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MicroRNAs modulate core-clock gene expression in pancreatic islets during early postnatal life in rats

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Abstract

Aims/hypothesis Evidence continues to emerge detailing a fine-tuning of the regulation of metabolic processes and energy homeostasis by cell-autonomous circadian clocks. Pancreatic beta cell functional maturation occurs after birth and implies transcriptional changes triggered by a shift in the nutritional supply that occurs at weaning, enabling the adaptation of insulin secretion. So far, the developmental timing and exact mechanisms involved in the initiation of the circadian clock in the growing pancreatic islets have never been addressed.

Methods Circadian gene expression was measured by quantitative RT-PCR in islets of rats at different postnatal ages up to 3 months, and by in vitro bioluminescence recording in newborn (10-day-old) and adult (3-month-old) islets. The effect of the microRNAs miR-17-5p and miR-29b-3p on the expression of target circadian genes was assessed in newborn rat islets transfected with microRNA antisense or mimic oligonucleotides, and luciferase reporter assays were performed on the rat insulin-secreting cell line INS832/13 to determine a direct effect. The global regulatory network between microRNAs and circadian genes was computationally predicted.

Results We found up to a sixfold-change in the 24 h transcriptional oscillations and overall expression of *Clock, Npas2, Bmal1, Bmal2, Rev-erbα, Per1, Per2, Per3* and *Cry2* between newborn and adult rat islets. Synchronisation of the clock machinery in cultured islet cells revealed a delayed cell-autonomous rhythmicity of about 1.5 h in newborn compared with adult rats. Computational predictions unveiled the existence of a complex regulatory network linking over 40 microRNAs displaying modifications in their expression profiles during postnatal beta cell maturation and key core-clock genes. In agreement with these computational predictions, we demonstrated that miR-17-5p and miR-29b-3p directly regulated circadian gene expression in the maturing islet cells of 10-day-old rats.

Conclusions/interpretation These data show that the circadian clock is not fully operational in newborn islets and that microRNAs potently contribute to its regulation during postnatal beta cell maturation. Defects in this process may have long-term consequences on circadian physiology and pancreatic islet function, favouring the manifestation of metabolic diseases such as diabetes.

Keywords Circadian clock · MicroRNAs · Pancreatic islets · Postnatal maturation

Cécile Jacovetti and Adriana Rodriguez-Trejo contributed equally to this study.

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Abbreviations

BMAL	Brain and muscle ARNT-like
CLOCK	Circadian locomotor output cycles kaput

Bmal1–luc	Bmal1–luciferase
GFP	Green fluorescent protein
miRNA	microRNA
P	Postnatal day
qRT-PCR	Quantitative RT-PCR
siRNA	Small interfering RNA
UTR	Untranslated region

Introduction

Early postnatal life is a critical period for the development of the endocrine pancreas and involves the maturation of secretory and mitogenic processes. We and others have demonstrated that newborn beta cells are still immature immediately after birth [1–3]. They lack the unique and essential feature of mature beta cells: the capacity to secrete insulin in response to elevated glucose concentrations. In contrast, amino acid-stimulated insulin release and insulin biosynthesis are already fully operational. At the same time, newborn beta cells proliferate intensively within the expanding pancreas to achieve an appropriate beta cell mass that will then be maintained throughout adult life. We found that beta cell maturation is associated with a major reprogramming in gene expression. This process is at least in part governed by microRNAs (miRNAs), a class of small non-coding RNAs that inhibit the translation and/or stability of target mRNAs by pairing to their 3′ untranslated region (UTR) [1]. Indeed, we demonstrated that modifications in islet miRNA levels that take place during the postnatal period directly control the expression of key metabolic and cell cycle genes, driving the maturation of glucose-responsive adult beta cells.

Rodent and human pancreatic beta cells, like most cells in the body, possess self-sustained molecular clocks that coordinate the timing of metabolism throughout the day and modulate insulin secretion to maintain blood glucose homeostasis [4–7]. The circadian oscillators operative in beta cells and other peripheral cells are synchronised by systemic signals (e.g. neural and endocrine signals, fasting/feeding and metabolic cycles) controlled by a central pacemaker in the suprachiasmatic nucleus of the brain. This master clock, itself synchronised by daylight, maintains phase coherence among the body's cellular clocks [8–10]. The clock machinery has a strong influence on gene expression programmes and orchestrates the timing of metabolic processes by regulating in a circadian manner about 10% of the transcripts [11, 12]. Cell-autonomous feedback loops repeated every 24 h are generated by the activation of the transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1; also known as aryl hydrocarbon receptor nuclear translocator-like protein [ARNTL]) that trigger the expression of their own repressors, periods (PER1, 2 and 3) and cryptochromes (CRY1 and 2). CLOCK and BMAL1

also drive the rhythmic expression of the nuclear receptor subfamily 1 group D genes Rev-erbα (also known as Nr1d1) and Rev-erbβ (also known as Nr1d2) [9]. Moreover, the expression of clock genes is regulated at the post-transcriptional level by many other factors, including miRNAs [13].

Studies of rhythmic gene expression based on the disruption of clock genes in liver, pancreas, muscle and adipose tissue suggest that peripheral clocks may play an important role in optimising metabolism by coordinating the function of key metabolic organs to nutrient availability and energy requirements [14–17]. Importantly, previous studies in humans and rodents favour the view that the synchronised rhythmicity of clock gene expression matures at rates that differ between tissues and is the result of specific changes occurring in the environmental conditions of each organ [18–21]. Indeed, in humans, plasma circadian variations of the glucocorticoid hormone appear after 3 months of age [22], which induces changes in the circadian gene expression phases in the liver, kidney and heart [23]. Consistently, a gradual completion of clock gene rhythmic expression during postnatal life has been reported in rat liver within 30 days after birth [24].

