Circadian Rhythms in Cell Maturation

Circadian rhythms are of major importance in mammalian physiology and disease. In this review, we give an overview of the present knowledge on origination of circadian rhythms. We discuss the development of both master and peripheral clocks and compare the origination of circadian rhythms in utero and in vitro.

Our planet turns around every 24 h. Anticipation to changes caused by this rotation, such as dark/light and temperature changes, has advantages on a population, organism, and cellular level. Information about the time of day allows preparation for an event before it starts and enables a rapid and adequate response. During the Babylonian, Egyptian, Greek, and Roman ages, people already understood the importance of knowing the time of the day and developed sundials (shadow clocks) to estimate the time. Diurnal patterns in organisms, such as closure of Tamarind tree leaves in the evening and diurnal differences in pulse rate, have been appreciated and documented over many centuries (11, 63). The idea of an endogenous clock was first raised in 1792. Jean-Jacques d’Ortous de Marain described that the 24-h pattern in the opening and closure of leaves of the Mimosa pudica plant continued in complete darkness (14). Other scientists, however, could not believe that a biological process can create a nonvariable period of 24 h over a wide variety of temperatures (known as temperature compensation) and attributed the findings of d’Ortous de Marain and others to changes in temperature, humidity, or an unknown factor X; it was only in the 1970s that there was consensus about the existence of an internal clock that anticipates diurnal environmental cues (9).

Recently, biological rhythms have gained a lot of attention in science. In the last 10 years, >20,000 articles involving circadian rhythms have been published, and numerous review articles have been written on the subject (79, 106). Knowledge about circadian rhythms in physiology and disease is accumulating. However, information about the origination of circadian rhythms during embryogenesis or in vitro maturation is scarce. Regenerative medicine, which aims to develop tissues in vitro, has renewed the interest in developmental processes. In this review, we provide an up-to-date overview on the origination of circadian rhythms during embryogenesis and in vitro differentiation. Although circadian rhythms are present in almost all organisms, we will focus on mammals. First, we provide a brief introduction on circadian rhythms, followed by a discussion of the literature on the origination of these rhythms. Difficulties and challenges in the study of the developing circadian rhythms are discussed, and origination of circadian rhythms in utero is analyzed. In the second part of the review, we introduce stem cells, provide information about origination of circadian rhythms during differentiation of stem cells, and compare origination of circadian rhythms in stem cells to those in the developing fetus. Finally, future perspectives are provided.

Circadian Rhythms

Circadian rhythms, diurnal rhythms, and circadian clocks are terms used in chronobiology, which are interchanged in literature and this review but have a different meaning. Diurnal rhythms are patterns that recur every 24 h, whereas circadian rhythms are biorhythms that persist with a period of ~24 h in the absence of external cues but remain responsive to environmental conditions such as light and exercise (22). Circadian rhythms thus differ from diurnal rhythms based on their self-sustainability. Circadian clocks are endogenous cell autonomous molecular mechanisms. These mechanisms consist of positive and negative molecular feedback loops that regulate the rhythmic expression of several core clock genes such as Bmal1/2, Clock, Cry1/2, and Per1/2/3 (77, 86). Clock and Bmal proteins form heterodimers, which bind to E-boxes within the promoters of numerous clock output genes, leading eventually to circadian regulation of ~10% of the cellular transcriptome. The Clock and Bmal heterodimer also binds to other core clock components such as Per and Cry. Per and Cry proteins slowly accumulate, form a complex, and inhibit the transcription of Clock and Bmal (FIGURE 1).

As such, oscillations in the expression of core clock genes within a cell are perceived as an indication that the cell possesses a functional clock, which in turn influences several signaling pathways leading to time-of-day-dependent cell function. Although Clock, Bmal, Per, and Cry form the main feedback
loop of the molecular clock, many more proteins are involved. Rev-Erb and ROR, for example, form an additional feedback loop with Clock/Bmal similar to Per/Cry, and Npas can functionally substitute Clock (98). In addition, posttranslational modifications are important for clock function and fine-tuning (57). Phosphorylation of core clock proteins, for example, determines their degradation and thereby keeps the 24-h period of the clock constant (52).

