Circadian networks in stem cells and their cardiac derivatives

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Cover art: front - oscillatory waves, back - modified human embryonic stem cell-derived cardiomyocyte structure; by Jan Willem Hoogstraten

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Circadian networks in stem cells and their cardiac derivatives

Circadiane netwerken in stamcellen en daarvan afgeleide cardiale celtypes

(met samenvatting in het Nederlands)

Proefschrift

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door

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Outline and scope of the thesis

Cardiovascular diseases remain a leading cause of death in Western countries, and are influenced by diurnal rhythms that result in oscillatory gene expression in the heart. Indeed, the circadian clock has been shown to determine the functionality of cardiomyocytes, with detrimental consequences when rhythmicity is disturbed. In vitro differentiation of pluripotent stem cells towards the cardiac lineage is well described, and allows for the derivation of an inexhaustible source of cardiomyocytes for the investigation of heart development and function. Moreover, cardiac stem cell derivatives can be used for drug screenings as well as be tested for their potential to regenerate the heart after injury such as myocardial infarction and heart failure. Whether and how knowledge on circadian rhythms could be applied to optimize applications of stem cell-derived cardiomyocytes in vitro as well as in vivo remains largely unknown, however. In this thesis, we study the role of clock factors and circadian networks in mouse and human embryonic stem cells and their cardiac derivatives.

In **chapter 1** a detailed overview of the role of the circadian clock in cardiovascular physiology is given. In addition, we describe how disturbance of circadian rhythmicity hampers proper cardiac function, and discuss how knowledge on the molecular clock could be used to improve cardiac therapy.
In **chapter 2** multiple differentiation strategies to obtain cardiomyocytes are reported. We elucidate on how to characterize, purify and use these cells that are attractive candidates for translational regenerative purposes.
In **chapter 3** the establishment and role of the circadian clock during directed differentiation of human embryonic stem cells towards cardiomyocytes is investigated. We uncover an oscillating stress-response network and show that this leads to a time-dependent response to stressors such as the anti-cancer drug doxorubicin.
In **chapter 4** we propose the use of neonatal rat cardiomyocytes as a straight-forward model to recapitulate in vivo circadian rhythmicity.
In **chapter 5** a synthetic REV-ERB ligand is shown to reprogram the transcriptome of mouse embryonic stem cells, which results in a metabolic switch as well as a slowdown of proliferation.
In **chapter 6** I discuss the (dis)advantages of different cardiac model systems as well as some considerations on the analysis of oscillatory gene expression.

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Circadian rhythms in stem cell biology and function

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Abstract

The circadian clock adapts the body physiology to light/dark cycles with a period of 24 hours. The main timekeeper, a group of neurons termed the suprachiasmatic nucleus (SCN), is located in the brain and is set by relayed signals from the eye after photoreception. Kept in synchrony by the SCN, peripheral tissues contain their own circadian clocks. Entrainment of these peripheral clocks is mediated by the SCN via humoral factors, metabolites and body temperature as well as by SCN independent determinants like food and physical activity. Therefore, peripheral clocks can also oscillate in a cell-autonomous way. At the molecular level, circadian oscillation is observed as an array of thousands of specific genes that are expressed in a 24-hour cosinor manner with different phases. This is obtained by the clock machinery, which is a transcriptional/translational feedback loop consisting of a dozen of core clock genes. Together with systemic cues, this core clock system initiates the oscillatory expression of tissue specific genes, termed clock-controlled genes. Oscillations in these output genes will render a 24-hour rhythmic output in body physiology. The circadian clock has been implicated in various processes such as cell cycle, cell differentiation, metabolism, aging, and regeneration. Impairment of the clock leads to varying defects ranging from sleep, metabolic and cardiovascular disorders to premature aging and even the development of cancer. Pluripotent embryonic stem cells do not show significant oscillations in clock gene expression but gradually start oscillating during in vitro differentiation, corresponding to the situation seen during embryonic development. The acquirement of a clock in differentiating cells contributes to their oscillatory physiology and is most likely linked with their regenerative capacity. In this chapter we review the function of circadian rhythms in stem cells and their derivatives. We outline their implication in regeneration, with a focus on how the circadian clock influences myocardial biology and cardiac regeneration.

1.1 Introduction

Terrestrial life revolves around the 24-hour cycle of day and night. The light-dark cycle has a direct influence on organismal functioning, dictating wake-sleep patterns in animals and cycles of photosynthesis in plants. An underlying mechanism termed the circadian clock regulates these processes at the molecular level. The word circadian is derived from the two Latin words *circa* and *dies* which mean “around” and “day” respectively. This mechanism is heavily conserved throughout evolution and allows organisms to adapt and to synchronize themselves to diurnal fluctuations in their environment. Circadian rhythmicity can be seen in many different life forms, ranging from unicellular organisms, like cyanobacteria, to highly specialized and complex multicellular organisms, such as mammals. Concomitantly, circadian rhythms regulate many body features of animals like behavior, metabolism, blood pressure, body temperature, tissue physiology, regeneration and homeostasis (Aschoff, 1983).

The central core clock in mammals is located in the suprachiasmatic nucleus (SCN), a group of approximately 20,000 neurons in the anterior hypothalamus in the brain. The SCN clock

is driven by light, the signal that is relayed after perception in the retina by photoreceptors. However, the clock processes are not driven by light per se. Light should be seen as the main external synchronizer (*also known as zeitgeber; time-giver*) forcing the body to adapt to a 24-hour period, rather than driving circadian rhythmicity in physiology directly. The SCN synchronizes peripheral clocks through neural and humoral factors like the serotonin-derived hormone melatonin (Cajochen et al., 2003). Peripheral clocks are present in almost all tissues in the mammalian body, including liver, lung, kidney, skin, and the heart. These clocks maintain circadian tissue physiology via controlling tissue-specific gene expression (Brown and Azzi, 2013). The molecular machinery behind this timekeeping system comprises multiple genes, termed *clock genes*, which products interact with each other ensuring stable and robust circadian rhythmicity.

The hallmark of circadian rhythms is that they keep on cycling with the same phase in the absence of an external input. This can be seen by the persistence of circadian rhythmicity when animals are kept in complete darkness. Another typical feature of the circadian clock is the fact that it is not altered by external perturbation or at mild variations of ambient temperatures, a process known as “temperature compensation” (Merrow et al., 2005). This is nicely illustrated by the fact that the clocks of warm-blooded animals are buffered against and maintained at different temperatures throughout the day.

The importance of maintaining a functional time-keeping system is shown by the fact that disruption of the clock has been associated with a vast array of malignancies, such as impairment of lipid homeostasis resulting in a fatty liver and obesity (Adamovich et al., 2014) (2014). A disturbed regulation of the clock has also been linked to the development of cardiovascular diseases, multiple sleep disorders, depression, inflammation, cancer, impairment of regenerative capacity and other metabolic disorders (Kennaway et al., 2013; Lumaban and Nelson, 2014; Rudic et al., 2004) like diabetes (Marcheva et al., 2010; Milagro et al., 2012). Furthermore, recent research has shown that circadian timekeeping can also be linked to developmental and physiological processes that are not necessarily associated with a 24-hour daily pattern. This can be observed in clocks regulating somatic stem cell heterogeneity (Janich et al., 2011), cell division (Feillet et al., 2014; Kowalska et al., 2013; Matsuo et al., 2003; Nagoshi et al., 2004; Unsal-Kaçmaz et al., 2005; Yang et al., 2009), damage induced regeneration (Janich et al., 2013), immune progenitor cell migration and differentiation (Scheiermann et al., 2013; Yu et al., 2013) as reviewed by Steven Brown (Brown, 2014).

In the heart specifically, the circadian clock modulates the response to induced damage, such as ischemia/reperfusion, as demonstrated by the fact that diurnal oscillations in infarct size are blunted in cardiomyocyte specific clock mutants (Durgan et al., 2010). However, the precise role of circadian clocks in cardiac regenerative medicine still needs to be determined. Stem cells can now efficiently be differentiated towards cardiac cells in vitro as reviewed in (Dierickx et al., 2012). Studying how circadian rhythms in cultured cells can enhance their

regenerative effects, but also elucidating the optimal administration time of these cells into the myocardium, will be areas of research that could boost the field towards more effective therapy.