However, the establishment of circadian rhythms during pancreatic beta cell development and its underlying mechanisms have not previously been investigated. Hence, in this study, we determined the expression levels and circadian oscillation profiles of several core-clock genes in maturing beta cells. Moreover, we assessed the role of nutrition-driven islet miRNA changes in modulating circadian clockwork during postnatal development. We investigated the expression and rhythmic oscillations of circadian genes in newborn rat islets, and analysed expression of that miR-17-5p and miR-29b-3p throughout beta cell maturation, in relation to islet clock gene expression.

Methods

Animals Male and pregnant female Sprague–Dawley rats were obtained from Janvier Laboratories (Le Genest-Saint-Isle, France) and housed under a 12 h/12 h light/dark cycle in a climate-controlled and pathogen-free facility. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and were approved by the Swiss Research Councils and Veterinary Office (authorisation VD2608.1). At birth, litters were adjusted to 10–12 pups per dam to standardise the mothers' milk availability. Newborn males and females were nursed until they were euthanised. At postnatal day (P)21, the pups were weaned on a standard chow diet containing 18.5% crude proteins, 4.5% fat and 54% carbohydrates.

Cell culture, transfection and lentiviral transduction Pancreatic islets were isolated as described [25] by collagenase

digestion followed by purification on Histopaque density gradients (Sigma-Aldrich, St Louis, MO, USA). The islets were cultured in RPMI 1640 Glutamax medium (Invitrogen, Carslbad, CA, USA) supplemented with 10% FCS (Amimed, London, UK), 50 U/ml penicillin, 50 μg/ml streptomycin, 1 mmol/l sodium pyruvate and 250 μmol/l HEPES. The rat insulin-secreting cell line INS832/13 [26], free from mycoplasma contamination, was cultured as described [27]. INS832/13 cells and dissociated rat islet cells were transfected using Lipofectamine 2000 (Invitrogen) [27]. To reduce miR-17-5p expression, approximately 250,000 cells were transfected with a specific single-stranded anti-miR (miScript miRNA inhibitor; Qiagen, Hilden, Germany), or with the miScript miRNA reference inhibitor (Qiagen) as a negative control. Overexpression of miR-17-5p and miR-29b-3p was achieved by transfecting cells with oligonucleotide duplexes (Eurogentec, Liege, Belgium; and Qiagen) using Lipofectamine 2000 (Invitrogen). A small interfering RNA (siRNA) directed against green fluorescent protein (GFP) was used as a negative control. The survival rate was over 90% in transfected cells. To monitor circadian bioluminescence, a Bmal1–luciferase (Bmal1–luc; Bmal1 is also known as Arntl) construct [28] was introduced into approximately 150,000 adherent cells by lentiviral transduction. To produce lentiviral particles, Bmal1–luc lentivectors were transfected into 293 T cells using the polyethylenimine method [5, 29]. Dissociated rat islet cells were transduced twice, with a multiplicity of infection of three for each transduction.

Measurement of miRNA and mRNA expression Total RNA was extracted with the miRNeasy kit (Qiagen). Mature miRNA and mRNA levels were assessed by quantitative RT-PCR (qRT-PCR) using the miScript II RT kit (Qiagen). miScript primer assays and primer sequences are provided in electronic supplementary material (ESM) Table 1. miRNA expression was normalised to the level of U6. mRNA expression was normalised to the amount of 18S. Pools of pancreatic islets from P1 (four pups), P5 (four pups) and P10 (two pups) were collected, while islets from P15, P20, P23, P31 and adult rats were extracted individually.

Luciferase assays Luciferase reporter plasmids were generated by cloning about 200 nucleotides of the 3′ UTR of rat Clock, Npas2 (encoding neuronal PAS domain protein 2) and Per3 containing the putative binding sites of miR-17-5p or miR-29b-3p into the psiCHECK-1 vector (Promega, Madison, WI, USA). Luciferase activity was measured in around 180,000 INS832/13 cells with a dual luciferase reporter assay (Promega) 2 days after transfection. Renilla luciferase activity from the psiCHECK-1 constructs was normalised for transfection efficiency to the SV40-driven Firefly activity generated from a co-transfected PGL3 promoter vector (Promega).

In vitro synchronisation and circadian bioluminescence measurement Adherent transduced islet cells were synchronised by a 1 h forskolin pulse (10 μmol/l; Sigma-Aldrich), and subjected to continuous bioluminescence recording with lumicycle equipment (made in house) in RPMI medium containing 100 μmol/l luciferin (D-luciferin 306–250; NanoLight Technology, Pinetop, AZ, USA) as previously described [5]. Photon counts from each well were integrated during 1 min over 24 min intervals. For detrended time series, raw luminescence signals were smoothened by a moving average with a window of 24 h, as previously described [7]. Average circadian amplitude and period length were calculated based on five consecutive peaks of detrended profiles starting from the second circadian cycle. The average timing of the positive peak phase was quantified starting from the second peak, relative to the 24 h day cycle [30].

Modelling of the core-clock mRNA–miRNA network during beta cell maturation We selected 62 miRNAs that are differentially expressed between newborn and adult rats [1]. For each miRNA of interest, we retrieved the target genes from several sources (DIANA-microT, ElMMo, miRBase, miRanda, miRDB, PicTar, PITA and TargetScan) using the MultiMir R package (http://multimir.ucdenver.edu; accessed Jan 2017) [31]. The retrieved interactions were filtered using the list of core-clock genes investigated in this study. The resulting network was visualised with Cytoscape 3.0 (www.cytoscape.org/cy3.html) [32]. The information about the expression changes between the islets of newborn and adult rats mapped on the network was inferred from the microarray data for the miRNAs [1] and the qRT-PCR data (present study) for the core-clock genes. The colour of the edges of the network indicated the sign of the log fold change of each connected node. Anti-correlated interactions, suggesting a functional link between the miRNA and the mRNA, were highlighted.