Endogenous clocks can be subdivided into a “master clock” that is located in the suprachiasmatic nucleus (SCN) of the brain and “peripheral clocks” that are present in essentially all mammalian cells (FIGURE 2). The core clock components in cells of the master clock and peripheral cells are similar, but input and output signals can differ. The master clock was discovered in 1972 by SCN lesion studies and is the most commonly studied endogenous clock (62, 90). Presence or absence of light is the main input signal (Zeitgeber or timekeeper) of the master clock in adults (26). Retinal photoreceptors in the eye receive photic information and convey this information via the retino-hypothalamic tract to the SCN. Nonphotic SCN Zeitgebers in adults include exercise and social behavior (60). The output of the master clock consists of several neurohumoral signals such as TGF-α, cardiotrophin-like cytokine, and prokineticin 2, which orchestrate circadian rhythms throughout the organism, either directly or by regulating peripheral circadian clocks (10, 47, 48).

Following their first description ~15 yr ago, it is now apparent that peripheral clocks are present in almost every mammalian cell. Perception of the relationship between peripheral clocks and the master clock has changed relatively recently (79, 80). Traditionally, peripheral clocks were considered to be “executors” of the central clock. In this view, peripheral clocks simply receive time-of-day information from the master clock and respond accordingly (“master-slave model”). However, peripheral clocks are now considered autonomous in nature, being able to function independently of the SCN. The master clock functions as a conductor and harmonizes peripheral clocks (“orchestra model”) (16). A good example of the autonomy of peripheral clocks is restricted feeding. When food is offered at “inappropriate” times (e.g., restricted to the sleep phase), peripheral clocks (e.g., in the liver) adjust their phase to the new feeding regime, while the master clock will remain in phase with the light/dark cycle (91).

Diurnal rhythms have a marked impact on health. Physiological parameters such as blood pressure, heart rate, body temperature, metabolism, and hormone levels have diurnal patterns, whereas at the cellular level ~10% of the transcriptome exhibits circadian oscillations in expression (19, 69, 92, 98). Disruption of diurnal rhythms is associated with disease in multiple organ systems, including the cardiovascular system, kidneys, gastrointestinal system, skeletal muscle, endocrine system, immune system, and reproductive system (79). In the cardiovascular system, for example, the occurrence of several diseases (e.g., the onset of myocardial infarction) has a diurnal pattern. Similarly, disruption of normal day-night rhythms (e.g., by shift work or mutation of clock genes) results in an increased cardiovascular risk, and outcome of cardiovascular events such as myocardial infarction is time-of-day dependent (20, 37, 44, 55, 71, 73, 102).

Development of Circadian Rhythms During Embryology

Considerations in Research of Developing Circadian Rhythms

As explained above, circadian rhythms are mediated by molecular clocks, a series of molecular feedback loops that drive diurnal oscillations in core clock and output gene expression. The presence of a circadian clock is therefore generally evaluated by quantifying the expression of oscillating clock genes such as Clock, Bmal, Per, and Cry.
(an example is provided in Figure 3). However, the focus on oscillating clock genes to determine presence of a fully functional clock has some caveats. O’Neill and colleagues showed that circadian rhythms are present in cells without a nucleus (and thus without transcription) and persist in nucleated cells when transcription of core clock genes is disabled, suggesting that mechanisms other than the classic molecular clock are able to maintain circadian rhythms (66, 67). Second, Paulose et al. demonstrated that development of diurnal metabolic rhythms precede rhythms in clock gene expression (70). It is, therefore, possible that, in early cell maturation, mechanisms other than the core clock genes regulate select diurnal patterns. Third, evaluating the presence of oscillating clock genes suggests that there is an abrupt start of oscillation during maturation. In fact, circadian rhythms originate progressively during maturation in several steps: initially, clock genes oscillate with low amplitudes, followed by higher amplitudes, but sometimes also periods without oscillations and phase shifts (82, 88). During various disease states, rather than a complete absence of circadian rhythms, there are often changes in the amplitude and phase of the rhythm (29). Oscillations in circadian clock genes therefore do not automatically mean the molecular clock is working properly. Last, when circadian rhythms are evaluated, often whole body or whole tissue samples are used. Results of clock gene expression are the average of many individual cells. Numerous studies suggest that circadian rhythms in one cell or tissue type might be in a markedly distinct phase compared with another region of the body, particularly during development (35, 84, 101). This is supported by the finding that, in some experiments, oscillations are found in vitro but not in vivo (18). Rhythms at the cell level might therefore not be detected when results are analyzed on a tissue scale due to the absence of synchronization. Despite these limitations, it is still believed that rhythmic expression of core clock genes is a good indication of clock function (67, 70, 101).