1.2 The core clock machinery

1.2.1 A complex transcriptional feedback loop defines the molecular clock

The molecular clock machinery comprises an interlocking activating and inhibiting transcriptional/(post)translational feedback loop (TTFL). This renders a 24-hour rhythmicity pattern in expression of clock-controlled genes (CCGs), resulting in a circadian functional output (schematic overview in Figure 1). Two of the main players in this complex system are BMAL1 (Brain and Muscle ARNT-Like 1) and CLOCK (Circadian Locomotor Output Cycles Kaput), encoded by *Arntl* and *Clock* respectively. They are both bHLH-PAS (basic helix-loop-helix, *Per-Arnt-Single-minded*) proteins and form the center of the activating limb of the circadian clock pathway. Upon heterodimerization via their PAS domains, they drive gene expression via docking on two types of enhancer box elements (E-Boxes): E-box (5'-CACGTG-3') and E'-box (5'-CACGTT-3'). These E-boxes lay near or in the promoter of their targets, termed CCGs (Gekakis et al., 1998; Hogenesch et al., 1997; Ohno et al., 2007; Yoo et al., 2005). It is now clear that both genes are inevitable to sustain a proper clock. For example, knocking out *Bmal1* results in the complete loss of behavioral rhythmicity (Bunger et al., 2000; Shi et al., 2010). *Clock* knockout mice do not show this phenotype, but this is probably because the role of CLOCK can be bypassed by NPAS2, a protein with an analogous function. Indeed, NPAS2 deficient mice still show rhythmic behavior (Dudley et al., 2003), which is lost in *Clock/Npas2* double knockouts (DeBruyne et al., 2007).

Transcription of two groups of CCGs, named Period (*Per*) genes and Cryptochrome (*Cry*) genes, are activated by BMAL1 and CLOCK. These output genes constitute the negative branch of the first autoregulatory feedback loop. Their necessary role in the clock pathway is underscored by the fact that *Cry1/Cry2* double knockout mice also show a complete loss of rhythmicity (van Der Horst et al., 1999; Vitaterna et al., 1999). PER and CRY proteins accumulate and dimerize in the cytoplasm, where their presence is tightly regulated. They can either be stabilized by phosphatase1 (PPI1) (Gallego et al., 2006) or phosphorylated by casein kinases (CKε/δ) (Camacho et al., 2001; Keesler et al., 2000; Lowrey et al., 2000) resulting in active degradation. For CRY1 this process occurs via ubiquitination by F-box and leucine-rich repeat protein 21 (FBXL21) (Dardente et al., 2008; Hirano et al., 2013; Yoo et al., 2013). Phosphorylated PER2 can be polyubiquitinated by β-TRCP1/2 resulting in proteasomal degradation (Eide et al., 2005). In general, dimerization of PER:CRY in the cytoplasm protects both proteins from degradation (Yagita et al., 2002). Upon stabilization, the PER:CRY dimer translocates into the nucleus (Yagita et al., 2000) forming a nuclear complex (Brown et al., 2005). There, the dimer binds to the NuRD (nucleosome remodeling deacetylases) transcriptional repressor complex and directs NuRD to BMAL1:CLOCK (Kim et

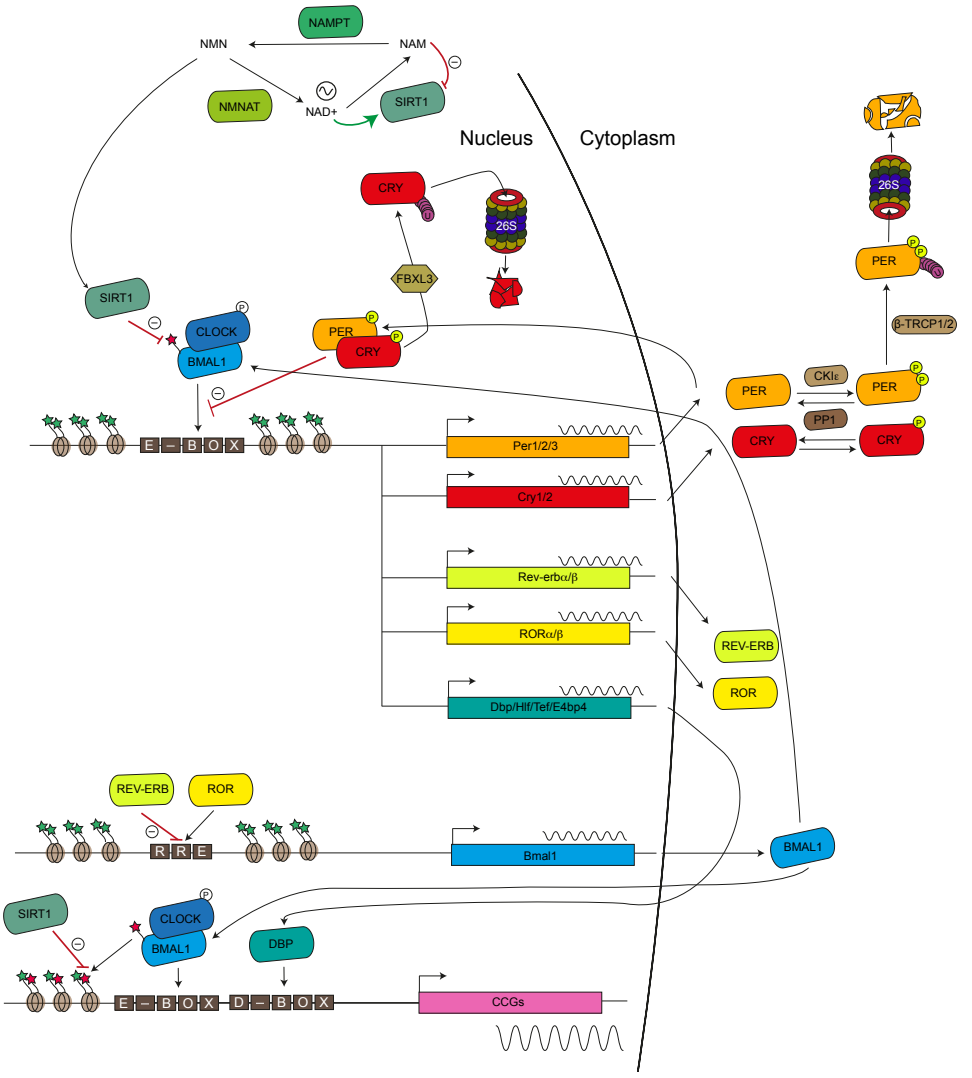

Figure 1. Schematic representation of the transcriptional/translational feedback loop of the circadian core clock pathway in mammals. The central proteins BMAL1 and CLOCK form a heterodimer and bind E-box elements in the promoter of period (*Per1/2*) and cryptochrome (*Cry1*/*Cry2*) genes. PER and CRY can be degraded by the 26S proteasome in the cytoplasm after ubiquitination (U) by β-TRCP1/2 after casein kinase ε mediated phosphorylation (P). If not degraded in the cytoplasm, PER and CRY can dimerize and shuttle to the nucleus, where they inhibit their own transcription via blocking BMAL1:CLOCK transcriptional activity. After gradual phosphorylation, the PER/ CRY complex is ubiquitinated by the F-Box protein FBXL3, and degraded in the nucleus. This lifts the repression on BMAL1:CLOCK, resulting in a new transcriptional cycle. In a secondary feedback loop, the BMAL1:CLOCK dimer drives transcription of *Rev-erbα/β* and *RORα/β*. Their proteins shuttle to the nucleus where they inhibit and activate *Bmal1* transcription, respectively via competing for a Rev-erb response element (RRE) in the *Bmal1* promoter. The result of one cycle, which takes approximately 24 h is the rhythmic transcriptional activation of clock-controlled genes (CCGs) by BMAL1:CLOCK. An oscillatory SIRT1 activity cycle integrates metabolism into the circadian clock. For additional information see text. *Stars*: histone tails

al., 2014). Then, a fully functional NuRD repressing complex is established, resulting in the inhibition of BMAL1:CLOCK driven transcription. Through this, PER and CRY inhibit multiple CCGs as well as their own transcription (Gekakis et al., 1998; Griffin et al., 1999; Kume et al., 1999). PER initiates this negative feedback loop by functioning as a molecular scaffold, that brings CRY into contact with BMAL1:CLOCK (Chen et al., 2009). In the nucleus CRY1 and CRY2 can be degraded by the proteasome, facilitated by F-box type E3 ubiquitin ligase (FBXL3). Its function is nicely illustrated by impaired degradation of CRY, as seen in the overtime (Ovtm) mutant, that causes enhanced inhibition of BMAL1:CLOCK- based transcription, resulting in period lengthening (Siepka et al., 2007). Upon decrease of nuclear PER and CRY levels, BMAL1:CLOCK inhibition is released, and a new transcriptional activation cycle of CCGs can start.

