{-/-}$, and *Per3*$^{-/-}$ mice are all prone to obesity and/or high percentage of fat by weight (Costa et al. 2011, Guo et al. 2012,Turek 2005). Meanwhile, *Bmal1*$^{-/-}$ and MSC-targeting Prx1Cre>Bmal1-deletion mice have progressive loss of bone density over the life span that is driven in part by mildly deficient osteogenic potential along with high turnover (Samsa et al. 2016, Tsang et al. 2019).

BMAL1's role in suppressing adiposity may be clock independent. While *Bmal1* shRNA enhances adipogenesis in 10T1/2 MSC-like cells, both *Clock* and *Per2* shRNA actually reduce the adipogenic potential of MSCs, likely at least in part through the downregulation of Wnt pathway components downstream of Bmal1 (Boucher et al. 2016, Guo et al. 2012). In addition, although *Per3* overexpression blocks and *Per3* deletion enhances MSC adipogenesis, the core clock was unaffected (Costa et al. 2011). Given the glucocorticoids used to stimulate the adipogenic differentiation of MSCs, which can directly bind the *Per3* promoter to regulate *Per3* expression, PER3 effects on adipogenesis may not be clock dependent (Costa et al. 2011, Pico et al. 2016). However, the circadian clock may play an instructive role later in adipocyte differentiation. For example, REVERBα expression is highly dynamic throughout pre-adipocyte differentiation in a manner that is likely regulated by both the TTFL and rhythmic degradation (DeBruyne et al. 2015, Wang & Lazar 2008).

Bmal1's role in promoting osteogenesis, in contrast, most likely reflects a clock-dependent function. Complementing the modest osteogenic deficiency of Prx1Cre>*Bmal1*-deleted MSCs, the overexpression of *Bmal1* in MSCs promotes osteogenesis in culture (He et al. 2013, Huang et al. 2020, Tsang et al. 2019). Conversely, knocking down the negative arm components *Cry2* or *Per2* in MSCs promotes osteogenesis, while the overexpression of *Reverb*α suppresses osteogenesis (He et al. 2015, Tang et al. 2020, Zhuo et al. 2018). Much as for adipogenesis, the transcriptional control of Wnt pathway components seems to be an important factor coupling the circadian TTFL to MSC osteogenesis (He et al. 2013, 2015). Interestingly, aged MSCs become less proliferative, less osteogenic, and more adipogenic while downregulating BMAL1 and upregulating REVERBα (Chen et al. 2012, Maryanovich et al. 2018, Yu et al. 2011). This suggests that degradation of the circadian TTFL may be relevant for maladaptive age-related changes in MSCs. Finally, two shRNA studies (Boucher et al. 2016, Zhuo et al. 2018) have reported a handful of results inconsistent with the rest

of the literature: increased osteogenesis under *Bmal1* shRNA and no effect on osteogenesis with *Clock* or *Per2* shRNA. At best guess, these anomalous results reflect insufficient knockdown and/or off-target effects that complicate the shRNAs' effects on osteogenesis.

3.4. Intestinal Stem Cells

Regrettably little is known about circadian control of adult stem cells of endodermal lineage, but of these, the intestinal niche is best studied (Figure 3). Intestinal stem cells (ISCs) reside within crypts at the base of the lumen. Signals—prominently including Wnts, especially from Paneth cells—regulate their division. Their differentiating daughters migrate into the intestinal lumen, where they constantly turn over intestinal epithelium damaged by the harsh digestive role of the intestine. The rate of this basal turnover becomes elevated in cases of unusual injury or inflammation, resulting in increased cell division (reviewed in Clevers et al. 2014, Parasram & Karpowicz 2020).