During embryogenesis, the developing circadian system is exposed to daily fluctuations in maternal signals including temperature, food intake, and hormones (e.g., melatonin) (84). In addition, the fallopian tubes and the uterus of the mother express clock genes (38). Some suggest that, because of their proximity, clock activity in these organs may interfere with developing fetal circadian rhythms, although this has not been demonstrated yet (38). The developing circadian system should therefore not be considered an immature adult system residing in a closed compartment but a system capable of anticipating diurnal circumstances in utero and preparing for life after delivery.

A last consideration in research of the developing circadian system is that most research is performed in nocturnal animals such as mice, rats, and hamsters. Humans, on the other hand, are diurnal, which implies that their circadian rhythms are different. In addition, Zeitgebers of rodents differ from Zeitgebers of primates (53, 65). These considerations should be taken into account when animal experiments are translated to the human situation.

**Origination of Circadian Clocks**

The molecular circadian clock originates during development in utero. The canonical clock genes Per, Cry, Clock, and Bmal are already nonrhythmically expressed in the unfertilized mouse oocyte (38, 45). Expression of these genes diminishes on day 2 after fertilization (two-cell stage), but restores on day 3 (16-cell stage) until day 4 (blastocyst). Next, expression of clock genes increases, and around mid- to end-gestation, diurnal oscillations in expression start. The shape, amplitude, and phase of clock gene rhythms change during
further development. This development is animal and tissue specific. In general, rodents have a more immature circadian clock at birth compared with primates (84). An overview of published findings to date regarding the origination of the molecular circadian clock in mouse, rat, hamster, and monkey is given in [FIGURES 4 AND 5] and Table 1, respectively. In FIGURE 6, a general model for the origination of circadian rhythms is provided.

Embryogenesis involves cell divisions. The occurrence of cell division is regulated by circadian rhythms, which restrict mitosis of the cell to specific time frames in the 24-h cycle in vivo and in vitro (cell division gating) (6, 7, 54, 64). During cell division, circadian rhythms of the mother cell are passed on to the daughter cell. In this process, circadian rhythms persist, although amplitude and phase of the rhythm sometimes change (31, 64). Amplitude changes are caused by a wider distribution of periods in proliferating cells compared with nonproliferating cells (64). Phase changes can be explained by reduced availability of clock proteins. During cell division, transcription halts and cellular components are divided over daughter cells. If cell division occurs in the accumulating phase of Per and Cry, reduction of these proteins will lead to circadian phase prolongation, whereas if this happens in the descending phase of Per and Cry, the circadian phase will be shortened (64).

Zeitgebers of the Developing Circadian Clock

Zeitgebers of the circadian clock during embryogenesis differ from Zeitgebers for adult cells. This is partly caused by the immature phenotype of clock-regulating components in the embryo. In rats, for example, the master clock (SCN) develops from embryonic day 14 (E14) to E17 and is only fully matured at postnatal day 10 (P10) (101). Synchronization of peripheral clocks during gestation must therefore depend on signals that do not come from the fetal master clock. Fetal Zeitgebers also differ because the environment of the fetus is not comparable to the environment of an adult. Light, for example, is the main Zeitgeber for the adult master clock but would seem less relevant in the uterus. Surprisingly, however, a recent study demonstrated that photic input influences eye development in the mouse fetus. This may also change the traditional view on the role of Zeitgebers and their signaling pathways in circadian development, although it remains unknown whether photic input in the fetus is strong enough to influence its circadian clocks (72). The established main Zeitgebers during development are maternal Zeitgebers, the most important one being maternal melatonin. Melatonin is mainly produced by the maternal pineal gland and able to pass the placenta unaltered, causing a diurnal rhythm in the fetal circulation. Melatonin receptors are present in the human fetus (78). Experiments in the hamster show that, when the maternal master clock is removed, fetal activity rhythms are desynchronized, but rhythms can be rescued by regular melatonin injections (13). Maternal steroids, the dopaminergic system, and feeding are also able to synchronize peripheral fetal clocks (28, 46, 68). Despite accumulating evidence of a correlation between maternal Zeitgebers and fetal circadian rhythms, phase and time necessary to adapt to changing Zeitgebers differs between maternal and fetal rhythms, indicating that the relation is indirect (23, 42).

