Embryonic development and maternal regulation of murine circadian clock function

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The importance of circadian clocks in the regulation of adult physiology in mammals is well established. In contrast, the ontogenesis of the circadian system and its role in embryonic development are still poorly understood. Although there is experimental evidence that the clock machinery is present prior to birth, data on gestational clock functionality are inconsistent. Moreover, little is known about the dependence of embryonic rhythms on maternal and environmental time cues and the role of circadian oscillations for embryonic development. The aim of this study was to test if fetal mouse tissues from early embryonic stages are capable of expressing endogenous, self-sustained circadian rhythms and their contribution to embryogenesis. Starting on embryonic day 13, we collected precursor tissues for suprachiasmatic nucleus (SCN), liver and kidney from embryos carrying the circadian reporter gene Per2::Luc and investigated rhythmicity and circadian traits of these tissues ex vivo. We found that even before the respective organs were fully developed, embryonic tissues were capable of expressing circadian rhythms. Period and amplitude of which were determined very early during development and phases of liver and kidney explants are not influenced by tissue preparation, whereas SCN explants phasing is strongly dependent on preparation time. Embryonic circadian rhythms also developed in the absence of maternal and environmental time signals. Morphological and histological comparison of offspring from matings of Clock-D19 mutant and wild-type mice revealed that both fetal and maternal clocks have distinct roles in embryogenesis. While genetic disruptions of maternal and embryonic clock function leads to increased fetal fat depots, abnormal ossification and organ development, Clock gene mutant newborns from mothers with a functional clock showed a larger body size compared to wild-type littermates. These data may contribute to the understanding of the ontogenesis of circadian clocks and the risk of disturbed maternal or embryonic circadian rhythms for embryonic development.

Keywords: Circadian clocks, embryonic development, mice

INTRODUCTION

Circadian clocks have evolved to allow organisms to anticipate and prepare for daily reoccurring changes in the environment. In mammals, these clocks are based on interlocked transcriptional–translational feedback loops (TTLs). In the core TTL, BMAL1 and CLOCK activate the transcription of Per1-3 and Cry1/2. PER and CRY proteins inhibit the BMAL1/CLOCK complex, and thus their own transcription (Koike et al., 2012; Lowrey & Takahashi, 2011). Such TTL-based circadian oscillations are found in most cells throughout the body. The major circadian pacemaker is located in the suprachiasmatic nucleus (SCN), which controls rhythms of peripheral downstream oscillators (Albrecht, 2012). Peripheral clocks locally induce rhythmic expression of 10–15% of active genes (Akhtar et al., 2002; Duffield et al., 2002; Panda et al., 2002; Storch et al., 2002). As a consequence, many critical physiological processes, like metabolic pathways and the cell cycle, are under circadian control (Eckel-Mahan & Sassone-Corsi, 2013; Masri et al., 2013).

Most studies on circadian function have focused on rhythms in adult animals, while the role of circadian clocks during embryonic development is still not well understood. The common view is that clock function emerges shortly before or around birth, but there is also evidence suggesting the existence of functional clocks during earlier stages of development in mammals (Du Pre et al., 2014). For instance, mouse embryonic fibroblasts collected at embryonic day 13 (E13) show rhythmic clock gene expression after synchronization in vitro, demonstrating that the cells already provide the molecular requirements for endogenous circadian timekeeping (Pando et al., 2002). Recent studies showed the ability of pluripotent stem cells and salivary glands
collected from early stages of PER2::LUC mouse embryos to develop circadian rhythms after several days in culture, while cell differentiation proceeds (Inada et al., 2014; Yagita et al., 2010). However, in these studies, rhythms needed to be induced by medium changes, and cell differentiation occurred under non-physiological conditions in vitro. Interestingly, rat fetuses carrying a Per1::Luc reporter transgene show bioluminescence signal already at E10 in utero with diurnal fluctuations starting several days prior to birth (Saxena et al., 2007). Whether these oscillations are driven by an endogenous embryonic clock or by rhythmic maternal signals was not shown. In fact, circadian rhythms of rat embryonic SCN glucose utilization are strongly dependent on maternal input (Reppert & Schwartz, 1983). Remarkably, pups born from SCN-lesioned hamsters or genetically arrhythmic mice are capable of developing circadian behavioral rhythms even when they are raised in the absence of external time cues (Davis & Gorski, 1988; Jud & Albrecht, 2006; Reppert & Schwartz, 1986).