An additional layer of transcriptional control of the circadian clock comprises the orphan nuclear receptors RORα/β and REV-ERBα/β, encoded by *Rorα/β* and *Nr1d1/2* respectively. The BMAL1:CLOCK dimer mediates their transcription via binding to their E-box, initiating a second feedback loop (Guillaumond et al., 2005; Preitner et al., 2002; Sato et al., 2004). Both RORs and REV-ERBs compete for retinoic acid-related orphan receptor response element (RORE) binding sites, also termed RRE (RevErbA response element), within the *Bmal1* promoter (Harding and Lazar, 1993; Ueda et al., 2002). Binding of RORs drives transcription of *Bmal1* while REV-ERBα inhibits its transcription (Akashi and Takumi, 2005; Guillaumond et al., 2005; Preitner et al., 2002). Although *Rorα* mRNA levels are only slightly oscillating, RORα is necessary for rhythmic *Bmal1* expression (Akashi and Takumi, 2005).

A last transcriptional loop involved in circadian clock oscillation consists of a number of proline and acidic amino acid-rich basic leucine zipper (PAR bZIP) transcription factors regulating circadian gene expression via binding D-box (DBP response element) elements (TTATG(C/T) AA) (Falvey et al., 1996). Albumin D-site-binding protein (DBP), thyrotroph embryonic factor (TEF), and hepatic leukemia factor (HLF) contribute to positive regulation, whereas nuclear factor interleukin 3 regulated (NFIL3 or E4BP4) provides negative regulation. Although this accessory loop is not strictly necessary for circadian oscillations, it provides robustness and precision to the period. An overview of the described core clock pathway is depicted in Figure 1.

1.2.2 Epigenetic regulation of the circadian clock

Besides the complex transcriptional/translational control system, circadian rhythmicity is also regulated by epigenetic mechanisms. Epigenetic control includes methylation of the DNA at CpG islands, non-coding RNAs and posttranslational modifications of histones. All of these epigenetic mechanisms have been implicated in driving and fine-tuning circadian rhythmicity in gene expression.

CLOCK, one of the core circadian rhythm proteins, can function as a histone acetyl transferase (HAT) that acetylates histone H3 on its lysine 9 (H3K9) and lysine 14 (H3K14) amino acid residues (Doi et al., 2006). H3K9ac and H3K14ac are both markers for permissive transcription. Therefore, BMAL1:CLOCK also regulates transcription of CCGs via modifying their histones (Etchegaray et al., 2003). This function is being neutralized by several histone deacetylases (HDACs), as described in more detail by Steven Brown (Brown, 2011).

In search for these balancing HDACs, Sirtuin 1 (SIRT1) was discovered by the group of Sassone-Corsi as a protein that counteracts the HAT function of CLOCK. In general, *Sirt1* is well studied in the context of aging, resistance to cellular stress, metabolism, inflammation and proliferation (Bordone and Guarente, 2005). Additionally, SIRT1 is now known to deacetylate the proteins BMAL1, PER2 (Asher et al., 2008) and histone 3 (H3) on the promoter of clock output genes like *Dbp* (Nakahata et al., 2008). The deacetylating activity of SIRT1 is NAD+ (nicotinamide adenine dinucleotide) dependent and circadian. In the absence of *de novo* NAD+ biosynthesis, NAD+ needs to be replenished to avoid cell death. This goes via the NAD+ salvage pathway, where the by-product of NAD+ usage, NAM (nicotinamide) is reconverted into usable NAD+ via NMN (nicotinamide mononucleotide). In this process, NAMPT (NAM phosphoribosyltransferase) is the rate-limiting enzyme. As the expression of NAMPT itself is under circadian control, NAD+ also oscillates as an available metabolite. By this, SIRT1 links the metabolic state of a cell with the epigenetic control of the clock gene transcription pathway. As a rheostat of the circadian clock, SIRT1 mainly controls the amplitude of CCGs (Sassone-Corsi, 2012).

Besides acetylation, methylation of histones is important to mediate circadian rhythmicity in gene expression. Histone H3 lysine trimethylation (H3K4me3) is a mark consistently associated with circadian transitions of the chromatin fiber, controlling CCG expression. One of these histone methyl transferases (HMTs) is mixed lineage leukemia 1 (MLL1) that can recruit the BMAL1:CLOCK dimer to the DNA of target genes and cause rhythmic H3K4 trimethylation (Katada and Sassone-Corsi, 2010). Histone H3 lysine 27 trimethylation (H3K27me3) on the other hand is a repressive epigenetic mark and has been shown to play a counteracting role in circadian regulation of gene expression. The *Per1* promoter for example shows rhythmic H3K27me3 marks, mediated by EZH2. The counteracting enzymes, histone demethylases, also play a role in circadian rhythms, where JARID1A is a known demethylase that inhibits HDAC1 and boosts BMAL1:CLOCK facilitated transcription of *Per* genes. Dynamic interaction between HDAC1 and JARID1A correlates with proper histone acetylation at the *Per* promoters (DiTacchio et al., 2011). The same holds true for LSD1, another histone demethylase whose activity depends on circadian phosphorylation by PKCα (Nam et al., 2014).

1.2.3 Additional regulatory systems fine-tune circadian rhythmicity

As described above, the genetic and epigenetic mechanisms underlying the circadian clock

are quite complex and have been studied extensively. In addition to all this, a handful of papers describe even more ways of circadian regulation of the core clock pathway. Most of these additional mechanisms enhance robustness of the clock, rather than being truly essential for rhythmicity. A first example of such an extra clock dimension is the oscillating cold inducible RNA-binding protein (CIRP). CIRP is a RNA binding protein, and is regulated by circadian rhythmicity in temperature oscillations. CIRP binds to Clock mRNA and stabilizes it, linking temperature to the circadian clock (Morf et al., 2012). Next, cyclic alternative splicing (McGlincy et al., 2012) as well as light inducible alternative splicing (Preußner et al., 2014) are common factors that regulate circadian rhythms. Third, rhythmic polyadenylation that stabilizes mRNA molecules, facilitates circadian rhythmicity in protein translation (Kojima et al., 2012). Furthermore, fluctuations in m(6)A-RNA methylation also affect the circadian transcriptome (Fustin et al., 2013). Finally, non-coding RNAs such as anti-sense RNAs and miRNAs affect circadian rhythms. This is demonstrated by a disrupted circadian transcriptome in cells that lack *Dicer*, a gene involved in miRNA processing (Chen et al., 2013; Du et al., 2014). In conclusion, the circadian clock is achieved by a complex orchestrated interplay between genetics, epigenetics and translational processes.

1.3 Circadian rhythms in tissue/organ physiology

1.3.1 Tissue specific control of clock output

The core clock machinery, as studied extensively in the SCN, is conserved in all peripheral organs. The basic signaling between the core oscillator and peripheral clocks involves both neuronal and humoral signals, such as melatonin. However, peripheral clocks can also respond independently to environmental cues such as body temperature and food metabolites (Damiola et al., 2000; Stokkan et al., 2001) (Figure 2). Nonetheless, there are significant differences between each tissue in the relative contributions of the clock components, as well as in the output pathways that are under their control. These endogenous cellular clocks drive extensive rhythms of gene transcription, with 3–10% of all mRNAs in a given tissue showing diurnal rhythms (Akhtar et al., 2002; Duffield et al., 2002; Miller et al., 2007). However, the genes that are under circadian control are largely non-overlapping in different tissues. This tissue specificity reflects the need for temporal control of the cellular physiology relevant to each unique cell type. As a result, the circadian clock exerts extensive control over many unique biological processes.