Whether ISCs possess an intrinsic TTFL in mammals is unclear. The intestine as a whole rhythmically expresses clock genes (Balakrishnan et al. 2010, Xu et al. 2017). However, the only study to closely examine mammalian ISC-specific rhythms with PER2::mCherry reported relatively flat expression (though still with a nonzero ~1.5X amplitude) from individual ISCs and low-amplitude, rapidly damping rhythms from organoids cultured in ISC-enriching conditions (Matsu-ura et al. 2016). In contrast, studies of invertebrate ISCs in *Drosophila melanogaster* reveal a clear cell-autonomous TTFL (Karpowicz et al. 2013, Parasram et al. 2018). Whether this reflects a bona fide evolutionary loss of ISC TTFL activity somewhere in the vertebrate clade, or whether mammalian ISCs simply have weak and/or PER2-independent TTFL activity that Matsu-ura et al. (2016) failed to detect, is of considerable interest for understanding the possible role of TTFL induction in early lineage specification (see Section 3).

In either case, the ISC cell cycle is regulated non–cell autonomously by circadian rhythms in a largely conserved manner. The mammalian ISC cell cycle runs on a 12-hour ultradian harmonic coordinated by circadian TTFL-controlled Wnt, TNF-α, and c-Jun N-terminal kinase (JNK) signaling (Matsu-ura et al. 2016, Stokes et al. 2017). These cues set the cell cycle period by gating the G_1/S checkpoint, at least in part by regulating p21 expression (Figure 1c) (Matsu-ura et al. 2016, Stokes et al. 2017). While the G_1/S transition seems to be generally rate determining in mammalian ISCs, systemic *Per1/2* is also required for a proper exit from the S phase, likely from enhanced *Wee1* expression that implies BMAL1 as a mediator (Figure 1c) (Matsuo 2003, Pagel et al. 2017). *Drosophila* ISCs show rhythmically increased proliferation on a circadian 24-hour period in response to injury, which relies upon both the ISC TTFL and circadian signaling from differentiated enterocytes (Karpowicz et al. 2013). As in mammals, Wnt and Hippo/JNK signaling is prominent among the extracellular clock-controlled cues involved (Karpowicz et al. 2013, Parasram et al. 2018).

To what extent circadian rhythms regulate specification and differentiation decisions after ISC cell division is unclear. *Bmal1*$^{-/-}$ intestine has no obvious morphological defects under physiological conditions, and while *Per1/2*$^{-/-}$ intestine is deficient in Paneth and goblet cells, this defect could be accounted for by elevated apoptosis (Pagel et al. 2017, Stokes et al. 2017). This question has not been directly asked in the fruit fly, although

interestingly, the differentiated enteroen-docrine lineage (which has some secretory function loosely similar to that of Paneth and goblet cells) and enterocyte lineage are distinguished by the deactivation and maintenance, respectively, of circadian TTFL activity (Parasram et al. 2018). We thus suspect circadian involvement in ISC cell fate decisions but definitive determination of this awaits further research.

4. TEMPORAL REGULATION OF LINEAGE COMMITMENTTHROUGHOUT DEVELOPMENT BY METABOLIC RHYTHMS EARLY AND THE CLASSICAL CIRCADIAN TRANSCRIPTIONAL-TRANSLATIONAL FEEDBACK LOOP LATER

At fertilization, de novo clock gene expression is blocked for the first several, rapid cleavage divisions of the cell cycle, and clock gene expression steadily drops (Amano et al. 2009, Ko et al. 2000). However, temporal cues are available, as fertilization coincides with an initial burst of ROS that develops an oscillatory pattern coupled to cell division, as shown in *Xenopus* (Han et al. 2018). As cell division proceeds, the developing organism first forms a dense, solid ball of cells at the morula stage, which then restructures into a hollow blastula by the approximately 100-cell stage. Somewhere in this morulation/blastulation time frame, the transcription of the embryo's own clock genes begins in mammals (Amano et al. 2009, Ko et al. 2000). Once the blastula successfully implants itself in the uterine lining, the hollow structure of the blastula allows involution to occur as division continues, forming a layered structure known as the gastrula. The inner layers form the primitive streak, the precursor of mesoderm and endoderm, while the outer layer goes on to become the ectoderm (Aires et al. 2018).