In spite of these findings, the question whether circadian clock function is established during or after embryogenesis is not conclusively clarified yet (Sumova et al., 2012). Investigating clock gene expression in fetal SCNs in vivo, several studies failed to detect significant circadian oscillations prior to birth, which lead to the concept that SCN rhythms only develop postpartum (Sladek et al., 2004; Sumova et al., 2008). Others reported rhythmic clock gene expression in the fetal rodent SCN at late gestation, which is in line with the rhythmic metabolic activity of the SCN at this stage (Ansari et al., 2009; Houdek & Sumova, 2014; Kovacikova et al., 2006; Reppert & Schwartz, 1983; Shimomura et al., 2001). A recent study provides evidence that cultured SCN explants from PER2::LUC embryos express circadian rhythms beginning at stage E15 (Wreschnig et al., 2014). In addition, at late gestation, the fetal SCN and peripheral tissues of capuchin monkeys show distinct rhythms, which are entrained by maternal melatonin (Torres-Farfan et al., 2006). Twenty-four-hour expression profiles did not reveal circadian oscillations of Per2 and Bmal1 in mouse embryonic peripheral tissues at stages E18-19 in vivo, and microarray transcriptomes of mouse embryonic livers at E18-19 did not detect rhythms of clock genes and many clock-controlled genes (Dolatshad et al., 2010; Li et al., 2012). However, organotypic tissue cultures of SCNs, lungs, livers, kidneys and hearts collected at late gestational stages display PER2::LUC rhythms showing that peripheral tissues harbor a principally functional circadian clock at late stages of gestation (Dolatshad et al., 2010; Nishide et al., 2014). The use of different animal models and clock read-outs may explain the different conclusions drawn from these studies (Seron-Ferre et al., 2012).

Still, several unanswered questions remain to be addressed. (i) Are – similar to what has been shown for the SCN (Wreschnig et al., 2014) – peripheral embryonic tissues at early developmental stages already capable of expressing circadian rhythms? (ii) Are such rhythms driven by maternal cues or do they arise from self-sustained embryonic clocks, which can be entrained by maternal signals? (iii) If so, can embryonic clock function develop already in utero in the absence of maternal and external time cues? (iv) What role does embryonic and maternal clock function play in the development of the embryo?

Because of its importance for normal physiology in the adult, we hypothesized that circadian clocks develop in early stages of embryogenesis and are involved in regulating this process. Using PER2::LUC mice, we show that the embryonic anterior ventral hypothalamus, which contains the future site of the SCN as well as developing peripheral organs are capable of generating self-sustained circadian rhythms from E13 on and that the ability to express rhythms emerges in the absence of external time cues. Furthermore, our data suggest that fetal and maternal clock function have differential influences on embryonic development.

**MATERIALS AND METHODS**

**Animals**

All experiments for this project were carried out in mice on a C57BL/6J genetic background. At all times, animals had ad libitum access to standard rodent chow and water. Generation of Per2<sup>tm1Jt</sup> (PER2::LUC) knock-in mice and Clock<sup>tm1Jt</sup> (Clock-A19) mice was described previously (Vitaterna et al., 1994; Yoo et al., 2004). Pregnant mothers were maintained in LD 12:12 cycles (12 hours light at 200 lux, 12 hours dark) unless mentioned otherwise. The time of “lights on” is defined as Zeitgeber time 0 (ZT0). Matings of wild-type females with homozygous PER2::LUC males were set up to produce embryos heterozygous for PER2::LUC. Clock-A19 matings were carried out by breeding either homozygous or heterozygous Clock-A19 animals or homozygous Clock-A19 females with homozygous PER2::LUC males. The duration of pregnancy was comparable in all groups of animals and all pups were born spontaneously. For PER2::LUC genotyping, the following primers were used: “Per2::Luc forward”: 5’-CGCTGTGTTTTACTGCGAGTGAAGG-3’; “Per2::Luc reverse 1”: 5’-CCACAGATCTTCCCCCTTCCCG-3’; and “Per2::Luc reverse 2: 5’-GTCCCTATCGAAGGACTCTGGAC’.” For Clock-A19 genotyping, the following primers were used: 0623_fw: 5’-AGACCTCTCTCTGTCCAG-3’; 0624_rev: 5’-TGAGTCTCAGACGAAATAGTA-3’; and 1013_rev: 5’-TGGGTTAAAAAGACCTCTTGGCC-3’; and 1014_fw: 5’-GTCAGGGCGCTACAAG-3’.