1.3.2 Transgenic animals provide novel insights to better understand the clock

Tissue-specific transgenic mice have been used to address the precise functions of peripheral clocks in physiological processes. For example, in liver-specific *Bmal1* KO mice there is a loss of rhythmicity of glucose regulatory genes, which leads to an accelerated glucose clearance during the course of the daily feeding cycle (Lamia et al., 2008). In the adrenal glands, many genes involved in the biosynthesis of corticosterone are clock-controlled. Therefore

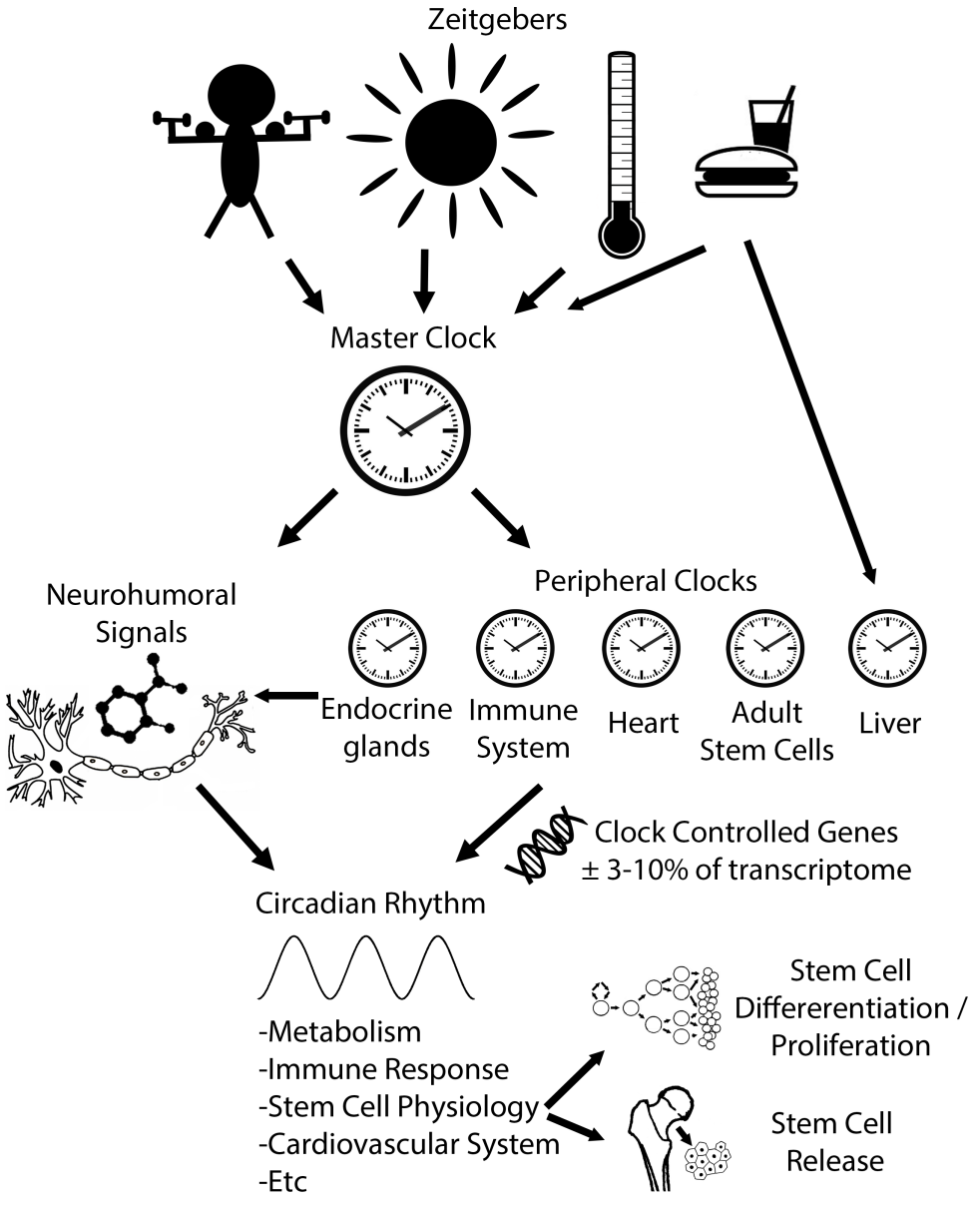

Figure 2. The circadian clock regulates rhythmic body physiology. A master clock in the brain and peripheral clocks in almost every cell/tissue of the body drive circadian rhythmicity. Clocks are entrained by different factors/*zeitgebers* such as light and food. These rhythms are propagated by electrical and neurohumoral signals resulting in a 24-hour rhythmic expression of clock-controlled genes. These genes render circadian rhythmicity in many functional processes ensuring proper body physiology and regeneration.

the tissue specific disruption of *Bmal1* affects the ability of the organ to maintain proper oscillatory secretion of corticosterone (Son et al., 2008). In pancreatic islets, the circadian clock helps regulating glucose-stimulated insulin secretion, the loss of which impairs glucose tolerance because of β-cell dysfunction (Marcheva et al., 2010).

The peripheral clock also plays a profound role in the cardiovascular system (Durgan and Young, 2010; Paschos and FitzGerald, 2010). In blood vessels, regulation of vascular function and tone has shown to be regulated by circadian rhythms. For example, deletion of *Bmal1* specifically in vascular endothelium leads to a reduction of blood pressure during the active phase of the day and increased heart rate throughout the 24-hour cycle (Westgate et al., 2008). The ability of endothelial cells from *Per2* mutant mice to proliferate and form vascular networks is substantially reduced, which is marked by increased senescence of the cells (Wang et al., 2008). In vivo, *Per2* mutant mice show decreased angiogenesis, as blood flow is impaired and combined with reduced recovery in response to ischemia characterized by a smaller increase in vessel formation.

Lastly, *Westgate et al* (Westgate et al., 2008) showed that the time to thrombotic vascular occlusion in response to a photochemical injury displays diurnal variation. Platelet aggregation factors, plasminogen activator inhibitor and tissue plasminogen activator, produced by the vascular endothelium show diurnal variability throughout the day/night cycle. In this regard, the deletion of *Bmal1* specifically in the vascular endothelium results in loss of the temporal pattern in susceptibility to thrombotic vascular occlusion.

1.3.3 Circadian rhythms in the heart

In the heart, rhythmic physiology has mostly been studied in mice harboring a dominant negative version of the CLOCK protein in cardiomyocytes specifically (CCM mice). In vivo radiotelemetry studies performed in wildtype (WT) and CCM mice for continuous 24 hour monitoring of physical activity, revealed a reduction in heart rate in the CCM mice (despite identical physical activity), which was especially pronounced during the awake/active phase. In this regard, the circadian clock seems to influence the generation and propagation of electric signals between adjacent cells in the heart. Although the precise mechanism remains unknown for this phenotype, the expression of various ion channels was found to be clock dependent. Furthermore, Connexin 40, a gap junction protein critical in atrial-ventricular conduction, shows oscillatory expression in WT hearts, that is absent in CCM hearts (Bray et al., 2008).

The metabolism of the heart is crucial for its contractile function, which has to meet the daily demand for increased workload during the active phase of the day. The two major components fueling the contraction of the myocardium are fatty acids and glucose (Taegtmeyer, 2000). Diurnal gene expression studies in CCM mice revealed that a large number of genes influencing triglyceride (fatty acids) and glycogen (glucose) metabolism are

controlled by the clock (Bray et al., 2008). The circadian regulation of lipolysis is exhibited in the diurnal variation of total triglyceride levels and synthesis in mouse hearts that peaks near the end of the active phase. These rhythms are essentially absent in CCM hearts. Furthermore, CCM mice display an altered response to high fat diet, showing a role of the cardiomyocyte circadian clock in the regulation of nonoxidative fatty acid metabolism (Tsai et al., 2010). Similarly, epinephrine-induced glycogenolysis has a time of the day dependent activity in WT hearts. However, these diurnal rhythms in cardiac glycogen metabolism are suppressed in CCM hearts (Bray et al., 2008).