Statistical analysis

Statistical differences were calculated using a Student's t test for unpaired comparisons, or ANOVA followed by a post hoc Dunnett test for multiple comparisons, with a discriminating p value of 0.05 (GraphPad Prism, La Jolla, CA, USA). The amplitude differences between the consecutive circadian cycles in each profile were assessed using one-way ANOVA.

Results

Rhythmic transcriptional oscillation of circadian genes is not yet established in newborn islets After birth, rat pancreatic beta cells are unable to secrete insulin specifically in response to glucose and undergo a postnatal maturation process

resulting in the acquisition of a functional adult beta cell mass [1]. Since islets possess self-sustained molecular clocks that optimise cellular functions, including insulin secretion, we determined whether the islet circadian rhythmicity and its machinery differed between newborn and adult islets. For this purpose, we assessed the mRNA levels of several core circadian genes in newborn and adult rat islets across 24 h (Fig. 1). Interestingly, we found that the transcriptional oscillations of *Npas2*, *Bmal1*, *Rev-erbα*, *Per1*, *Per2*, *Per3* and *Cry2* over 24 h were strikingly attenuated in P10 compared with 3-month-old adult islets (Fig. 1b, c, e–h, j). Data are expressed as means ± SD.

These findings can be explained either by an intrinsic incapacity of the genes to oscillate in neonatal islets or by the lack of synchronisation between newborn cells. To investigate the cell-autonomous properties of the circadian clocks in newborn islets, the oscillation profile of the bioluminescent circadian reporter *Bmal1*–luc was recorded in cultured P10 and adult islet cells, following in vitro synchronisation. When synchronised by forskolin, both P10 and adult islet cells

exhibited self-sustained circadian oscillations of *Bmal1*–luc expression (Fig. 2a, b), but the characteristics of these profiles were distinct (Fig. 2c–e). Newborn islet cells displayed an earlier first peak (Fig. 2c; 5.51 ± 1.31 h in P10 islet cells compared with 10.10 ± 0.74 h in adult cells) and a circadian phase delay of almost 4 h (Fig. 2d; 12.68 ± 1.37 h in P10 vs 8.79 ± 0.64 h in adults). Moreover, circadian period length was about 1.5 h longer in P10 islet cells (25.08 ± 0.16 h vs 23.81 ± 0.22 h in adults; Fig. 2e). The average amplitude of *Bmal1*–luc oscillations in P10 islet cells tended to be lower compared with their adult counterparts, but this did not reach statistical significance (Fig. 2f; 0.19 ± 0.03 h vs 0.27 ± 0.046, $p = 0.248$). However, desynchronisation was faster in P10 than adult islet cells, as shown by a decreased circadian amplitude starting from the fifth cycle compared with the second cycle in P10 cells (Fig. 2g; $p = 0.0061$). Taken together, these data suggest that although the rhythmic oscillations of *Bmal1*–luc reporter were observed in P10 islet cells synchronised in vitro, their properties differed significantly from those of their adult counterparts.