All animal experiments underwent ethical assessment and were licensed and approved by the Office for Consumer Protection and Food Safety of the State of Lower Saxony and executed in accordance with the German Law on Animal Welfare.
Behavioral analysis
Homzygous Clock-Δ19 females were singly housed in running wheel-equipped cages in constant darkness (DD) until behavioral arrhythmicity was observed by χ² periodogram analysis over seven consecutive days. Arrhythmic females were taken out of the cage for about 72 hours during which they were mated with a PER2::LUC male in DD. Vaginal plug-positive females were put back to the running wheel cages for 15 days until embryos were isolated and dissected. Wheel-running activity was analyzed using ClockLab software (Actimetrics, Willmette, IL).

Tissue culture and bioluminescence measurement
Timed pregnant females were killed by cervical dislocation, and embryos were isolated from the uterus, decapitated immediately and kept in 4°C phosphate buffered saline. If not stated otherwise, animals were killed and dissected between ZT2-ZT6. Morning dissections (07:00; ZT0) were staged as EX.5, while evening dissections (19:00; ZT12) were staged as EX.0 (“X” is a placeholder for the embryonic day). The ventral part of the hypothalamus, which includes the developing embryonic SCN, and embryonic liver and kidney were dissected. The livers from stages E13.0–E16.5 were cut in half with a scalpel, while kidney and hypothalamus were cultivated in toto. Livers and kidneys from later stages were embedded in a block of 4% low-melting agarose, 10-min intervals using a LumiCycle bioluminometer to enhance rhythmicity. Measurements were done at long-day conditions (DD) until behavioral arrhythmicity was observed by χ² periodogram analysis over seven consecutive days. Arrhythmic explants were taken out of the cage for about 72 hours during which they were cultured in 35-mm Petri dishes containing 1 ml of culture medium (high glucose DMEM without phenol red, 352.5 m d-glutamine, 2% B-27 and 0.1 mM luciferin). The tissues were sliced with a vibratome (Campden, Lafayette, IN). Tissues were immediately transferred to tissue culture inserts (Millipore, Darmstadt, Germany) and cultured in 35-mm Petri dishes containing 1 ml of culture medium (high glucose DMEM without phenol red, 352.5μg/ml sodium carbonate, 10 mM HEPES, 25 U/ml Pen/Strep, 2 mM L-glutamine, 2% B-27 and 0.1 mM luciferin). The tissues of all experiments received no further treatment in order to enhance rhythmicity. Measurements were done at 10-min intervals using a LumiCycle bioluminometer (Actimetrics) that was placed inside a 37°C incubator with 5% CO₂. Period, peak phases, damping time and amplitude were determined by fitting a sine wave (Sin fit (Damped) for period, damping time and phase, LM fit (Sin) for amplitude) to 24-h baseline-subtracted data of culture days 2–7 using the LumiCycle software (Actimetrics). The first PER2::LUC peak was excluded from analyses. Outliers were excluded based on the Grubbs’ test. In order to take embryo growth and variations of sample sizes due to technical reasons into account, amplitudes were normalized to total brightness of individual specimens (amplitude of days 2–7 divided by total average brightness of the same time range). Explants failing to show significant χ² periodogram values or goodness of fit values >0 were determined to be arrhythmic.

Oil red O staining
Neonates (P0) were embedded in OCT (Tissue-Tek, Sakura, Torrance, CA), and 12-μm cryosections were prepared. Oil red O (Sigma, St. Louis, MO) stock solution was 1% in isopropyl alcohol. Staining solution was 60 % stock solution + 40% dextrin solution. Sections were incubated for 20 min and washed with 60% isopropyl alcohol until dye excess was removed and then washed in water. Nuclei were counterstained for 2 min in Mayer’s hematoxylin solution (Sigma) and washed with water. Stained sections were mounted with Kaiser’s glycerol gelatin (Merck, Darmstadt, Germany).