4.1. Possible Requirement for a Primordial Metabolic Clockfor Mesoendodermal Specification

A pair of recent papers (Ameneiro et al. 2020, Gallardo et al. 2020) beautifully demonstrate that while *Bmal1*$^{-/-}$ ESCs are resistant to differentiation in general, they are particularly poor substrates for primitive streak formation. In both adherent and gastruloid conditions compatible with multilineage differentiation,*Bmal1*$^{-/-}$ ESCs produced cells with much lower expression of mesodermal and endodermal lineage markers, while ectodermal lineage markers were relatively unaffected. This is consistent with data obtained in zebra fish, in which constitutive *Clock1a* knockout causes a selective defect in the formation of mesodermal lineages due to a loss of the rhythmic *Smad3* expression required to transduce promesoderm nodal signals (Bian et al. 2017). Clock is able to similarly regulate *Smad3* expression in mammalian cell culture (Bian et al. 2017).

Furthermore, Bmal1 may be epistatic to the effects of ROS accumulation on the mesoendodermal specification of ESCs. ESCs experiencing elevated ROS under differentiating conditions typically yield progeny enriched for the mesoendodermal lineage (Ji et al. 2010). Nonetheless, elevated ROS resulting from reduced glycolysis and enhanced OXPHOS in *Bmal1*$^{-/-}$ ESCs fails to prevent the ectodermal enrichment of their progeny (Ameneiro et al. 2020). One tempting hypothesis for this disconnect is the possibility that

ROS timing makes the difference; while constant levels of ROS inhibit the mesodermal cardiovascular differentiation of ESCs, pulsatile ROS actually promotes such differentiation (Sauer & Wartenburg 2005). Indeed, ROS cycles in early dividing embryos are rhythmically phase locked to the cell cycle (Han et al. 2018).

The cell cycle also provides a potential means for stem cells to reorganize their metabolic cycles upon mesoendodermal specification. A Wee1-dependent mitotic pause occurs during the selection of mesodermal and endodermal fates in hESCs, providing sustained exposure to the S/G_2/M cell cycle phases that are relatively conducive to selecting these lineages over ectoderm (Pauklin & Vallier 2013,Van Oudenhove et al. 2016). This pause is particularly important for the formation of endoderm, having more subtle effects on mesodermal differentiation (Van Oudenhove et al. 2016). Because mitosis freezes transcription, it is a signal that has been proposed to mediate entrainment of the TTFL oscillator by the cell cycle (Paijmans et al. 2016, Yan & Goldbeter 2019). But the slow induction of circadian TTFL cycling in PSC culture, as well as the late appearance of cell-autonomous rhythms in mesodermal tissues such as the heart sometime after E9.5, suggests that a cell-autonomous circadian oscillator is likely not yet present in the gastrula (Umemura et al. 2017, 2019; Yagita et al. 2010).

However, the cell cycle pause during mesoendodermal specification could instead serve as an instructive cue for reorganizing rhythmic metabolism. In addition to transcriptional arrest, mitochondrial OXPHOS is transiently increased during the G_2/M transition to provide adequate energy to complete mitosis (Wang et al. 2014). Blocking either this cell cycle–coupled OXPHOS enhancement or the concomitant ROS production arrests the cell cycle at G_2/M (Han et al. 2018, Wang et al. 2014). Timing of the G_2/M pause in gastrulation may therefore be related to the uterine implantation that precedes it, which results in decreased uterine oxygen content and thus an overall increase in glycolysis and decrease in OXPHOS that presumably raises the barrier to mitosis (Fischer & Bavister 1993, Houghton et al. 1996). However, one outstanding question is why this pause only occurs in the newly specified mesoendoderm.

One possibility is that the extended pause allows an opportunity for the more extensive epigenetic remodeling of the chromatin required for ESCs to avoid defaulting into the ectodermal lineage (see Section 4.2). Consistent with this hypothesis, DNA methylation profiling of ESCs and precursor cells across the three germ layers has revealed that mesoderm precursors undergo 35% more promoter DNA methylation than ectodermal precursors (Isagawa et al. 2011).Broader profiling of chromatin modifications comparing these cell types will be necessary to definitively evaluate this possibility.