In adults, shift work leads to an increased incidence of several diseases (44). The exact effect of changes in maternal Zeitgebers, such as shift work on pregnancy and the developing embryo, however, is unclear. Maternal diurnal rhythms change during pregnancy (43, 83). Experiments in rat and hamster indicate that, although there is asynchrony between offspring, normal circadian rhythms arise in the absence of maternal Zeitgebers (12, 39, 75). Second, when comparing full and preterm babies, presence of circadian rhythms seems to be related to the degree of maturation instead of presence of Zeitgebers (101). In contrast, studies investigating the effects of maternal shift work during pregnancy show adverse effects on the offspring’s health. A meta-analysis from 2011 concluded that infants of pregnant women that do shift work are small for gestational age, but no effects of shift work on the incidence of preeclampsia and preterm delivery were found (8). Animal studies indicate that shift work leads to glucose intolerance and insulin resistance in pups (99). In addition, a dramatic reduction of successful term pregnancy after copulation was observed in female...
mice subjected to light-dark cycle phase shifts (93). (Studies in humans that analyze postnatal effects of maternal shift work during pregnancy are currently lacking.)

**Output of the Developing Circadian Clock**

Several physiological parameters such as heart rate and breathing movements have diurnal patterns during late gestation (15, 100). Animal models also show that fetal hormone levels, such as GH, IGF-I, ACTH, and cortisol show time-of-day-dependent oscillations, mainly at end-gestation (3, 4). It is unclear, however, whether these oscillations are regulated by autonomous fetal circadian clocks, by fetal circadian clocks that function as an executor of maternal signals, or by maternal factors directly (4, 59). Some studies suggest that this differs between developmental stages (12). The idea of functional fetal circadian clocks is supported by two findings. First, circadian rhythms are present in preterm infants, even if they are kept under constant external condition and without their mother (101).

<table>
<thead>
<tr>
<th>Author</th>
<th>Mode</th>
<th>Tissue</th>
<th>Gene</th>
<th>Start oscillation</th>
<th>Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johnson et al, 2002</td>
<td>PCR</td>
<td>ESC</td>
<td>Clock Bmal1, Per1, Per2, Per3, Cry1, Cry2</td>
<td>Not measured</td>
<td><img src="image1" alt="Graph" /></td>
</tr>
<tr>
<td>Ko et al, 2000</td>
<td>PCR</td>
<td>ESC</td>
<td>Bmal1</td>
<td>Not measured</td>
<td><img src="image2" alt="Graph" /></td>
</tr>
<tr>
<td>Dolatshad et al, 2010</td>
<td>PCR Whole</td>
<td>body</td>
<td>Clock Bmal1, Per2, Cry1</td>
<td>In vivo no oscillations, in vitro oscillations at E18 (=only time-point measured)</td>
<td><img src="image3" alt="Graph" /></td>
</tr>
<tr>
<td>Ansari et al, 2009</td>
<td>Immuno blot</td>
<td>SCN</td>
<td>Clock Bmal1, Per1, Per2, Cry1</td>
<td>No oscillation, No oscillation</td>
<td><img src="image4" alt="Graph" /></td>
</tr>
<tr>
<td>Shimomura et al, 2001</td>
<td>ISH</td>
<td>SCN</td>
<td>Per1, Per2</td>
<td>E17, P6</td>
<td><img src="image5" alt="Graph" /></td>
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</tbody>
</table>