Furthermore, hearts from 22-month-old CCM mice exhibited increased ejection fraction, fractional shortening, and left ventricular mass compared to WT. These are all characteristic of physiological hypertrophy and a strong link that the intrinsic clock of the heart also regulates myocardial growth. Growth factors, such as insulin-like growth factor-1 (IGF-1), are at the basis of physiological hypertrophy and can signal through the PI3K-Akt pathway. In this regard, the phosphorylation status of key components of this signaling pathway (Akt, GSK-3β, and p70S6K) all oscillate in hearts over the course of the day, and are continually elevated in CCM hearts (Durgan et al., 2010). Myocardial growth is also closely associated with protein synthesis, which involves initiation factors (eIFs), several of which have been found to be under cardiomyocyte circadian regulation (Bray et al., 2008).

In summary, the peripheral clocks allow for individualized rhythmic gene expression in order for organs to be able to anticipate their diurnal tasks. In the cardiac context, heart rate, metabolism, and growth are all parameters that are under tissue specific circadian control. This enables the heart to be used most efficiently during periods of activity and rest.

1.4 Ontology of circadian rhythms and the role of clocks in cell cycle

1.4.1 Circadian clocks during embryonic development

While the embryo develops, conditions within the uterus vary throughout the day. Concentrations of glucose and other metabolites for example, are relatively high during daytime compared to at night. A mature circadian clock to anticipate to these diurnal differences is not yet present in the embryo, but develops during gestation. The proteins of the circadian clock, including CLOCK, BMAL, PER, and CRY are already present in the unfertilized oocyte (Johnson et al., 2002). However, their expression is non-rhythmic and low compared to adult cells. During development *in utero*, the expression of those genes gradually increases until birth (Saxena et al., 2007). Around the period of mid- to end gestation, diurnal oscillations of the core clock proteins commence. The amplitude of expression increases until the end of pregnancy and upon delivery, there is a 12-hour phase shift, reversing 24-hour clock gene expression (Saxena et al., 2007). In some species, such as rodents, development of the molecular clock even continues after birth.

Circadian clocks rely on Zeitgebers for synchronization to external surroundings. Light for example, is received by the retina and via internal cues, synchronizes circadian clocks within the body. The fetus does not directly receive light input. Also, many of the maternal Zeitgeber signals do not reach the fetus because they do not pass the placenta. Therefore, the regulation of molecular clocks within the fetus rely mainly on melatonin, which is produced by the maternal pineal gland and is able to pass the placenta (Reppert and Weaver, 2002). The obtained 24-hour rhythms play an important role in the physiology of the developing fetus. Metabolic activity and parameters such as breathing movements and heart rate show diurnal oscillations within the uterus (de Vries et al., 1987; Visser et al., 1982). When those rhythms are disrupted during gestation, this can have detrimental effects as indicated by shift work during pregnancy. This results in relatively small babies and animal experiments show that it may also lead to glucose intolerance and insulin resistance in newborns (Bonzini et al., 2011; Varcoe et al., 2011). A detailed overview of *in utero* development of circadian rhythms was previously published (Pré et al., 2014).

1.4.2 Oscillation of core clock genes upon differentiation of embryonic stem cells

Previously, researchers have reported the absence of a clear functional core clock in pluripotent embryonic stem cells (ESCs). Via bioluminescent reporter systems and the analysis of expression levels of clock genes, no clear circadian rhythms could be identified. However, when pushing these stem cells to leave their pluripotent, proliferative state through the withdrawal of leukemia inhibitory factor (LIF), circadian rhythms slowly started to emerge. By day 28, clear and robust rhythms were observed in D28 differentiated embryoid bodies (EBs) (Umemura et al., 2013; Yagita et al., 2010). The same holds true when stem cells are differentiated in a more directed way towards the neuronal lineage by addition of retinoic acid (Kowalska et al., 2010; Yagita et al., 2010). When differentiated cells are reprogrammed towards induced pluripotent stem cells (iPSCs), they lose their established oscillatory clock gene expression pattern. However, upon re-differentiation, they acquire an active circadian clock again. This suggests that an inherent circadian clock is linked to the differentiation status of a cell, and that this clock might be established during early phases of development (Brown, 2014).

Albeit not clearly oscillating, most clock genes are expressed in ESCs. However, expression levels are different in comparison to differentiated cells. *Per1*, *Per2* and *Clock* are expressed at a lower level, *Cry2* is expressed at a higher level, and *Bmal1* shows similar transcript levels (Umemura et al., 2014). Apart from being molecular oscillators, this implies a different function of clock genes in ESCs. A possible explanation for the lack of clock gene oscillation in ESCs, is proposed in a recent paper of the Yagita lab. They show that the decline of Importin α2, encoded by *Kpn2*α plays a key role in the acquirement of a circadian clock in vitro. Importin α2 is a nuclear transporter and shuttles specific pluripotency factors, like OCT3/4, into the nucleus. Additionally, it keeps differentiation linked factors, like OCT6, in the cytoplasm. Through this, a pluripotent state is retained (Yasuhara et al., 2013). Importin

α2 also keeps clock factors like PER1 and PER2 in the cytoplasm (Umemura et al. 2014). Therefore, the absence of a functional clock in murine ESCs might be accountable to the fact that PER2 stays in the cytoplasm in pluripotent cells. The start of circadian oscillation is proposed as the timed entry of PER2 into the nucleus (Yagita et al., 2000).

1.4.3 Role of the circadian clock in cell cycle

Not only the circadian clock system, but also the cell cycle is an important biological oscillator. Researchers have intensively studied whether both processes are related and what the possible link between them might be. The cell cycle is represented by four different consecutive phases that ultimately lead to the division of proliferative cells. The S-phase is the replicative phase, in which the DNA is being duplicated. Next, G2 is a growth phase in which the cell contains a fully doubled genome. This leads to the M- (or mitotic) phase, where the DNA is propagated to the newly formed cell through actual division. M-phase leads to G1, which is another growth phase after which the cell re-enters the S-phase. However, non-dividing (somatic) cells can escape the cell cycle at G1 and reside in G0. However, they can switch back to a proliferative state via re-entering the cell cycle. The transition between cell cycle phases is facilitated by cyclin dependent kinases (CDKs) and cyclins, establishing so called “cell cycle checkpoints”.

In the group of F. Naef, the influence of the cell cycle on the circadian clock and *vice versa*, have been studied at a single cell level in NIH3T3 cells. In the absence of a synchronizing stimulus, cell division precedes the peak of *Reverbα* expression with 5 hours, concluding a clear 1:1 coupling between the cell cycle and the circadian clock (Bieler et al., 2014). This process is termed “Phase locking”. When disturbing the cell cycle length, the circadian period is impacted but the 1:1 coupling remains. In reverse, lengthening the circadian period is not affecting the cell cycle, concluding a clear unidirectional coupling between cell cycle and the circadian clock (Bieler et al., 2014). On the other hand, cells in organs are under influence of synchronizing stimuli (e.g. light, temperature, hormones, feeding). Therefore, Feillet et al. found that synchronizing in vitro cultured cells leads to the occurrence of two subpopulations in culture: one in which the 1:1 phase locking is sustained, and another in which two circadian clock periods coincide with three complete cell cycles (Feillet et al., 2014). This suggests that the cell cycle is synchronized via physiological stimuli with the circadian clock (Feillet et al., 2015). Both studies indicate a clear interaction between cell cycle and the circadian clock, with the dominating factor being dependent on the environment (Feillet et al., 2015).