Another possibility is that the mesoendodermal lineage is selectively sensitive to entraining maternal cues. Implantation implies more direct exposure to these cues immediately prior to gastrulation, as the uterus communicates extensively with the embryo during and after implantation. The uterus expresses an estrogen-receptive circadian TTFL, and uterine receptivity to implantation is both entrained by systemic circadian cues such as melatonin and impaired by unfavorable photoperiod or jet lag (Mahoney 2010, Nakamura et al. 2008, Zhang et al. 2017). Later in development, the fetus may function in many ways as a

peripheral oscillator of the maternal circadian system (Serón-Ferré et al. 2012). Mitotic/metabolic cycling in the mesoendoderm, which unlike ectoderm goes on to form exclusively peripheral oscillators in the adult animal, could be more responsive to maternal entrainment at this early stage.

4.2. Contribution of the Lack of a Circadian Transcriptional-Translational Feedback Loop to the Default Neural Stem Cell Fate

A long-controversial topic in stem cell biology has been whether a neural cell fate is the default specification for pluripotent ESCs, first hypothesized from spontaneous neural differentiation of *Xenopus* and mammalian cells in culture (Grunz & Tacke 1989, Wiles & Johansson 1999). Various extrinsic cues influence the ectodermal/mesoendodermal specification decision for cells in an intact gastrula. However, both progressive changes in intracellular transduction of those cues and an initial innate bias also seem to play roles, and these cell-autonomous factors do seem to initially favor neural ectodermal cell fate (Smukler et al. 2006, Turner et al. 2014, Zirra et al. 2016).

We propose that the seeming lack of a circadian TTFL in ESCs and iPSCs is one cell-autonomous factor that biases them toward the NSC lineage (Umemura et al. 2017, 2019; Yagita et al. 2010). Notably, NSCs are unusual among adult PSCs in that they lack an autonomous TTFL, a distinction they may only (arguably) share with ISCs out of the adult stem cells we discuss (Malik et al. 2015a,b; Matsu-ura et al. 2016; but see also Karpowicz et al. 2013) (Figure 3). Particularly marked is NSCs' uniqueness within their lineage in lacking a TTFL, as all other stem cell types we review from the ectodermal (HFSC and EKSC) and related neural crest (MSC and MnSC) lineages have detectable circadian TTFLs (Buhr et al. 2019; Janich et al. 2011, 2013; Wu et al. 2008) (Figure 3). A consequence of this is that NSCs are also unusual in that the initial neural/glial specification decision for their progeny seems to be most directly influenced by the clock-independent functions of Bmal1. Aside from the pro-adipogenic influences of Bmal1 in MSC specification, most other cell fate decisions that Bmal1 contributes to in ectodermal/neural crest lineage adult stem cells appear to occur through Bmal1's cell-autonomous TTFL function (see Section 3).

4.3. Probable Circadian Transcriptional-Translational Feedback Loop Involvement in Later Lineage Specification

Retinal progenitor stem cells (RSCs) give rise to differentiated cell types in a well-defined order across retinal development (reviewed in Cepko 2014). Chx10Cre>*Bmal1* knockout in retinal progenitors early in retinogenesis profoundly reshapes the pool of final cells, depleting early-born populations, such as acetylcholinergic starburst amacrine cells and Brn3b+ retinal ganglion cells, and disrupting the proper distribution of opsin subtypes in the early-born cone population (Sawant et al. 2017, 2019). Moreover, late-born populations such as recoverin-positive bipolar cells and Müller glia are overproduced, and the lamination and morphology of the structurally critical Müller glia population are badly disrupted (Sawant et al. 2019). More Chx10Cre>*Bmal1*-depleted RSCs appear to undergo symmetrical, self-renewing division at E15 when asymmetrical divisions responsible for producing early-born retinal subtypes should be occurring (Sawant et al. 2019). This suggests that, like neuron/glia balance in the hippocampal niche, clock effects on RSC daughter fates may be

secondary to the clock regulation of mitosis. This view is supported by the effects of cyclin D1 loss, which similarly prompt RSCs to overproduce late-born bipolar cell and Müller glia populations (Das et al. 2012). The developmental defects, especially defective Müller glial architecture, described by Sawant et al. (2019) likely contribute to the morphological and functional defects in the eyes of mice with *Bmal1*-deficient retinas, including thinned retinas and axial elongation of the eye that produces a myopic refractive index, poor visual acuity, and impaired b-wave electroretinogram activity (Baba et al. 2018, Stone et al. 2019).