**Figure 4.** Expression of core clock components during embryology in the mouse
E, embryonic day; P, postnatal day; SCN, suprachiasmatic nucleus; ESC, embryonic stem cell; ISH, in situ hybridization.
<table>
<thead>
<tr>
<th>Author</th>
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<th>Tissue</th>
<th>Gene</th>
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<th>Pattern</th>
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<tr>
<td>Saxena et al, 2007 (82)</td>
<td>Luciferase construct</td>
<td>Whole body</td>
<td>Per1</td>
<td>E12</td>
<td>![Graph]</td>
</tr>
<tr>
<td>Seron-Ferre et al, 2011 (84)</td>
<td>PCR</td>
<td>Whole body</td>
<td>Bmal1</td>
<td>No oscillation</td>
<td>E16 (only time-point measured)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Headless body</td>
<td>Per2</td>
<td></td>
<td>E18 (only time-point measured)</td>
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<tr>
<td></td>
<td></td>
<td>- Head</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>- Pars Tubularis</td>
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<td>- Hippocampus</td>
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<td>- Pineal</td>
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<td></td>
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<td>- Adrenal</td>
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<td>- Heart</td>
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<tr>
<td></td>
<td></td>
<td>- Liver</td>
<td></td>
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<tr>
<td>Sladek et al, 2004 (88a)</td>
<td>ISH/immuno histochemistry</td>
<td>SCN</td>
<td>Clock</td>
<td>No oscillation</td>
<td>Expressed E19-P10 (all time-points measured)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Bmal1</td>
<td>P3</td>
<td>No quantitative data in article.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Per1</td>
<td>P3</td>
<td></td>
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<td></td>
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<td></td>
<td>Per2</td>
<td>P3</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Cry1</td>
<td>P3</td>
<td></td>
</tr>
<tr>
<td>Sladek et al, 2007 (88)</td>
<td>PCR</td>
<td>Liver</td>
<td>Clock</td>
<td>P30</td>
<td>Expressed E20-adult (all time-points measured)</td>
</tr>
<tr>
<td></td>
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<td>Bmal1</td>
<td>P2</td>
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<td></td>
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<td></td>
<td>Per1</td>
<td>P10</td>
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<td></td>
<td></td>
<td></td>
<td>Per2</td>
<td>E20</td>
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<td></td>
<td></td>
<td>Cry1</td>
<td>P30</td>
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</tr>
<tr>
<td>Kováčiková et al, 2006 (46a)</td>
<td>ISH</td>
<td>SCN</td>
<td>Clock</td>
<td>After P2</td>
<td>Expressed E20-P2 (all time-points measured)</td>
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<td></td>
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<td>Bmal1</td>
<td>P1</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td>Per1</td>
<td>E20</td>
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<td></td>
<td>Per2</td>
<td>P1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cry1</td>
<td>P2</td>
<td></td>
</tr>
<tr>
<td>Sakamoto et al, 2002 (80a)</td>
<td>Northern blot</td>
<td>Heart</td>
<td>Bmal1</td>
<td>PS</td>
<td>Expressed P2-P30 (all time-points measured)</td>
</tr>
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<td></td>
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<td></td>
<td>Per1</td>
<td>P5</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Per2</td>
<td>P14</td>
<td></td>
</tr>
<tr>
<td>Torres-Farfan et al, 2011 (96a)</td>
<td>PCR</td>
<td>Adrenal</td>
<td>Bmal1</td>
<td>E18 (only time-point measured)</td>
<td>Not measured</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Per2</td>
<td></td>
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</tr>
</tbody>
</table>

Figure 5. Expression of core clock components during embryology in the rat and hamster
Second, intrinsic circadian oscillations in metabolic activity and transcription of clock output genes are present before birth (74, 76). A summary of the interaction between maternal and fetal circadian rhythms is depicted in FIGURE 7.

Circadian rhythms serve several purposes during embryogenesis. Just like in the outside world, the environment of the unborn fetus has diurnal changes. Glucose levels, for example, peak during the day. Anticipation to this peak allows the fetus to optimally use maternal energy. Second, for normal development, well timed proliferation of different tissues is essential. As described above, circadian rhythms play an important role in timing of proliferation. Third, circadian rhythms are involved in differentiation, an important step in physiological development. In the stem cell section of the present review, this issue will be discussed. Finally, origination of circadian rhythms in utero enables immediate anticipation of diurnal changes after birth.

### Table 1. Origination of circadian rhythms in primates

<table>
<thead>
<tr>
<th>Author/Animal</th>
<th>Mode</th>
<th>Tissue</th>
<th>Gene</th>
<th>Start Oscillation</th>
<th>Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torres-Farfan et al., 2006 (96b)</td>
<td>PCR</td>
<td>SCN adrenal, pituitary, thyroid, brown fat, pineal</td>
<td>Capuchin monkey Clock Bmal 1 Per2 Cry2</td>
<td>E142 (only time point measured, = 90% gestation)</td>
<td>Not measured</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Exception: Pineal Per2: no oscillating expression</td>
</tr>
<tr>
<td>Suter et al., (93a)</td>
<td>PCR</td>
<td>Liver</td>
<td>Japanese macaque Per1 Npas2 (comparable to Clock)</td>
<td>Not measured</td>
<td>Expression at E130 (only time point measured)</td>
</tr>
</tbody>
</table>

Expression of core clock components during embryology in nonhuman primates. E, embryonic day; P, postnatal day; SCN, suprachiasmatic nucleus. Objects in bold pertain to one another.