Alcian blue/Alizarin red skeletal staining
Neonates (P0) were killed in liquid nitrogen and macerated in water at room temperature (RT) for two hours and then transferred to 65°C warm water for 1–2 min. Skin and peripheral organs were removed. This was followed by incubation in 100% ethanol (EtOH) for four days at RT and in Alcian blue cartilage staining solution (150 mg/l Alcian blue 8GX (Sigma) in 80% EtOH, 20% acetic acid) for three days at RT, slowly rocking, followed by washing in 100% EtOH for six hours at RT and overnight incubation in 2% potassium hydroxide (KOH) to digest tissue. Next, specimens were consecutively incubated in Alizarin red (Sigma) bone staining solution (50 mg/l Alizarin red in 1% KOH) for 24 hours at RT, for another six hours in 1% KOH at RT, and in 1% KOH/20% glycerol until tissue was transparent. Specimens were stored in 50% glycerin/50% EtOH.

Measurement of neonate body part size and organ weight
Neonates (P0) were killed in liquid nitrogen. Body part size was measured with the help of a sliding caliper. Afterwards, organs were dissected and weighed with precision scales.

Data analysis
Statistical analysis was carried out with GraphPad Prism (GraphPad Software, La Jolla, CA). Used statistical tests and errors shown are indicated in the figure legends.

RESULTS

Rhythmic PER2::LUC activity in the developing SCN, liver and kidney from E13.5 until birth
To test whether embryonic tissues are capable of expressing endogenous circadian rhythms, we cultured liver and kidney tissues and the ventral part of the terminal hypothalamus, which contains the developing embryonic SCN, from heterozygous PER2::LUC mouse embryos. Tissues were collected from staged embryos at E13.5 and on each subsequent day until birth and, for comparison, on postnatal day 7 (P7). At all stages, embryonic hypothalamus, liver and kidney explants showed distinct circadian rhythms (Figure 1A). Long-term measurements over the course of 12 days of tissues from E15 show that these rhythms are stable and last for many days (Figure S1). To investigate the development of circadian traits during embryogenesis, we determined...
FIGURE 1. Circadian rhythms in explants from mouse embryonic ventral part of the hypothalamus, which includes the developing embryonic SCN, liver and kidney before and after birth. (A) Representative PER2::LUC oscillations in organotypic slice cultures from SCN, liver and kidney explants of different embryonic stages (E13.5-E19.5) and postnatal stage P7. Black: raw data, grey: smoothed data. (B and C) PER2::LUC period lengths (B) and normalized amplitude (C) of organotypic slices of SCN, liver and kidney were determined at early embryonic stages and were not significantly different from period and amplitude of tissue explants from postnatal stage P7, except of period from kidney explants at E18. Data are shown as average ± SEM; *p ≤ 0.05, (one-way ANOVA with Bonferroni post test comparing all data sets to P7); n values are shown in brackets. Data groups with n < 3 were excluded from statistical analysis.
period and amplitude of the cultured tissues. Except of a significant period difference between kidneys from E18 and P7, PER2::LUC period lengths and total luminescence-adjusted amplitudes of embryonic tissues were comparable to those of P7 explants (Figure 1B and C).

Embryonic phase of PER2::LUC rhythms of liver and kidney reflects endogenous rhythms rather than preparation times

To test whether luciferase rhythms in explants reflect endogenous embryonic clocks or are initiated by processing the tissues during culture preparation, we sacrificed pregnant mice on days 14 to 16 post coitum at either ZT0 or ZT12 and cultured embryonic tissues immediately. If explant phasing was determined by in vivo rhythms, the first PER2::LUC peak in culture for both groups should appear at roughly the same external time. In contrast, in case rhythms were initiated by the preparation procedure, the first PER2::LUC peaks of the two preparation groups should appear approximately 12 hours apart, at comparable times in culture after the preparation. PER2::LUC peak phasing of embryonic SCN
explants was relatively instable and strongly influenced by preparation time (Figure 2A). In contrast, for liver and kidney explants, we found that tissue preparation had no prominent influence on the appearance of the second PER2::LUC peak at all investigated embryonic stages (Figure 2B and C), arguing that for these tissues and stages explant PER2::LUC oscillations reflect the presence of circadian rhythms in embryos.