Fig. 1 Rhythmic expression profile of genes involved in the circadian clock in newborn and adult islets. The islets of 10-day-old (black circles) and adult (white circles) rats were isolated and the RNA extracted at 4 h intervals across the 12 h/12 h light/dark cycle. Transcript expression was determined by qRT-PCR. Fold-change values are displayed as relative abundance to that of 18S amplified within the same sample. Two-way ANOVA was used to determine variance with respect to time and group (mean ± SD, $n = 4$ rats per group per time point, *$p < 0.05$ between groups)
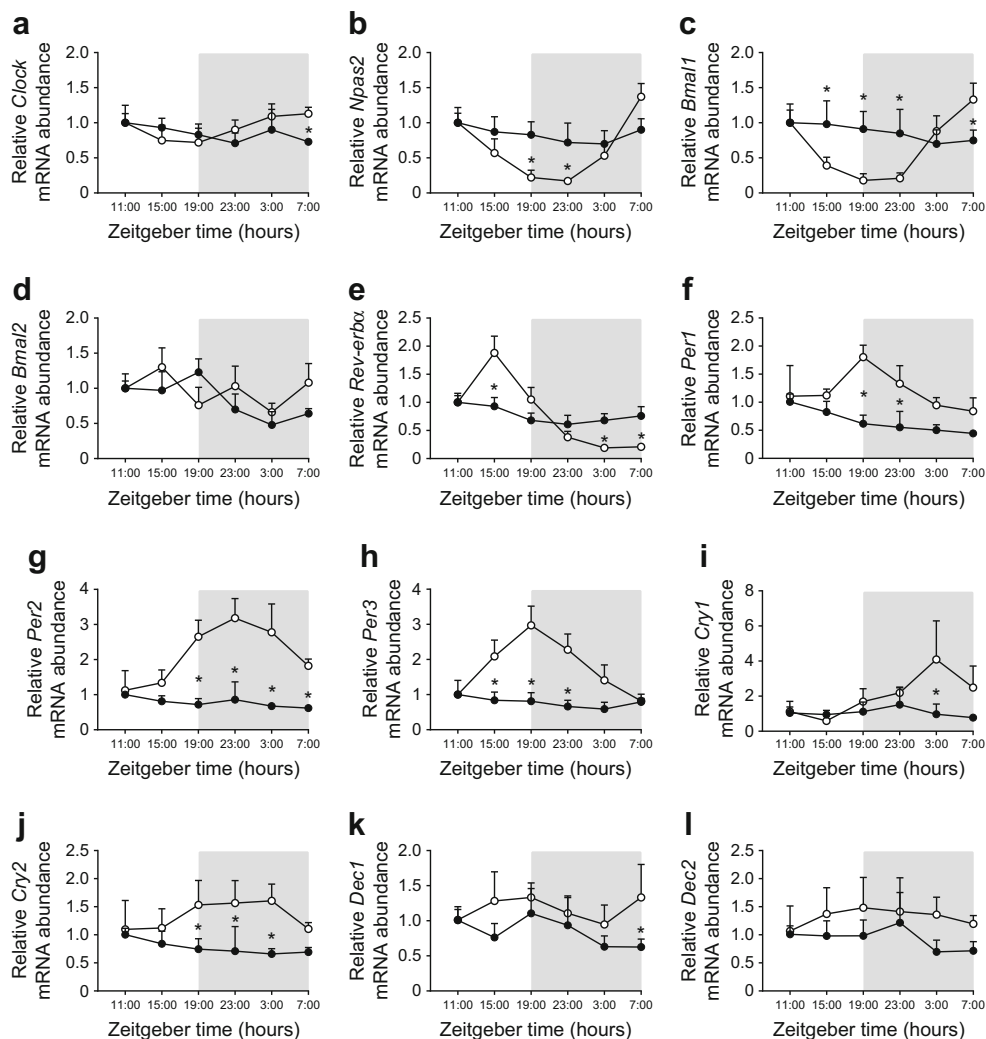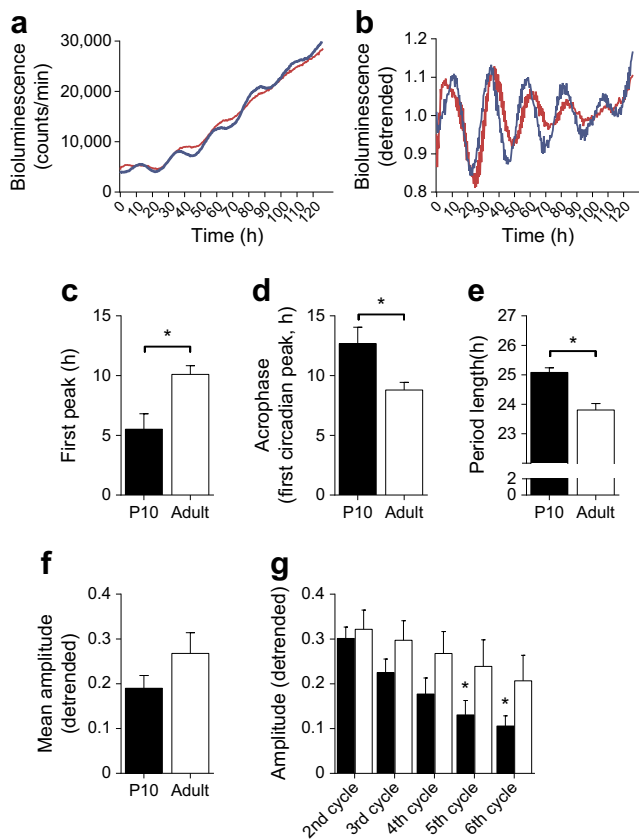
Fig. 2 Newborn and adult islet cells synchronised in vitro exhibit distinct circadian oscillation profiles. Pancreatic islets were isolated from P10 and 3-month-old adult rats. Islet cells were transduced with lentiviral particles expressing *Bmal1*–luc, synchronised by a forskolin pulse, and bioluminescence was recorded. (**a**) Raw data profiles (counts/min) from a parallel recording of islet cells derived from P10 (red line) and adult (blue line) animals. (**b**) Average detrended bioluminescence profiles for *n* = 3 independent experiments (one pooled litter of 10–12 pups per experiment) for P10 islet cells (red line), and *n* = 3 independent experiments (one animal per experiment) for adult islet cells (blue line). (**c**) Zenith (peak) time for the first peak of *Bmal1*–luc detrended bioluminescence (**b**) following forskolin synchronisation. (**d**) Average circadian phase of three consecutive peaks starting from the second peak and normalised to a 24 h day period. (**e**) Circadian period length. (**f**) Circadian amplitude, averaged for all the cycles starting from the second cycle, or (**g**) presented individually for each cycle (black bars, P10 islet cells; white bars, adult cells). Data in (**c–g**) are expressed as mean ± SEM, *n* = 3 independent experiments (one pooled litter of 10–12 pups per experiment) for P10 islet cells, and *n* = 3 independent experiments (one animal per experiment) for adult islet cells. *$p < 0.05$ unpaired, two-tailed Student's *t* test. (**g**) One-way ANOVA was applied to the P10 and adult graphs separately, indicating a significant ($p = 0.0061$) decrease in amplitude in P10, but not in adult ($p = 0.5390$), islet cells