### Stem Cells

In utero, a single cell can develop into a full organism. The fertilized cell duplicates into two cells, followed by numerous other duplications and differentiation steps, which eventually lead to a complex organism with several specialized tissues. In the first stage of development, cells are able to differentiate into cells of all three germ layers (pluripotency). Later, cells can only differentiate into cells of a single germ layer (multipotency), followed by terminal differentiation into adult, somatic cells. Cells that are able to proliferate and differentiate are called stem cells. Pluripotent stem cells are able to proliferate indefinitely, multipotent stem cells generally have a more limited proliferation capacity, and most fully differentiated cell types show very little proliferation or none at all. In adults, under physiological conditions, only multipotent stem cells and fully differentiated cells are present. Multipotent stem cells play an important role in tissue renewal and repair. In tissues such as intestines (intestinal stem cell), skin (hair follicle stem cell), and blood (hematopoietic stem cell), multipotent stem cells create a constant renewal of cells (61). In other tissues such as the heart (cardiac stem cell), multipotent stem cells reside and become active after injury (5, 103).

In addition to in vivo studies, stem cells have been extensively studied in vitro. Pluripotent stem cells used for in vitro research can be derived from embryos [embryonic stem cells (ESCs)] or can be reprogrammed from somatic cells by forced expression of defined transcriptional genes [induced pluripotent stem cells (iPSCs)] (94). Pluripotent stem cells proliferate indefinitely when cultured under the appropriate conditions, thus forming a stable cell line, and can be differentiated into somatic, adult cells. Using pluripotent stem cells, large amounts of somatic cells, such as heart, kidney, or liver cells can be created. Because primary adult human cells are difficult to obtain in suffi-
cient numbers and many cell types cannot be expanded in culture for a prolonged period, stem cell-derived cells provide a better alternative for research and therapy in many situations. Stem cells and their derivatives are now being used to study embryonic development, to mimic adult physiology and pathophysiology, and for pharmacological efficacy/toxicity screenings, as well as being evaluated for their regenerative potential in diseases like heart and liver failure. A short summary of the developmental hierarchy of different stem cells is depicted in **FIGURE 8**.

**Circadian Rhythms in Stem Cells**

**Oscillations in Adult Stem Cell Mobilization and Trafficking**

Circadian rhythms have been described in different kinds of stem cells. A molecular circadian clock was found in human mesenchymal stem cells (MSCs) and epidermal stem cells in vivo and in vitro after synchronization (32, 36). In addition, studies in human volunteers show that hematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs) have diurnal rhythms in mobilization (50, 96). Oscillating numbers of HSCs in vivo are accompanied by diurnal rhythms in hematopoietic growth factors, which together lead to diurnal variations in the number of circulating blood cells (25, 50). Mouse studies show that amplitudes of these rhythms are largest at young age and diminish in the aged (89). Mechanistic studies have shown that stem cell mobilization, via neural signals, is orchestrated by the master clock (58). Using a circadian mutant mouse model, Mendez-Ferrer et al. (58) showed that diurnal patterns in stem cell mobilization disappear when the master clock is disrupted and that the regulation of mobilization is mediated by circadian rhythms in noradrenalin secretion. Some speculate that diurnal differences in the outcome of cardiovascular disease, such as myocardial infarction, might be caused in part by diurnal rhythms in homing of stem cells (96).

**Stem Cell Proliferation**

During embryonic development, circadian rhythms initiate at the end of gestation. In vitro studies show comparable results. Oscillations of clock genes are not present in pluripotent stem cells, develop during maturation, and disappear again when differentiated cells are reprogrammed to their pluripotent state in vitro (104). Circadian rhythms thus seem to be associated with stem cell proliferation and maturation. This hypothesis is supported by several other findings. Core circadian clock proteins and hypoxic transcription proteins, important in stem cell maturation and migration, are both part of the basic helix-loop-helix/Per-Arnt-Sim homology (bHLH/PAS) protein family and have a similar protein structure (25, 40). Second, transcription of nuclear hormone receptors involved in adult stem cell proliferation shows diurnal oscillation (105). Third, several enzymes that are involved in proliferation and reprogramming of stem cells, such as histone acetyltransferase/deacetylase and glycogen synthase kinase 3 beta (GSK3B), also play an important role in the regulation of circadian clocks (1, 17, 33, 81, 97). GSK3B is part of the Wnt signal transduction pathway and is responsible for phosphorylation of clock genes (1). Inhibition of GSK3B leads to a lengthened circadian period and modified stem cell differentiation and function (34, 97). Last, the development of tumors is associated with polymorphisms in core clock genes (25). Recent evidence even suggests that circadian rhythms do not only arise during differentiation but play a causal role in this process. Janich at al. (36) found that epidermal stem cells display circadian gene expression rhythmicity with heterogeneous phases. The phase of the stem cell was correlated with their predisposition to differentiate or activate; dormant stem cells were in opposite phase compared with proliferating stem cells (36). In these stem cells, deletion of core clock