Embryonic circadian rhythms develop in absence of maternal or external time cues
To determine the impact of maternal and environmental time cues on the development and traits of embryonic circadian rhythms, we cultured tissues from PER2::LUC embryos, which developed in arrhythmic mothers maintained in constant environmental conditions. Homozygous Clock-D19 females were kept in running-wheel cages in constant darkness until no circadian locomotor activity rhythms were detectable any more. At this stage, they were introduced to a homozygous PER2::LUC male. After mating was confirmed by plug check under dim red light, females were returned to their home cage and recording of locomotor activity was resumed. There was no indication that mating had any restorative effect on behavioral rhythmicity in Clock-D19 mutant mice (Figure 3A). Fifteen days post coitum, dams were killed and embryonic tissues were isolated under dim red light and cultured as described. Although mothers were behaviorally arrhythmic and were kept under constant conditions before and during pregnancy, both fetal liver and SCN explants of all embryos showed distinct circadian rhythms in culture. Fetal kidney explants showed signs of rhythms in the circadian range, but failed to fulfill our criteria of stable rhythmicity since the sine wave goodness of fit was <0 (Figure 3B). In adult Clock-D19, heterozygous mice behavioral rhythms are characterized by lengthened period (Vitaterna et al., 1994). In line with this, SCN explants of heterozygous Clock-D19 embryos of mothers kept in DD showed a slightly lengthened period compared against wild-type embryos from mothers kept in LD12:12, although this effect was not significant (Figure 3C). In addition, the damping time of heterozygous Clock-D19 liver explants was lower than that of WT explants. Compared to wild-type SCNs, the SCNs of heterozygous Clock-D19 embryos also showed tendencies a lower damping time, but this difference was not significant (Figure 3D). Although the damping time in tissues from heterozygous was lower than in tissues from wild-type animals, a more detailed peak-to-peak analysis confirmed that the period of both genotypes was equally stable and not significantly different from each other (data not shown).

Embryonic and maternal circadian clocks play different roles during embryogenesis
To get a first idea about the potential impact of embryonic and maternal clock function on embryonic development, we morphologically and histologically characterized homozygous Clock-D19 and wild-type neonates (P0) from mothers of the same genotype, respectively. Because obesity had been reported in adult Clock-D19 mutants (Turek et al., 2005), we estimated adiposity in newborns by oil red O staining on horizontal cryosections through the whole gluteofemoral region. We found that when both embryonic and maternal clocks were not functional, neonates showed marked amounts of adipose tissue in the gluteofemoral area. In contrast, wild-type neonates from wild-type mothers showed no detectable fat tissue in that region (Figure 4A). We also investigated the development of skeletal structures. To determine the degree of ossification, we stained cartilage and ossified bone tissues with Alcian blue and Alizarin red. In comparison to wild-types, Clock-D19 neonates showed advanced ossification (Figure 4B). This difference was particularly apparent in the skull, the limbs, the caudal spine and the thoracic cage. Unlike in wild-type neonates, in mutants, the skull was almost completely ossified at P0. Moreover, ossification of the phalanges of mutants had already initiated, which was not the case for wild-type neonates. In addition, ossification of the spine and thoracic cage of neonates was more advanced in Clock-D19 newborns. We further found that mutant neonates showed disturbed limb and organ development. The foreleg was significantly shortened (Figure 4C), while liver, heart and spleen were significantly larger compared to the respective organs of wild-type neonates (Figure 4D). Of note, torso length, head and brain size were not significantly altered, indicating that growth effects were tissue-specific (Figure 4D).