Islet circadian genes are differentially expressed during postnatal maturation As beta cell postnatal maturation involves drastic changes in the expression of numerous islet genes, mainly involved in stimulus–secretion coupling and cell cycling [1], we determined whether the expression of circadian genes also differed between newborn and adult islets. qRT-PCR measurements revealed that half of the tested genes displayed different levels in adult compared with immature newborn islets (Fig. 3a). The expression of *Clock*, *Npas2*, *Bmal1* and *Bmal2* were higher in fully mature islet cells, whereas the levels of *Rev-erbα* and *Per3* were lower. We next examined the kinetics of the changes in expression of these circadian genes by measuring their levels at different time points during postnatal life. We observed that immediately after birth the expression profiles were stable, becoming modified after the third postnatal week (Fig. 3b–g), i.e. at the time the islet cells became fully functional. Indeed, we previously demonstrated that the nutritional shift occurring at weaning (postnatal day 21) leads to the acquisition of a mature insulin secretory phenotype. This adaptation ensures the release of appropriate amounts of insulin in response to an increased carbohydrate dietary intake [1]. These data reveal that the adult circadian gene expression profile in islets is established once beta cell maturation has occurred.

miRNAs regulate circadian gene expression Nutrient-induced postnatal miRNA modifications promote beta cell maturation by regulating the expression of metabolic and cell cycle genes [1]. To further determine the contribution of miRNAs to the regulation of islet circadian genes in postnatal life, we used the computational algorithms miRSystem and TargetScan to search for miRNAs targeting the 3′ UTR of circadian mRNAs. We included in our computational analysis 17 miRNAs that we had previously investigated for their role in beta cell maturation [1]. The compiled results revealed that the predicted targets of miR-17-5p and miR-29b-3p were enriched in genes belonging to the circadian machinery. To experimentally verify the computational predictions, we mimicked in islet cells obtained from 10-day-old rats the reduction of miR-17-5p (ESM Fig. 1a) and the upregulation of miR-29-3p (ESM Fig. 1b) that occurred during the postnatal maturation of beta cells. The decrease in miR-17-5p resulted in an increase of *Clock* and *Npas2* mRNA levels (Fig. 4a, b). Dual luciferase reporter assays in the rat beta cell line INS832/13 confirmed that miR-17-5p directly targeted the 3′ UTR of *Clock* but not *Npas2* (Fig. 4c; ESM Fig. 1c). Thus, the effect of miR-17-5p on *Npas2* expression is most likely to be indirect. As predicted by the computational programs, the increase in miR-29b-3p in newborn islet cells resulted in a reduction in *Per3* expression (Fig. 4d) and suppressed the luciferase activity produced from the *Per3*–3′ UTR construct (Fig. 4e; ESM Fig. 1d). These findings confirm that miR-29b-3p directly inhibited the expression of *Per3*, the antagonistic regulator of the CLOCK–BMAL1 heterodimer complex. We then assessed whether the expression of miR-17-5p and miR-29b-3p followed an oscillatory pattern across 24 h. As expected, the levels of miR-17-5p were lower and those of miR-29b-3p higher at all time points in adult islets [1]. However, none of the miRNAs displayed a circadian oscillatory pattern (Fig. 4f, g).