At a molecular level, the circadian clock has been linked to the cell cycle in several ways. The two main cell cycle phase windows controlled by clock genes can be found at the G2/M and the G1/S phase transition. Cell cycle regulators such as *Wee1* in the murine liver (Matsuo et al., 2003), *p20* and *p21* in a developing zebrafish embryo (Laranjeiro et al., 2013), *c-Myc* and *cyclin D1* (Fu et al., 2002) are under control of several clock proteins and show circadian

rhythmicity in their expression. It has been shown that the key component of the negative limb of the clock pathway, PER2, can regulate the cell cycle via inhibiting c-MYC activity. C-MYC directly blocks cyclin D1 activity, which is a roadblock for G1/S phase transition in the cell cycle. Overexpression of *Per2* concomitantly leads to cell cycle arrest in certain cancer cell lines and pushes these cells into apoptosis (Hua et al., 2006; Oda et al., 2009; Sun et al., 2010). Recently, a relatively new clock player termed NONO has also been implicated in cell cycle regulation. This multifunctional nuclear protein partners with PER2 and regulates expression of the cell cycle checkpoint gene *p16-Ink4A* in a circadian fashion. Since p16-INK4A facilitates G1 exit, NONO couples cell cycle to the circadian clock, which is argued to be a way to segregate cell proliferation from tissue organization in a time-based manner (Kowalska et al., 2013). A growing amount of evidence links the circadian clock to the cell cycle and *vice versa*. A detailed description of the specific core players in this cell cycle/circadian clock network can be read in the following reviews (Feillet et al., 2015; Kelleher et al., 2014).

1.5 Circadian rhythms in adult stem cells

1.5.1 Regulation of adult stem cells by oscillatory systems

Adult stem cells are multipotent stem cells that are present in adult organisms. They can proliferate and differentiate, but their potential is limited to specific cell types. Hematopoietic stem cells for example can differentiate into multiple blood cells, but not into skin cells. Adult stem cells are present in many organs. In some tissues they supply a constant renewal of cells (intestines, skin, blood), whereas in others they only become active after injury (heart, skeletal muscle). In the heart, adult stem cells of cardiac and non-cardiac origin are being investigated for their use in regenerative therapy.

24-hour rhythmicity plays an important role in adult stem cells. First, there are 24-hour oscillations in stem cell mobilization and trafficking (Figure 2). Hematopoietic stem cells (HSCs) in the circulation for example, show 24-hour oscillations in both humans and mice (Laerum, 1995; Méndez-Ferrer et al., 2008). In humans, there is a HSC release peak 5 hours after the day-to-night transition. This rhythm is orchestrated by the circadian central clock, via diurnal noradrenalin secretion and local, sympathetic nerves to the bone marrow (Méndez-Ferrer et al., 2008). Oscillations in the number of circulating HSCs lead to 24-hour oscillations in hematopoietic growth factors and the number of blood cells in the circulation (Gimble et al., 2009; Laerum, 1995). As a consequence, 24-hour oscillations are present in processes mediated by blood cells: diurnal rhythms in leukocytes for example cause oscillations in immune responses and rhythms in thrombocytes causes rhythms in coagulation time. Almost all adults have 24-hour oscillations in HSC release. In aging and some diseases, however, these oscillations are dampened (Sletvold and Laerum, 1988). Other adult stem cells in the circulation, such as endothelial progenitor cells, also show 24-hour rhythms (Thomas et al., 2008).

1.5.2 Circadian clock gene expression in adult stem cells

Adult stem cells themselves also have molecular circadian clocks. Mesenchymal stem cells (MSCs), adult stem cells that can differentiate into cells of the mesoderm, such as osteoblasts, chondrocytes, and adipocytes, provide a good example of this. When MSCs are cultured in vitro, 24-hour oscillations in gene expression of core clock genes such as *Bmal1*, *Cry1*, *Per2/3*, *Rev-erbα/β*, and *Dbp* are found, indicating that the peripheral circadian clock is present in these cells, independent of the presence of the central clock (Wu et al., 2008). Differences in expression between MSCs of old and young animals are observed (Yu et al., 2011). These rhythms seem to play an important role in physiology and pathophysiology. RORα for example, a component of the circadian clock present in MSCs, influences MSC differentiation (Meyer et al., 2000). When RORα is disrupted, bone mass parameters and bone geometry are impaired. Other studies have demonstrated that the circadian clock regulates bone metabolism, one of the functions of MSCs. Mice with altered circadian rhythms display bone remodeling and more than 26% of the bone transcriptome exhibits circadian rhythmicity (Fu et al., 2005; Zvonic et al., 2007). Other functions of MSCs, such as adipose tissue homeostasis, also showed to be under circadian control (Guo et al., 2012).

Circadian rhythms in adult stem cells are associated with proliferation and differentiation (Figure 2). Proliferation and differentiation are main characteristics of adult stem cells and vital for regenerative medicine. When MSCs are cultured in vitro and circadian rhythms are disrupted by translocation of CRY1 and PER1 from the nucleus to cytoplasm using laser irradiation, this leads to a change in differentiation from adipogenesis to osteogenesis (Kushibiki and Awazu, 2008). In addition, genes that regulate stem cell proliferation and differentiation often show circadian rhythms in gene expression. There are genes, such as *GSK3* and *Sirt1*, which play an important role in both the circadian molecular clock and proliferation (Trowbridge et al., 2006; Yang et al., 2006). In addition, the phase of circadian rhythms seems to play a role in differentiation. In human epidermal stem cells the phase of circadian rhythms determines proliferation predisposition (Janich et al., 2013). When differentiation is induced by TGFβ or calcium, the core clock gene phase determines whether the epidermal stem cells respond to these cues. Based on circadian clock phase, stem cells can be divided into 'dormant' or 'active' stem cells (Janich et al., 2011). Disruption of clock genes such as *Bmal1* or *Per1/2* leads to accumulation or depletion of dormant stem cells.

In tissues that do not face a significant amount of proliferation during homeostatic conditions, such as the liver, heart, and skeletal muscle, circadian rhythms play a role in proliferation and differentiation after injury. After muscle injury for example, satellite cells from the basal lamina of muscle fibers become active to regenerate the muscle (Chatterjee et al., 2014). When the core clock gene *Bmal1* is disrupted, muscle regeneration is impaired because satellite cells cannot expand. In the liver, the circadian clock regulates cell cycle genes, which in turn regulate mitosis (Matsuo et al., 2003).

In summary, 24-hour rhythms are important in the physiology of adult stem cells. The master clock orchestrates oscillations of adult stem cell numbers in the circulation, whereas peripheral clocks within stem cells regulate 24-hour stem cell physiology. Specifically, circadian rhythms play an important role in proliferation and differentiation, both in normal physiology and after injury. Therefore, it would be interesting to determine if the dormancy/activity of cardiac stem cells is also clock mediated.

1.6 The role of circadian rhythms in tissue regeneration for cell-based therapy

The previous paragraphs have illustrated the importance of circadian rhythms in humans and specifically in the context of embryonic- and adult stem cells and their derivatives. Although research in this research field is scarce, it is likely that circadian rhythms will influence cell-based therapy.

First, circadian rhythms influence the number of adult stem cells present in different parts of the body. In (cardiac) regeneration therapy, sometimes stem cells from patients are collected and injected (Perin et al., 2014). When cells are harvested, their amount and quality could depend on the time of collection. Also, homing of the patient's own stem cells after injury, for example cardiac surgery, may be affected by 24-hour rhythms. Depending on the time of injury, the body its regenerative capacity will differ, implying optimal regenerative time windows.

Second, the recipient body has 24-hour rhythms in functions that are important in cell-based therapy. The immune response for example differs between day and night as seen in the pulmonary epithelium (Gibbs et al., 2014), but other important parameters fluctuate as well. Using cell-based therapy at the time-point that conditions in the recipient patient are best could benefit results.

Third, circadian rhythms of donor cells could be enhanced. Several types of stem cells have 24-hour rhythms that influence their function. Injecting stem cells at a time-point optimal for the donor cells could also benefit regenerative therapies. This can be done by "resetting" cells in vitro to an optimal time-point using chemical substances (Izumo et al., 2006) or subjecting them to a serum shock (Balsalobre et al., 1998). This way, circadian rhythms between donor cells and recipient patient could be harmonized, possibly resulting in better engraftment of the donor cells.