In order to discriminate between the impact of embryonic and maternal clock function on embryogenesis, we next characterized morphological parameters of neonate offspring from heterozygous Clock-D19 breedings. In this case, neonates were either wild-type, heterozygous or homozygous for Clock-D19, while their mothers harbored a largely functional, Clock-D19 heterozygous clock. Interestingly, when born from the same mother, heterozygous and homozygous Clock-D19 neonates did not show increased fat deposits in the gluteofemoral region compared to wild-type littermates (Figure 5A). Furthermore, organ size was not affected by the embryonic genotype (Figure 5B). Although the livers and the hearts of heterozygous or homozygous, Clock-D19 neonates were slightly enlarged, this difference did not reach statistical significance. However, the torso lengths of homozygous Clock-D19 neonates were significantly larger compared to wild-type littermates, indicating that embryonic clock function may impact on skeletal growth regulation during embryonic development (Figure 5C).

DISCUSSION
In juvenile and adult mammals, the physiological importance of the circadian clock is well established,
and postnatal development of clocks has been investigated in many organs (Mateju et al., 2009; Sakamoto et al., 2002; Sladek et al., 2004, 2007; Sumova et al., 2012; Yamazaki et al., 2009). However, the existence and potential physiological relevance of circadian clocks during embryogenesis is still a matter of debate. While there are studies providing evidence of the existence of circadian rhythms in fetuses, conclusions are considerably inconsistent and strongly depend on the acquired methodology and species (Dolatshad et al., 2010; Polidarova et al., 2014; Sladek et al., 2007; Sumova et al., 2012). In addition, most investigations were restricted to the days around or shortly before birth. Studies using stem cells collected at early embryonic stages show that the cells differentiate in vitro and eventually develop circadian clock rhythms without
maternal input (Inada et al., 2014; Yagita et al., 2010). However, these rhythms needed to be initiated by medium changes, leaving the question open whether the cells have been rhythmic before. Furthermore, stem cell differentiation in vitro differs greatly from natural differentiation in an embryo developing in the mother. Thus, it is still unknown whether embryos in vivo develop an endogenous circadian clock and which role circadian rhythms play during embryogenesis.

In this article, we provide evidence that embryonic tissues harbor the ability to express endogenous circadian clocks at early developmental stages. A previous study showed that fetal SCN explants only show PER2::LUC rhythms when prepared at E15 or later (Wreschnig et al., 2014). However, in our study, at E13.5, the earliest embryonic stage we have investigated, the fetal liver, the fetal kidney and the ventral part of the fetal hypothalamus containing the developing SCN show distinct circadian PER2::LUC rhythms immediately after starting the culture. Of note, at this stage, these organs are not yet fully developed. The first morphological precursors of the liver are detectable at E9, but only at around E15, hepatoblasts differentiate into hepatocytes and build a functional liver (Zorn, 2008). Kidney development starts at around E8 and continues until 7–10 days after birth (Davidson, 2008). SCN neurogenesis occurs at stages E13.5/E14.0 and ends at E15 (Kabrita & Davis, 2008; Okamura et al., 1983). However, the dense synaptic network of the SCN, which enables the SCN to act as the dominant pacemaker, mainly occurs at later gestational stages and goes on until postnatal stages (Aton & Herzog, 2005; Moore & Bernstein, 1989). Since the SCN structure is not fully developed at most embryonic stages that we have investigated, we cannot rule out that the hypothalamus samples we have collected contained other developing...
brain areas than the SCN that may have contributed to the detected rhythms. Interestingly, our data show that period and amplitude of all three tissues are determined at very early gestational stages and stay stable over the course of embryogenesis and until after birth. Since differentiation of SCN, liver and kidney is not completed at E13.5, our data suggest that circadian rhythms may occur already in oligopotent progenitor cells. This result is in line with previous findings that, in contrast to totally undifferentiated stem cells, neuronal precursor cells already have the potential to be rhythmic in vitro (Kowalska et al., 2010).