Fig. 3 Modifications of islet clock gene expression throughout postnatal life. (**a**) qRT-PCR was performed using islet RNAs from 10-day-old vs 3-month-old rats isolated between 8:00 and 10:00 hours. The expression of the indicated genes in adult islets is presented as the ratio to the level measured in newborn islets (dotted line) and represent the mean ± SD of four rats per group. *$p < 0.05$ vs newborn rats, ANOVA. (**b**–**g**) qRT-PCR was performed using samples from rats at the indicated ages. Data are mean ± SD ($n = 4$ per group). Statistical difference from 10-day-old rats was assessed by one-way ANOVA with a Dunnett post hoc test: *$p<0.05$. *Npas2* expression: $p = 0.06$ between P10 and adult islets
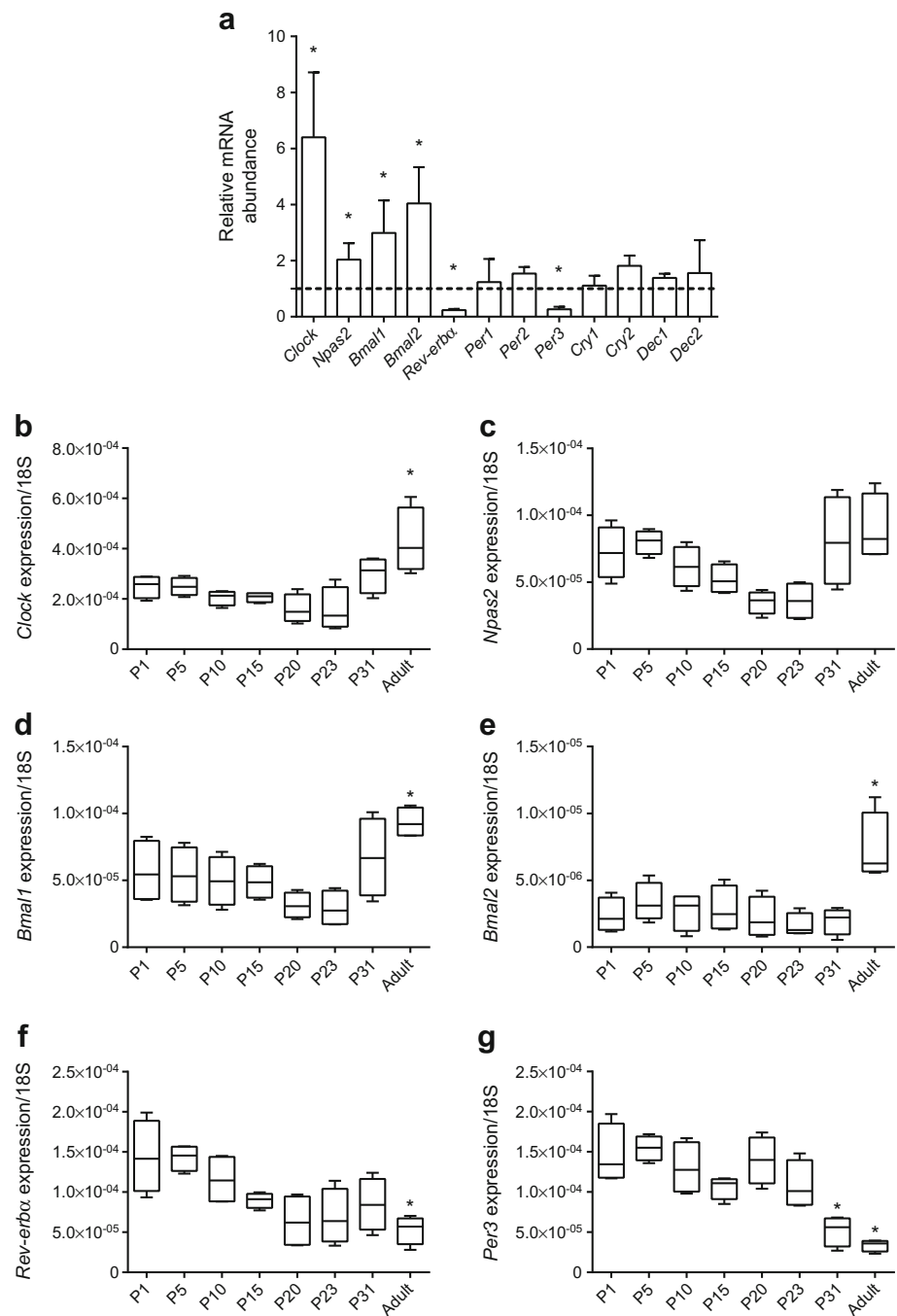
Besides miR-17-5p and miR-29b-3p, several other miRNAs displaying expression changes during postnatal beta cell maturation are predicted to target at least one core-clock gene. To obtain a comprehensive picture of the potential impact of miRNAs on the expression of core-clock components, we used a computational approach to search for all the predicted interactions between the 62 miRNAs displaying expression changes during the functional maturation of beta cells [1] and the 12 clock genes investigated in this study (Fig. 1). MiRNAs and core-clock genes were found to be connected by a complex regulatory network (Fig. 5), with more than 40 miRNAs showing anti-correlated expression changes with their respective targets. As expected, most miRNAs were predicted to regulate the level of multiple clock genes, and several clock genes were found to be targeted by multiple miRNAs. Of note, 20 different miRNAs displaying reduced expression changes upon weaning were found to potentially contribute to the observed rise in the level of *Clock* in mature beta cells (Fig. 5). These findings indicate that the islet circadian gene expression profile is probably not determined by a single
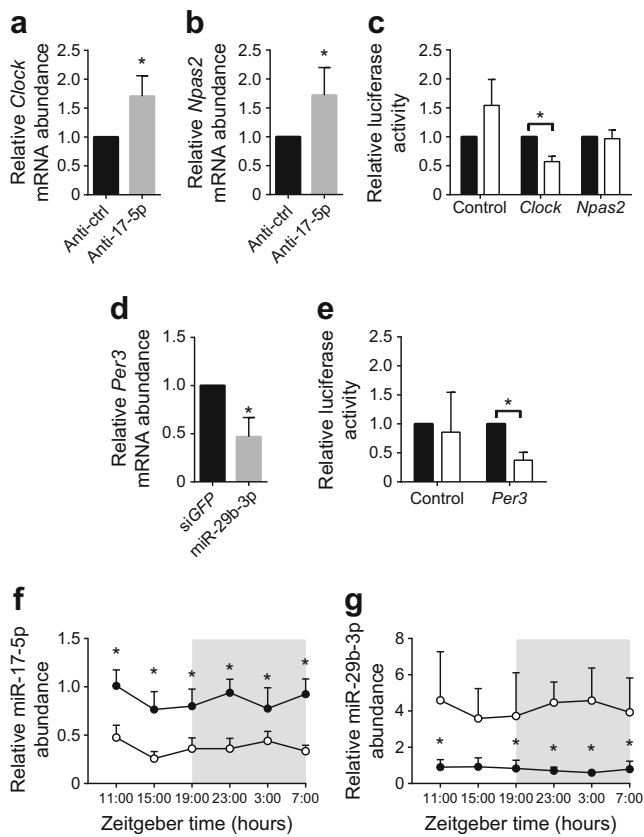
Fig. 4 miR-17-5p and miR-29b-3p regulation of clock genes and oscillation profiles. (a, b, d) Dispersed 10-day-old rat islet cells were transfected with anti-miRNAs to inhibit (a, b), or with oligonucleotide mimics to overexpress (d), the miRNAs. An miRNA reference inhibitor (Qiagen) and an siRNA against GFP were used respectively as negative controls (ctrl). mRNA levels assessed by qRT-PCR are shown as fold changes (n = 4–6). Data are mean ± SD. *p<0.05, Student's t test. (c, e) Direct interaction of miR-17-5p (c) and miR-29b-3p (e) with their putative targets was assessed by luciferase reporter assays in INS832/13 cells. Vectors containing the 3′ UTR of *Clock*, *Npas2* and *Per3* were co-transfected with either a control oligonucleotide or oligonucleotides mimicking the miRNAs of interest. An empty vector was used as the control. Data are mean ± SD (n = 4), and statistical significance was calculated by ANOVA. (f, g) Level of miR-17-5p and miR-29b-3p in 10-day-old (black circles) and adult (white circles) rat islets isolated at 4 h intervals across the 12 h/12 h light/dark cycle was determined by qRT-PCR. The data (mean ± SD, n = 4 per group per time point; *p < 0.05 between groups) are displayed as abundance relative to that of U6 amplified within the same sample

miRNA but results from the cooperative action of multiple miRNAs involved in the functional maturation of beta cells.