Finally, circadian rhythms could be modified to enhance regenerative therapy, for example by targeting the molecular clock or its downstream pathways. Many techniques are available for this purpose, engaging on different levels ranging from genetic engineering of the molecular clock to altering clock in- or output signals (Bray et al., 2008; Tong et al., 2015). The effects of these alterations differ significantly. Some modifications for example will only slightly influence circadian phase or amplitude, whereas others completely abolish rhythmicity,

with major impact on function (Bray et al., 2008; Sack et al., 2000). Modifications can be made both in donor cells and in the recipient patient.

In summary, optimal use of the knowledge on circadian rhythms and potentially modifying circadian rhythms or clock components could enhance stem cell differentiation and the effect of stem cell based cardiac repair.

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2

Embryonic template-based generation and purification of pluripotent stem cell-derived cardiomyocytes for heart repair

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Abstract

Cardiovascular disease remains a leading cause of death in Western countries. Many types of cardiovascular diseases are due to a loss of functional cardiomyocytes, which can result in irreversible cardiac failure. Since the adult human heart has limited regenerative potential, cardiac transplantation is still the only effective therapy to address this cardiomyocyte loss. However, drawbacks, such as immune rejection and insufficient donor availability, are limiting this last-resort solution. Recent developments in the stem cell biology field have improved the potential of cardiac regeneration. Improvements in reprogramming strategies of differentiated adult cells into induced pluripotent stem cells (iPSCs), together with increased efficiency of directed differentiation of pluripotent stem cells towards cardiac myocytes have brought cell-based heart muscle regeneration a few steps closer to the clinic. In this review, we outline the status of research on cardiac regeneration with a focus on directed differentiation of pluripotent stem cells towards the cardiac lineage.

2.1 Introduction

Heart failure can be caused by several factors such as aging, hypertension, valvular dysfunction or ischemic events, such as myocardial infarction (MI). After acute MI, up to a billion cardiomyocytes (CMs) can be destroyed by necrosis or apoptosis, which accounts for one quarter of the whole myocardium (Eefting et al., 2004; Laflamme et al., 2005). The lost functional CMs are replaced by fibroblasts, which, unlike CMs, proliferate quickly. The fibroblasts form scar tissue, resulting in ventricular wall thinning and loss of contractility. This subsequently impairs the myocardial function and therefore supply of blood, nutrients and oxygen to the peripheral organs. Heart failure is characterized by a poor prognosis with gradual deterioration; the five-year mortality rate in severe heart failure is 50% (Roger et al., 2012).

The mammalian heart is a terminally differentiated organ. However, recently, this paradigm has been challenged by the discovery that some cardiomyocytes retain their self-renewal potential (Beltrami et al., 2001). In addition, C-kit positive cells, termed cardiac progenitor cells (CPCs), have been described to reside in the heart. These cells are able to differentiate into cardiomyocytes in vivo, as well as into endothelial cells and vascular smooth muscles cells required for revascularization (Anversa et al., 2006; Beltrami et al., 2003; Bergmann et al., 2009; Goumans et al., 2007; Raikwar et al., 2006; Urbanek et al., 2005). This endogenous regenerative potential however, seems to be insufficient to replenish the lost pool of cardiac cells caused by major injury.

Currently, there are two potential cell-based therapies for restoring heart function: the enhancement of endogenous regenerative potential (Bollini et al., 2011); and the delivery of exogenous cells to repopulate the heart (Steinhauser and Lee, 2011). Notably, a combination of both strategies may prove an even better alternative. Moreover, exogenous cell

transplantation on itself may already lead to the enhancement of endogenous regeneration. In this review, we focus on exogenous cell-based cardiac therapy. In particular, we investigate the use of pluripotent stem cells (PSCs) as a source for directed differentiation towards CMs. Studying this directed differentiation may unravel signaling clues prone to targeting by small compounds to activate the resident CPCs (Vidarsson et al., 2010) to proliferate and differentiate into CMs. The ideal source for transplantation would be a proliferative cell type that is able to generate all the different cells of the heart. PSCs meet these requirements and can be isolated without the need of a heart biopsy, expanded indefinitely and differentiate into cardiac cells. This would establish a sufficient number of *bona fide* CMs to cope with the enormous amount of myocardial cells required for transplantation.

Currently multiple research groups (Burridge et al., 2011a; Kattman et al., 2011b; Willems et al., 2011) are optimizing protocols to direct the differentiation of PSCs to the cardiac lineage in a reproducible, quick and efficient manner. Emerging knowledge of cardiac embryology has lead to specialized protocols, leading to the efficient generation of PSC-derived CMs reminiscent of primary CMs (Burridge et al., 2011a; Elliott et al., 2011; Hudson et al., 2012; Kattman et al., 2011b; Laflamme et al., 2007; Uosaki et al., 2011; Willems et al., 2011). In rodents, several transplantation experiments have shown that PSC-derived CMs can enhance cardiac function in MI models (Laflamme et al., 2007; Passier et al., 2008; van Laake et al., 2008) although no cardiomyocyte-specific long-term beneficial effects have been reported yet. Although this novel approach seems promising, there are challenges that hinder its efficacy. It is difficult to produce the appropriate amount of cells from a well characterized PSC population; purification processes are inefficient; and functional integration post transplantation is still suboptimal (Passier et al., 2008). Additionally, there are challenges with the maturation of premature CMs, characterization of specific CM-subtypes and the introduction of other cardiovascular cells in order to optimize in vivo functionality after transplantation (Kattman et al., 2011a).

On the horizon there are alternative approaches for generating large quantities of CMs for cell replacement in injured hearts. Application towards drug discovery through cardiac differentiation in vitro has been initiated (Davis et al., 2011). There is a growing understanding of fundamental cardiac developmental processes, in order to recapitulate cardiac differentiation in vitro (Kattman et al., 2011b). Combing a variety of methods and novel approaches together with a greater understanding of how heart development and repair works, we will be poised to better create personalized heart failure therapies. The loss of functioning CMs and the inability of the heart to fully regenerate itself on its own, demonstrates a great need to properly generate pure populations of CMs appropriate for therapeutic application.

2.2 Pluripotent stem cells: ESCs vs iPSCs

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of a

blastocyst (Evans and Kaufman, 1981). This cell population can give rise to all cells of an entire embryo. In vitro, established ESC lines can proliferate indefinitely and are pluripotent, contributing to derivatives of all three embryonic germ layers (ectoderm, mesoderm and endoderm). The molecular network underlying pluripotency is complex and identifying key players that support these functional properties is of great interest in developmental and stem cell biology areas. About 20 years after the establishment of mouse ESCs (Evans and Kaufman, 1981; Martin, 1981), the first human ESC line (Thomson et al., 1998) was derived. Like their murine counterparts, hESCs are able to recapitulate embryonic events in vitro by differentiating into all cell types of the body, including cells from the cardiac lineage (Gai et al., 2009; Kehat et al., 2001; Mummery, 2003; Xu et al., 2002). This is promising for the relatively new field of cardiac regenerative medicine, since PSCs can provide the many CMs needed for restoring heart function after a myocardial trauma. However, the use of hESCs is inevitably accompanied with ethical and societal considerations. Other drawbacks include the technical difficulty of deriving hESCs and immune rejection due to allogenicity. Impediments such as these may limit the use of hESCs for regenerative purposes.

Recent findings with a newer type of cell, the induced pluripotent stem cell (iPSC) are boosting the field of regenerative medicine and these cells are showing much promise as a potential source. IPSCs are pluripotent cells generated from a differentiated cell (for example, a skin fibroblast) by means of ectopic overexpression of defined reprogramming factors (Oct3/4, Klf4, Sox2, c-Myc, Nanog and Lin28), all factors expressed in their embryonic counterparts, ESCs (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Reprogramming specialized somatic cells to the embryonic state reverses cell specification via epigenetic modification through erasing the epigenetic profile of the differentiated cell, while reestablishing the embryonic methylome (Cherry and Daley, 2012; Kim et al., 2011). IPSCs have the same proliferative and pluripotent characteristics as ESCs. Moreover, by coaxing them towards a specific lineage, they can differentiate into a desired cell type, including cells from the cardiovascular lineage (Schenke-Layland et al., 2008; Zhang et al., 2009). Human iPSCs (hiPSCs) have already been implanted in MI mouse models, resulting in regeneration of the myocardium, endothelial tissue and smooth muscle, all ameliorating the heart's contractibility and electric stability (Nelson et al., 2009).