Tellingly, both systemic *Per1/2*$^{-/-}$ and CrxCre>*Per2*–C knockout retinas have complementary S-cone phenotypes to the Bmal1 studies, suggesting that retinogenesis specification events driven by these genes reflect bona fide circadian TTFL activity (Ait-Hmyed et al. 2013, Sawant et al. 2017). This is consistent with the timing of retinogenesis, which begins in mid-gestation and ends in early postnatal life (reviewed in Cepko 2014). While we are unaware of any study examining clock ontogeny specifically within the retina, developmentally related tissues of the SCN—the master light-entrained circadian oscillator of the brain—exhibit circadian TTFL ontogeny at gestation points similar to the onset of retinogenesis (reviewed in Bedont & Blackshaw 2015; see also Carmona-Alcocer et al. 2018, Landgraf et al. 2015, Wreschnig et al. 2014). Indeed, the SCN itself may also feature circadian TTFL involvement in later lineage specification and/or differentiation, as the numbers of certain neuropeptidergic neuron lineages within the nucleus are skewed in *Clock*$^{-/-}$ mutants (Herzog et al. 2000, Silver et al. 1999). The SCN also provides an elegant example of how developmental neurogenic findings can inform the field of adult neurogenesis, as there is considerable evidence that the SCN may itself contain quiescent NSC potential in adulthood (reviewed in Carmona-Alcocer et al. 2020).

This work suggests that the developmental effects of the circadian TTFL on later branch points in lineage specification and differentiation are worth investigating. Elsewhere in the brain, for instance, cortical progenitors have a developmentally stereotyped progression of distinct earlyand late-born daughter cell types, and this program seems to be largely cell autonomous, much like that in RSCs (Duan et al. 2015, Shen et al. 2006).
Such developmental programs could easily be guided by circadian rhythms as the cell-autonomous clock comes online in differentiating progenitors. In another recent example from the endodermal lineage, artificial circadian food entrainment imposed on pancreatic stem cells enhanced the final step of their development, which together with transcriptomic profiling supports a role for a food-entrained TTFL in terminal islet differentiation (Alvarez-Dominguez et al. 2020). More generally, as we discuss, most multipotent stem cells in adult niches express intact circadian TTFLs that regulate daughter cell fate. We propose that, similarly, an active TTFL in mid-embryonic to early-postnatal multipotent stem cells plays a role in regulating proliferation and daughter cell fate during development. Research on the role of the circadian TTFL and related factors on finer cell fate decisions within developmental lineages is unfortunately sparse, and we believe this is a major opportunity for future research.