Discussion

Emerging evidence indicates that the cell-autonomous circadian clocks operating in the endocrine pancreas play an essential role in coordinating insulin secretion and islet cell transcriptome in a cyclic-dependent manner [5, 6, 33–36]. However, the rhythmicity and expression profiles of clock

genes in pancreatic islets and the mechanisms regulating them throughout postnatal beta cell maturation have so far not been investigated. The present study highlights drastic differences in the expression and temporal profiles of the core-clock genes between 10-day-old and 3-month-old rat islets. Importantly, we found that miR-17-5p and miR-29b-3p directly regulated the core circadian genes *Clock* and *Per3* throughout postnatal islet maturation, and are likely to be involved in establishing a functional autonomous molecular clock. Our data suggest that the regulatory network connecting miRNAs and clock genes is most probably far more complex, and the establishment of a fully operational circadian clock is unlikely to be explained by single miRNA–target associations. Indeed, several other miRNAs involved in the functional maturation of beta cells are predicted to regulate at least one of the core-clock genes and display anti-correlated expression changes with their putative targets. Additional studies will be needed to experimentally verify these computational predictions and to precisely delineate the interconnection between miRNAs and the circadian clock in the context of postnatal beta cell maturation.

We demonstrated that the circadian expression patterns of clock components are not fully established in immature newborn islets. Of note, although in vivo circadian oscillations in newborn islet cells were strongly dampened, oscillations of the circadian reporter could still be induced in vitro in P10 islet cells by forskolin synchronisation (compare Fig. 1 with Fig. 2). These findings suggest that the lack of oscillations observed in newborns is a consequence of the desynchronisation of the cells rather than an intrinsic incapacity of clock genes to undergo circadian expression changes. However, the oscillatory profiles observed in vitro have distinct characteristics in P10 cells compared with their adult counterparts. These changes comprised the phase advance of the first circadian peak (Fig. 2c), which may suggest an altered immediate resetting response. The differences between P10 and adult islet cell clocks were not limited to the kinetics of the first circadian cycle, but were extended to the circadian cycle characteristics such as phase, amplitude and period length (Fig. 2d–f), including a faster decrease in the amplitude of the P10 oscillations, which may suggest more rapid desynchronisation for these cells following the initial pulse. In agreement with our observations, the reported phase shift of the circadian oscillator Per1 in rat liver, thyroid and pineal gland throughout development [19] may highlight a ubiquitous delay of the clock gene oscillations in the maturating peripheral tissues after birth. Despite the fact the circadian clock machinery is already expressed at a cellular level at very early developmental stages—although initially only at low basal levels—it was proposed that circadian rhythm ontogenesis is strongly influenced by changes in the environmental conditions and in organ function occurring during development [19, 37, 38]. It is well established that both mature beta and alpha cells display cell-autonomous circadian rhythms of core-clock and functional metabolic genes [6, 7, 39]. Consistently, we
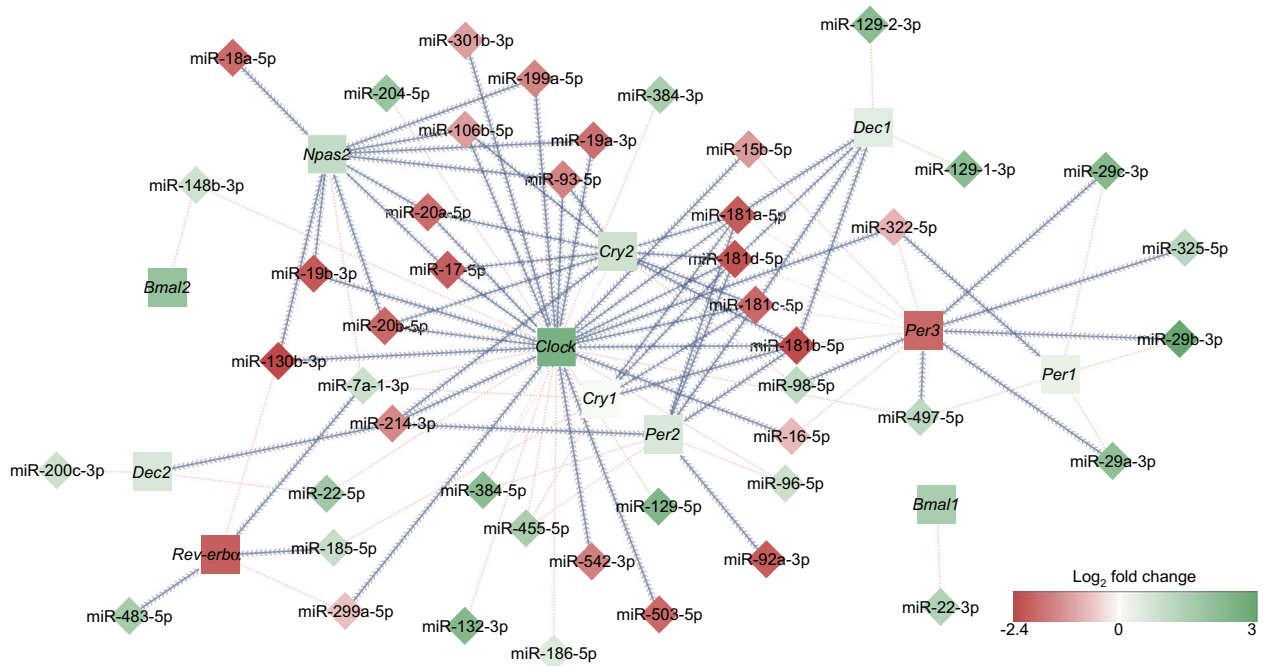
Fig. 5 Core-clock mRNA–miRNA network during beta cell maturation. Potential interactions between miRNAs displaying expression changes during postnatal beta cell maturation and circadian clock genes were computationally predicted as described in the experimental procedures. Green and red symbols indicate up- and downregulation (log$_2$) between adult and newborn rat islets, respectively. Opposite expression changes between the miRNAs and their putative targets suggestive of functional interactions are depicted with thick blue lines, while changes in the same direction are shown with pink lines. Clock genes are shown with square symbols, while miRNAs are presented as diamond symbols