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**Figure 7. Circadian rhythms in the fetus**
genes completely arrested the circadian clock and caused diminished self-renewal, resulting in premature aging and a reduced tumor risk. The exact mechanism that links phase differences in circadian rhythms to differentiation is unknown, but promotor analysis revealed that proteins in the Wnt signaling pathway and TGF-β regulators are likely involved. These results correspond with previous studies, which showed that disruption of the circadian clock leads to reduced proliferation and premature aging, exemplified by a reduction of muscle and bone mass (24, 41, 56). In addition, in vitro studies found that the ability of cells to differentiate reduces when circadian rhythms are disrupted and in vivo, and clinical studies link disruption of circadian rhythms to tumor susceptibility (2, 27, 87, 95). Lin and colleagues (51) thoroughly investigated premature aging in circadian disrupted animals. In their first study, they found a correlation between Bmal1 protein levels and the proliferative capacity of MSCs, progenitors of osteoblasts. They hypothesized that disrupted circadian rhythms result in a reduced proliferative capacity of MSCs, which in turn leads to reduced osteoblast formation and reduced bone formation, and eventually to reduced bone mass and premature aging. In a recent publication, they demonstrated that the Wnt pathway might mediate the effect of circadian rhythms on stem cell proliferation (51). In a mouse model, overexpression of Bmal1 led to increased levels of β-catenin, a core component of the canonical Wnt pathway, and an increased proliferation rate. The exact mechanism, however, and why disruption of circadian rhythms can lead to both increased and reduced tumor risk is still unclear.

**Future Directions**

The importance of time and circadian rhythms in biomedical research has been elucidated in the past several decades. In most preclinical and clinical studies, however, time is still not considered important. In preclinical research, for example, many experiments are performed in nocturnal animals, such as mouse and rat during working hours, which is their inactive (sleeping) period. When the same therapy is administered in the active (awake) period of humans, results are often disappointing. Circadian research shows that effects of treatments or stimuli differ between the active and inactive period, and poor translation of preclinical studies to the clinic might be partly due to this difference (21). In clinical studies, timing of a treatment is hardly analyzed, whereas studies that do take this into account find that therapies work better if they are administered at specific times (30). Taking time serious in biomedical research should in the future increase translation from laboratory to bedside and maximize the effect of treatments in clinic, such as pharmacological therapies, invasive therapies, and upcoming therapies, such as stem cell therapy and other types of regenerative medicine.

Research about the development of circadian rhythms and the role of circadian rhythms in development is slowly accumulating, and much knowledge is still missing. First of all, the origin of circadian rhythms needs to be studied in much more detail. Evidence, for example, that oscillating clock genes are not present in pluripotent stem cells and the first stages of development is mainly based on conventional PCRs that analyze large amounts of cells. New techniques enable more accurate, real-time measurement of oscillations on a single-cell level (49). Experiments using these techniques should hopefully elucidate whether circadian rhythms are truly absent in the first stages of development or just not synchronized/prominent yet.

Second, the exact role of circadian rhythms in maturation/differentiation needs further investigation. Several papers showed that in both pluripotent and adult stem cells, circadian rhythms are related to differentiation and maturation, but their exact role remains unknown. Is it, for example, possible to induce differentiation by affecting the circadian clock? Does the absence of Zeitgebers in in vitro research prevent or temper maturation of stem cells? Do changes in maternal Zeitgebers, such as shift-working mothers, affect physiological development of unborn children? Do interventions such as imposing strict day/night cycles on preterm babies in the neonatal intensive care unit

**Figure 8. Stem cells**

iPSC, induced pluripotent stem cell; ISC, intestinal stem cell; CSC, cardiac stem cell; HSC, hematopoietic stem cell.
References