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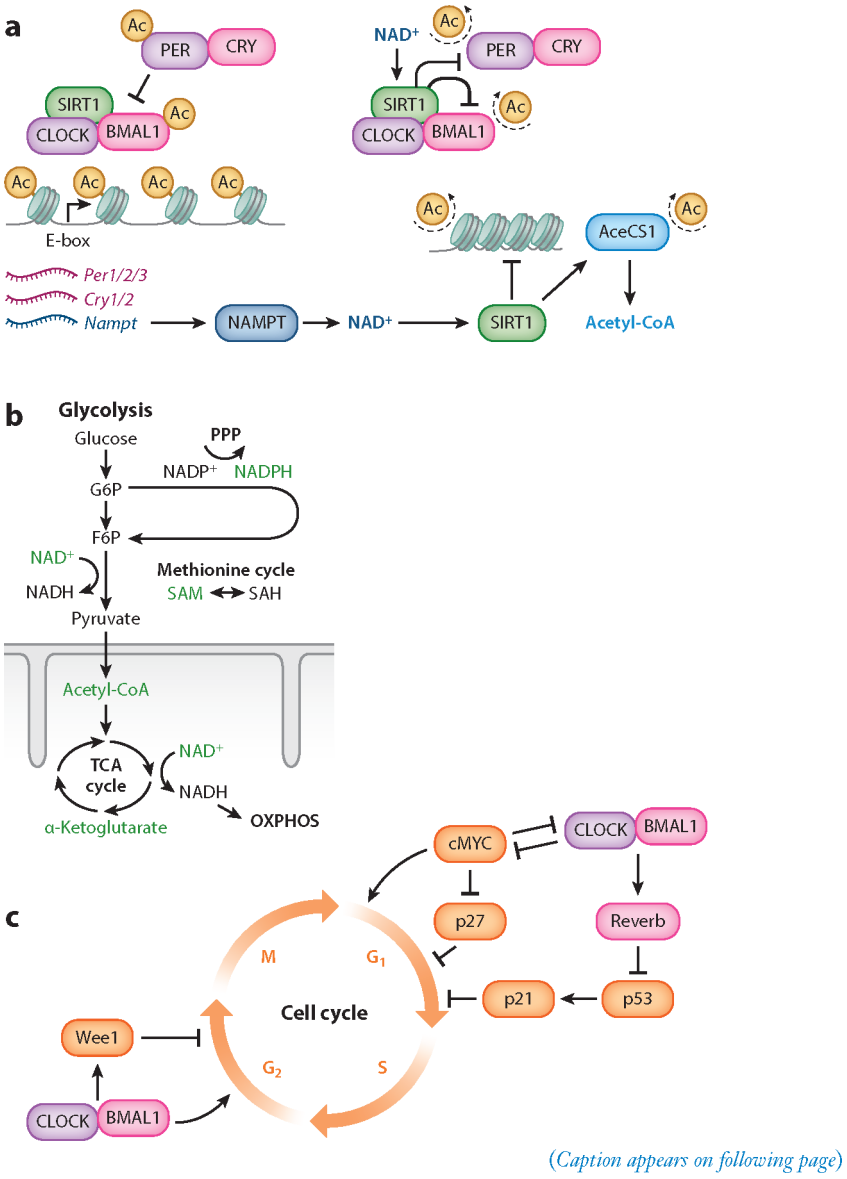

(Caption appears on following page)

Figure 1.

Circadian clock interaction with metabolism, cell cycle, and chromatin remodeling. (*a*) The core circadian TTFL is composed of the heterodimer CLOCK:BMAL1, which drives the transcription of their repressors PER1/2/3 and CRY1/2; these repressors dimerize in the cytoplasm and translocate to the nucleus to repress CLOCK:BMAL1. CLOCK:BMAL1 also drives the rhythmic accumulation of NAD^+ by driving the transcription of the gene encoding the protein NAMPT, the rate-limiting enzyme in the NAD^+-salvage pathway. The cyclic synthesis of NAD^+ corresponds to the rhythmic activity of NAD^+-dependent HDACs including SIRT1. Rhythms in SIRT1 activity over 24-hour periods also contribute to the cyclic synthesis of the universal acetyl donor acetyl-CoA through the enzyme AceCS1, which is activated by deacetylation. (*b*) Major catabolic pathways conserved across living cells include glycolysis, the TCA cycle, and OXPHOS. Each of these pathways produces metabolites that interact with circadian rhythms through the production

of metabolites that act as substrates for posttranslational modifications or can activate protein-modifying enzymes (labeled in *green*). Among anabolic pathways, the PPP and the methionine cycle generate metabolites that interact with circadian rhythms. (*c*) Prominent connections of the circadian clock (*purple*) with the cell cycle (*orange*), emphasizing the checkpoints that are especially important for their coupling in stem cells. Abbreviations: Ac, acetyl; AceCS1, acetyl-CoA synthetase 1; CRY, Cryptochrome; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; HDAC, histone deacetylase; NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NAMPT, nicotinamide phosphoribosyltransferase; OXPHOS, oxidative phosphorylation; PER, Period; PPP, pentose phosphate pathway; SAH, S-adenosylhomocysteine; SAM, S-adenosyl methionine; TCA, tricarboxylic acid; TTFL, transcriptional-translational feedback loop.