observed that forskolin-induced oscillations of clock genes are elicited ex vivo despite the absence of in vivo circadian rhythms in newborn islets. These data were obtained in whole islets. Hence, these changes may occur and have an effect on one or more islet cell types. Nevertheless, beta cells constitute the main cell type in both P10 and adult islets, suggesting that modifications in the circadian gene oscillation profiles and overall mRNA levels contribute to beta cell maturation. To further elucidate the mechanism of beta cell clock maturation, in vitro experiments implying single-cell analysis of P10 and adult islet circadian bioluminescence profiles will be required.

Taken together, our results suggest that the initiation of islet circadian rhythms might rely on environmental factors, comprising blood-borne signals. Although further investigations are needed to determine the potential involvement of hormonal factors and neural inputs in the establishment of circadian rhythms, our study reveals that miRNAs are part of the mechanisms contributing to the post-transcriptional control of clock genes in immature islets. We have shown previously that miRNA modifications are driven by the shift in food supply occurring at weaning when high-fat maternal milk is replaced by a carbohydrate-enriched diet. In view of this, it is tempting to speculate that the nutritional switch is responsible not only for islet miRNA changes and the subsequent functional beta cell maturation, but also for adaptive circadian clock ontogenesis. Indeed, the attenuated clock gene expression and phase in the islets during early postnatal

life suggest that the circadian regulation of insulin secretion is not yet necessary for maintaining blood glucose homeostasis as newborn animals fed a low-carbohydrate diet are normoglycaemic. Accordingly, disruption of the circadian rhythmicity of clock genes induces beta cell dysfunction, impairment of glucose metabolism and the release of inappropriate amounts of insulin [33, 36]. Before weaning, the low glucose responsiveness of newborns is thought to be the consequence of reduced glucose oxidation and metabolite production leading to an imbalanced NADPH/NADP ratio [40]. NADPH and the subsequent activation of essential transcription factors is a strong regulator of clock gene activation and has the potential to phase-shift circadian rhythms [41, 42]. This supports our hypothesis that circadian clock ontogenesis is rather needed once the beta cells have to face the rise in insulin needs required to match the increased carbohydrate supply, and hence the islet clock is required to coordinate beta cell function with circadian energy homeostasis.

Our study also reveals that the expression of core-clock genes in postnatal rat islets reaches levels similar to those of adults within 30 days of age. The differential rhythmicity and expression of clock genes observed in immature compared with adult islets have been suggested to apply also to other metabolic organs in early life. Indeed, previous studies have reported a gradual development of the circadian clock during the postnatal period, with temporal differences in the

appearance of clock amplitude oscillations between the central nervous system and heart in mice [43]. This raises the hypothesis that the time frame of islet clock establishment might be cell type-specific and directly related to the capacity of the beta cells to release insulin in response to nutrients, in particular to glucose, allowing the increased insulin needs caused by food transition to be met.

The islet miRNA changes taking place during postnatal beta cell maturation, which we detailed in a previous study [1], are unlikely to be dictated by the central clock pacemaker in the suprachiasmatic nucleus, which is itself activated by light exposure [44]. In contrast, miRNA modifications occur between postnatal days P20 and P31, when the transition from fat-enriched maternal milk to a high-carbohydrate diet takes place. In line with this, Sladek and colleagues showed that the amplitude of the oscillations of clock genes in rat liver are not yet established at the age of 20 days but achieve a mature state only at around P30 [24]. Similarly, the circadian expression of intestinal disaccharidase enzymes achieves the levels observed in adult rats only several days after weaning [45]. Previous findings suggest that the establishment of clock gene expression and rhythmicity in peripheral tissues such as the endocrine pancreas is entrained by the periodicity of meals, which is acquired during adulthood, rather than by light [46]. Indeed, photo-entrainment is already functional at birth [47], whereas the rhythmicity of timely regulated food intake seems to be established after the shift to solid food, reinforcing the need to achieve a regulated insulin release in a circadian manner after the transition of nutritional supplies that occurs at weaning. Food-anticipatory activities are defined as food-seeking behaviours in anticipation of mealtimes, including physiological and hormonal activations in response to daily temporal windows of feeding time [48, 49]. Notably, global and brain-specific deletions of the clock component *Rev-erbα* have been reported to impair the 24 h pattern of food entrainment in mice [50]. We could speculate that, in newborns, the lack of a regulated temporal pattern of food intake justifies the absence of a circadian control of beta cell secretory activity. This raises the possibility that circadian clock entrainment and feeding time dictate the already described phasic insulin release from adult islets [7].

In conclusion, we have revealed the contribution of miRNAs to circadian clock regulation in pancreatic islets during the postnatal period. The suggested plethora of known and yet-to-be-discovered signalling pathways linking the chronobiology of insulin secretion with miRNAs indicates that we have just begun to scratch the surface of understanding the interplay between the circadian clock and postnatal beta cell maturation.

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