Importantly, our data indicate that circadian bioluminescence rhythms of liver and kidney were not merely initiated by preparing the tissue cultures, but rather reflect endogenous embryonic rhythms that continue cycling in culture. Although using PER2::LUC explants to assess the internal phase is less accurate than direct in vivo observations, other studies have shown that organotypic tissue cultures from adult PER2::LUC mice reliably reflect endogenous in vivo phases (Pezuk et al., 2010; Ruan et al., 2008; Yoo et al., 2004). Liver and kidney rhythms from embryonic stages E14.0–E16.5 show stable phases independent of preparation time. Relatively prominent phase differences in liver were observed when comparing E15.5 and E16.0. This finding is consistent with a previous study in which a relatively strong impact of preparation time on the phasing of embryonic livers at these two stages was demonstrated (Dolatshad et al., 2010). However, in our study, the difference between the phases of E15.5 and E16.0 were less pronounced than in this study, and the inclusion of additional embryonic stages shows that the phasing of in vitro PER2::LUC rhythms of embryonic livers and kidneys is robust against preparation time. It is likely that this apparent discrepancy reflects experimental differences during isolation or cultivation of tissues. In contrast, the phase of the embryonic SCN is strongly influenced by preparation time, which leads to a prominent phase difference between samples prepared at ZT0 and ZT12. Previous data show that the adult SCN may be more sensitive to preparation and drastically shifts dependent on the preparation time, whereas peripheral oscillators appear more robust against such perturbations (Yoshikawa et al., 2005). Thus, one explanation for our result is that in vivo fetal SCN rhythms were reset during dissection and cultivation. Alternatively, fetal SCN PER2::LUC rhythms were induced by the preparation procedure. Dissecting the tissues and expose it to culture medium might be strong synchronization cues and therefore may lead to induction of detectable PER2::LUC rhythms in embryonic SCN samples.

Animals born from arrhythmic mothers and raised under constant conditions have been shown to reveal circadian rhythmic behavior several weeks after birth (Davis & Gorski, 1988; Jud & Albrecht, 2006; Reppert & Schwartz, 1986). In this study, we show for the first time that liver and SCN from fetuses from completely arrhythmic mothers and under continuous constant environmental conditions are already capable of generating coherent rhythms at E15. Although the kidney of embryos from arrhythmic mothers showed signs of rhythmicity, these oscillations did not reach statistical
significance when mothers were arrhythmic. This suggests organ specific differences in the ability to develop endogenous circadian rhythms independent of maternal and other external signals or the degree of coupling of the oscillators varies between tissues, which may lead to different qualities of rhythms. Alternatively, circadian PER2::LUC rhythms of heterozygous Clock-D19 mice were shown to be less stable than rhythms of wild-type mice (Yoo et al., 2005). Hence, the sensitivity of organs to the mutation may be different in various tissues. This may also explain why SCN and liver samples from heterozygous Clock-D19 embryos from arrhythmic mothers appear less stable than rhythms from wild-type embryos from rhythmic mothers. In line with the long free-running period of heterozygous Clock-D19 mice, the embryonic SCN from stage E15 showed a slightly lengthened period (Vitaterna et al., 1994; Yoo et al., 2005). However, the liver period of the same embryos was less affected by the mutation and showed a period close to 24 hours. It is possible that during embryonic development the loss of CLOCK in the liver is compensated by other circadian clock components, like NPAS2, which appears not to be the case in the embryonic SCN (Debruyne et al., 2006).

Our data suggest that the embryonic and the maternal clock play different roles during embryogenesis. We have measured histological and morphological traits of neonates and found that homozygous Clock-D19 neonates from homozygous Clock-D19 mothers have larger fat depots, show enlarged peripheral organs and a higher degree of ossification compared to wild-type embryos from wild-type mothers. However, in the case of Clock-D19 heterozygous mothers, which are capable of providing rhythmic time signals, physiological abnormalities of homozygous Clock-D19 neonates were less distinct, indicating that the observed phenotypes are largely depending on the genotype or clock function of the mother. Adult homozygous Clock-D19 mice were shown to be hyperphagic and obese (Turek et al., 2005). Besides increased food uptake, disturbed rhythms and general decrease of lipolysis, the Clock-D19 mutation promotes triglyceride accumulation in white adipose tissue, which may contribute to their obese phenotype (Shostak et al., 2013). In addition, homozygous Clock-D19 mice show elevated levels of serum triglycerides, cholesterol and glucose (Turek et al., 2005). Embryonic fat deposits are strongly regulated by maternal lipid and glucose levels (Desoye et al., 2011). The main source of embryonic glucose is derived by the mother and regulates embryonic insulin production, which in turn stimulates embryonic fat tissue growth (Czech et al., 2013). In line with this, in humans gestational diabetes is correlated with higher fat amounts in fetuses (King, 2006). In addition, fat storage is enhanced by transplacental lipid transfer from the mother (Desoye et al., 2011). Thus, increased fat storage in homozygous Clock-D19 neonates from homozygous Clock-D19 mothers is likely to depend on disturbed circadian clocks and the resulting obesity of the mothers. However, pleiotropic effects of CLOCK, which are not involved in circadian mechanisms, may also explain the observed effects.