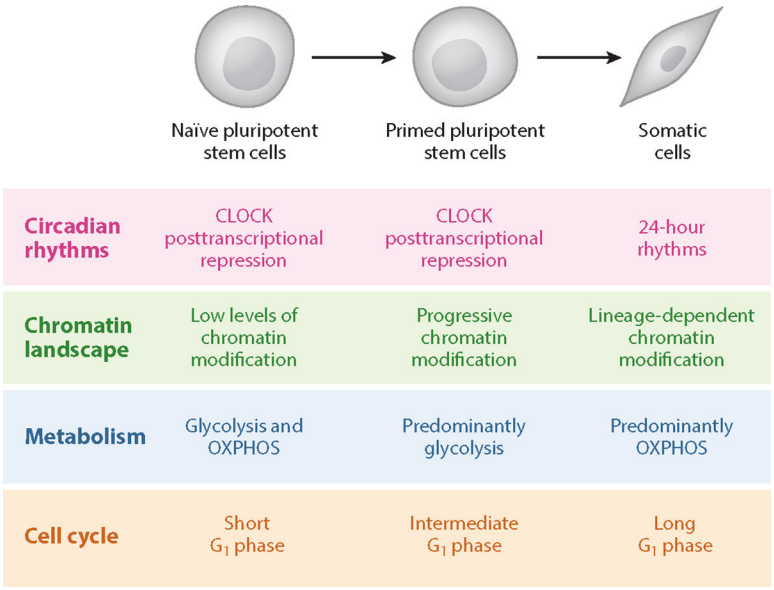

Figure 2.

Pluripotent stem cell differentiation and clock development involves sequential changes to cellular metabolism and chromatin remodeling. Distinct phases of pluripotency can be captured in vitro and have characteristic epigenetic, metabolic, and cell cycle profiles. Both naïve and primed pluripotent ESCs lack circadian gene expression, and the core TTFL protein CLOCK is posttranscriptionally repressed. Naïve ESCs have very little chromatin modification across the epigenetic landscape, and progressive DNA methylation is associated with sequential lineage commitment. Naïve ESCs are able to perform OXPHOS, glycolysis, and fatty acid oxidation, whereas primed ESCs rely primarily on glycolysis to generate ATP. Differentiated cells rely primarily on OXPHOS. Abbreviations: ESC, embryonic stem cell; OXPHOS, oxidative phosphorylation; TTFL, transcriptional-translational feedback loop.