Although total weight of wild-type and Clock-D19 mice is similar in the first five weeks after birth (Turek et al., 2005), we show that liver, heart and spleen are significantly heavier in homozygous Clock-D19 neonates when the mothers have no functional clock, but not when the maternal clock was functional.

Obesity is correlated with an increase of liver and heart size based on increased liver fat storage and increased demands on the heart muscle in obese adult mice and humans (Avery & Bumpus, 2014; Dong et al., 2006; Molina & DiMaio, 2012; Saito & Bray, 1984). A relationship between body weight of the mother and organ size of offspring was already shown for embryonic hearts during early gestation (Kandadi et al., 2013) and livers of 14-day old offspring of mice (Mischke et al., 2013). Hence, increased liver and heart size in homozygous Clock-D19 neonates might be related to the obese phenotype of their homozygous Clock-D19 mothers. However, a correlation between obesity and organ size was not found for the human spleen, and the spleens of obese mice are even smaller (Meade et al., 1979). We also observed advanced ossification in Clock-D19 neonates from homozygous Clock-D19 mothers. Osteogenesis was previously shown to be under circadian control and lack of BMAL1 leads to disturbed bone development in juvenile Bmal1<sup>−/+</sup> mice (Takarada et al., 2012). Thus, a disturbed circadian clock is likely to lead to abnormal ossification already during embryogenesis.

Interestingly, when comparing wild-type, Clock-D19 heterozygous and homozygous littermates from mothers with a functional clock, we found that the torso size is significantly bigger in homozygous Clock-D19 neonates. Since these pups were born from the same mother as their wild-type littermates, this strongly suggests that the embryonic clock is involved in embryo growth regulation. The cell cycle is under circadian control and thus, cell division is restricted to certain times of the day (Fu et al., 2002; Grechez-Cassiau et al., 2008; Matsuo et al., 2003). In the absence of a circadian clock, cells may divide more often, which may explain the bigger size of homozygous Clock-D19 neonates compared to their wild-type littermates. Alternatively, altered metabolic or endocrine regulation in the embryo or the placenta, which is partly formed by embryonic cells, may promote growth or nutrient uptake from the placenta. Although the effect of the embryonic genotype on torso length is moderate, it suggests an involvement of endogenous fetal circadian clocks in regulation of embryonic development.

In summary, our data suggest the presence of endogenous circadian clocks in the developing mouse SCN, liver and kidney at early gestational stages. Period and amplitude of embryonic circadian rhythms are determined early and stay relatively stable throughout embryogenesis and beyond birth. Our results suggest
that already at early embryonic stages, circadian rhythms in liver and kidney are endogenous. In contrast, rhythms of the embryonic SCN may have been induced by the preparation of the tissue. We further show that embryonic circadian rhythms are capable of developing in the absence of maternal or other environmental time cues. Genetic disruption of maternal and fetal clocks differentially affects embryonic development. Together, these data imply that external circadian disruption as occurs in shift workers may present a risk for normal embryonic development. However, further investigations are needed to more specifically dissect the roles of both maternal and fetal clocks in this context. Future experiments investigating physiological parameters of rhythmic fetuses from arrhythmic mothers may provide insights into the extent to which functional embryonic circadian clocks may compensate for disruptions caused by an arrhythmic maternal environment. Furthermore, examination of different circadian clock mutants may help to exclude pleiotropic effects of the Clock gene on embryonic development in Clock-Δ19 animals. In addition, studying the activity of the embryonic clock controlled genome, including genes regulating metabolism, cell division, apoptosis, differentiation and growth factors, may provide more mechanistic insight into the ontogenetic function of the circadian system.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary material available online
Supplementary Figure S1.