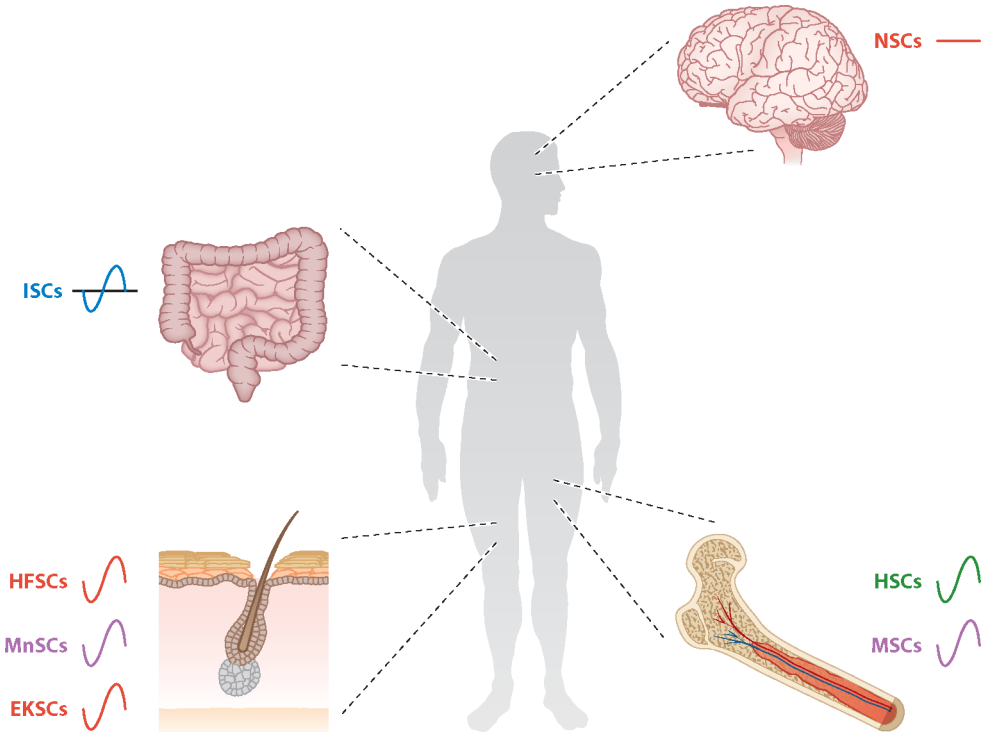

Figure 3.

Adult stem cells derived from each germ lineage exhibit different degrees of circadian function. Four body parts (brain, intestine, bone marrow, and skin) containing adult stem cell niches are shown. Each stem cell type is color coded by developmental lineage: ectoderm (*red*), neural crest (*purple*), mesoderm (*green*), or endoderm (*blue*). The lines adjacent to each stem cell type indicate either the presence (*sine wave*) or absence (*straight line*) of the circadian TTFL in those cells. Abbreviations: EKSC, epidermal keratinocyte stem cell; HFSC, hair follicle stem cell; HSC, hematopoetic stem cell; ISC, intestinal stem cell; MnSC, melanocyte stem cell; MSC, mesenchymal stem cell; NSC, neural stem cell; TTFL, transcriptional-translational feedback loop.

Table 1

Clock-regulated metabolites that participate in chromatin remodeling contribute to cellular differentiation prior to clock development

Metabolite	Metabolic pathway	Chromatin modification	Circadian interaction	Stem cell fate change
Acetyl-CoA	Glycolysis Tricarboxylic acid (TCA) cycle	Histone acetylation	Activity of acetyl-CoA synthetase 1 (AceCS1) requires cyclic acetylation dependent on a functional circadian clock and the NAD^+-dependent deacetylase SIRT1 (Sahar et al. 2014). Catabolism of glucose to acetyl-CoA depends on ATP-citrate lyase (ACLY); ACLY protein levels are cyclic in the mouse liver (Mauvoisin et al. 2014).	Maintenance of pluripotency (Moussaieff et al. 2015)
α-Ketoglutarate	TCA cycle	Histone demethylation DNA demethylation	Inhibits JumonjiC domain–histone demethylase 1a (JARIDla), which associates with CLOCK:BMAL1 to facilitate *Per2* transcription (DiTacchio et al. 2011).	Maintenance of pluripotency (Carey et al. 2015)
Nicotinamide adenine dinucleotide (NAD^+)	Glycolysis	Histone deacetylation	In mouse liver, 24-hour cycling rhythm is exhibited (Krishnaiah et al. 2017). CLOCK:BMAL1 drives transcription of *Nampt*, a crucial enzyme for NAD^+ production (Nakahata et al. 2009, Ramsey et al. 2009).	Acquisition and maintenance of pluripotency (Calvanese et al. 2010, Y. Lee et al. 2012, Lees et al. 2020, Tang et al. 2014)
S-adenosyl methionine (SAM)	One-carbon metabolism (SAM and methionine cycle)	Histone methylation DNA methylation	In mouse liver, 24-hour cycling rhythm is exhibited (Krishnaiah et al. 2017). Accumulation of SAM by-product SAH (S-adenosyl homocysteine) hinders transmethylation and elongates circadian period (Fustin et al. 2013).	Maintenance of pluripotency (Shiraki et al. 2014, Shyh-Chang et al. 2013)