Ethically, iPSCs are less controversial because they circumvent the need for embryo sacrifice. Since they can be derived autologously from a specific patient, immunogenicity is potentially omitted. However, iPSCs have recently been described to evoke a T-cell dependent immune response upon transplantation in syngeneic recipient mice, while mouse ESCs (mESCs) were immunotolerated (Zhao et al., 2011). Still, it should be noted that this response could be due to an immune reaction against the undifferentiated cell population specifically. In a clinical setting, such undifferentiated pluripotent cells should never be transplanted; their differentiated derivatives will be used for functional engraftment (Okita et al., 2011). On the other hand, autologous application of cells is not always possible (in acute situations) or desired (in genetic disease, unless preceded by gene editing of donor cells). Currently the

greatest advantage of iPSCs is their use in disease modeling, since they can be derived from patients with a known phenotype and, optionally, a known gene mutation. However, it has to be noted that iPSCs can retain minor epigenetic signatures of their cell type of origin, a concept previously described as 'epigenetic memory'. This epigenetic memory is thought to influence the differentiation potential of an iPSC, meaning that iPSCs as such are potentially more heterogeneous. Therefore the use of ESCs complements the use of iPSCs, as ESCs may be more suitable for studying molecular mechanisms implicated in cardiac cell-face specification and optimizing the differentiation processes to CMs in vitro.

While different labs have reported variations on the combination of reprogramming factors used to generate iPSCs, the most widely used combination is still ectopic expression of the four original "Yamanaka factors" (Oct4, Klf4, Sox2 and c-Myc). This can result in the disruption of endogenous genes causing defects ranging from mild aberrations to drastic tumorigenic mutations (Ghosh et al., 2011; Lin et al., 2010; Moriguchi et al., 2010). After iPSC formation these reprogramming factors are normally inactivated. Occasional reactivation of these factors, and in particular the proto-oncogene c-Myc, can give rise to tumor formation after transplantation (Okita et al., 2007). Currently, various reprogramming cocktails have been developed, as well as different delivery methods, that in contrast to widely used retrovirus based reprogramming method, do not leave footprints in the genome (Okita et al., 2008; Stadtfeld et al., 2008) such as transfection with episomal vectors (Yu et al., 2009) or minicircles (Jia et al., 2010; Narsinh et al., 2011), synthetic mRNA (Warren et al., 2010), miRNA (Anokye-Danso et al., 2011) or the transposon based *Piggybac* system (Woltjen et al., 2009). Currently the preferred methods for generating iPSCs for translational research are episomal vector based (Hu et al., 2011) and Sendai virus based reprogramming (Ban et al., 2011; Fusaki et al., 2009; Seki et al., 2012), since they have a high efficiency and do not disrupt the host genome (Rao and Malik, 2012). For a detailed overview, we recommend the following reviews by Stadtfeld & Hochedlinger, Lai et al, Okita & Yamanaka, and Rao & Malik (Lai et al., 2011; Rao and Malik, 2012) (Okita and Yamanaka, 2011; Stadtfeld and Hochedlinger, 2010).

2.3 Embryonic cardiomyocyte differentiation (cardiomyogenesis)

Generating cardiac myocytes from PSCs in vitro has been hampered by the paucity of known inductive clues regulating cardiomyogenesis. It is essential, therefore, to understand cardiac embryonic development and its molecular hierarchy in order to determine the most efficient method of generating cardiac cells from PSCs. Key players like transcriptional regulators, cytokines and other signaling molecules in this complicated process can be targeted and overexpressed or administered exogenously in order to effectively drive PSCs to the cardiac lineage. The mammalian heart is the first organ developed during embryogenesis and consists of three major mesodermal cell types that originate from the mid- to anterior streak: endothelial cells, vascular smooth muscle cells and CMs (Lawson et al., 1991). Cardiomyogenesis consists of four consecutive steps: mesoderm formation, mesoderm

patterning towards anterior or cardiogenic mesoderm, cardiac mesoderm formation and CM maturation (Willems et al., 2009).

2.3.1 Mesoderm formation

Induction of mesoderm via gastrulation is a process well studied in mouse embryos. Mesoderm induction in cardiac differentiation is characterized by signaling of the transforming growth factor β (TGF-β) family member NODAL (or Activin A), which is first activated, and then inhibited as gastrulation progresses. Mesendoderm, including cardiac mesoderm, is formed only where NODAL is highly expressed; lower levels of NODAL stimulate lateral and posterior parts (Mercola et al., 2011). NODAL signaling in the proximal epiblast in mice maintains BMP4 expression in the adjacent extraembryonic ectoderm, which then acts via WNT3 expression in the proximal epiblast (Burridge et al., 2012). Following Wnt, BMP and NODAL expression, the canonical Wnt-signaling antagonist Dkk1, and NODAL inhibitors *Lefty* and *Cer* (which is also a BMP inhibitor), become expressed, shutting down their targeting pathways in a spatially restricted manner. This mesoderm induction stage can be witnessed by expression of Brachyury (also called T), a transcription factor expressed in primitive streak mesoderm (Brachyury is an early mesoendodermal marker) (Figure 1).

2.3.2 Mesoderm patterning and cardiac mesoderm formation

Subsequently, genes involved in mesodermal patterning, like MESP1, are induced by Brachyury. MESP1 is considered the key regulator of cardiomyogenesis, and more specifically in cardiac progenitor specification or cardiovascular lineage commitment (Bondue and Blanpain, 2010; Bondue et al., 2008a). ESCs expressing Brachyury early in development, followed by Mesp1 expression, are indispensible for the later generation of fully matured cardiac cells (Bondue et al., 2008a; David et al., 2008; Lindsley et al., 2008). MESP1 expression, which marks cardiogenic mesoderm, then triggers a cascade of heart-forming interacting factors including Nkx2.5, Tbx 5, Hand 1/2, Gata4, Mef2c, Myocd, Foxh1 and LIM-homeobox transcription factor Isl1, which mark the cardiac mesoderm (Bondue and Blanpain, 2010; Plageman and Yutzey, 2004; Riley et al., 1998) (Figure 1). This cardiac mesoderm gives rise to the first heart field (FHF), the second heart field (SHF), the endocardium and the proepicardial mesenchyme (Buckingham et al., 2005). Differentiation of the FHF, which gives rise to the linear heart tube (forming the left ventricle, atria and nodal conduction system) runs slightly ahead of the SHF's differentiation and is modulated by BMP4 (Klaus et al., 2007). The SHF, distinguished by Isl1, Tbx1, Fgf8 and Fgf10 expression (Cai et al., 2003; Ilagan et al., 2006; Kelly et al., 2001; Meilhac et al., 2004; Xu et al., 2004), stays in a proliferative state until it is required to form the outflow tract and the right ventricle (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). This proliferation is mediated by Hedgehog signaling (Dyer and Kirby, 2009), as shown by premature differentiation upon ablation of its receptor, Smoothened (Goddeeris et al., 2008), and by canonical WNT signaling. Downregulation of this latter pathway is needed then for myocardial progenitors in order to differentiate (Ai et

al., 2007; Cohen et al., 2007; Kwon et al., 2007; Mercola et al., 2011). When the heart fields have been established, pathways like FGF and Notch signaling (via its transmembrane Notch receptor) control proliferation and fate determination of competent cardiac progenitors. More specifically, Notch signaling drives expression of Neuregulin, BMP10 and EphrinB2 in the endocardium (Grego-Bessa et al., 2007; Watanabe et al., 2006), while epicardially produced retinoic acid (RA) acts via FGF-signaling (Lavine et al., 2005). Epicardially produced RA also stimulates the expansion of the myocardium through induction of the secreted hormone erythropoietin (EPO), which subsequently activates insulin growth factor 2 (IGF2) (Brade et al., 2011). These pathways all promote proliferation of the cardiac progenitors.

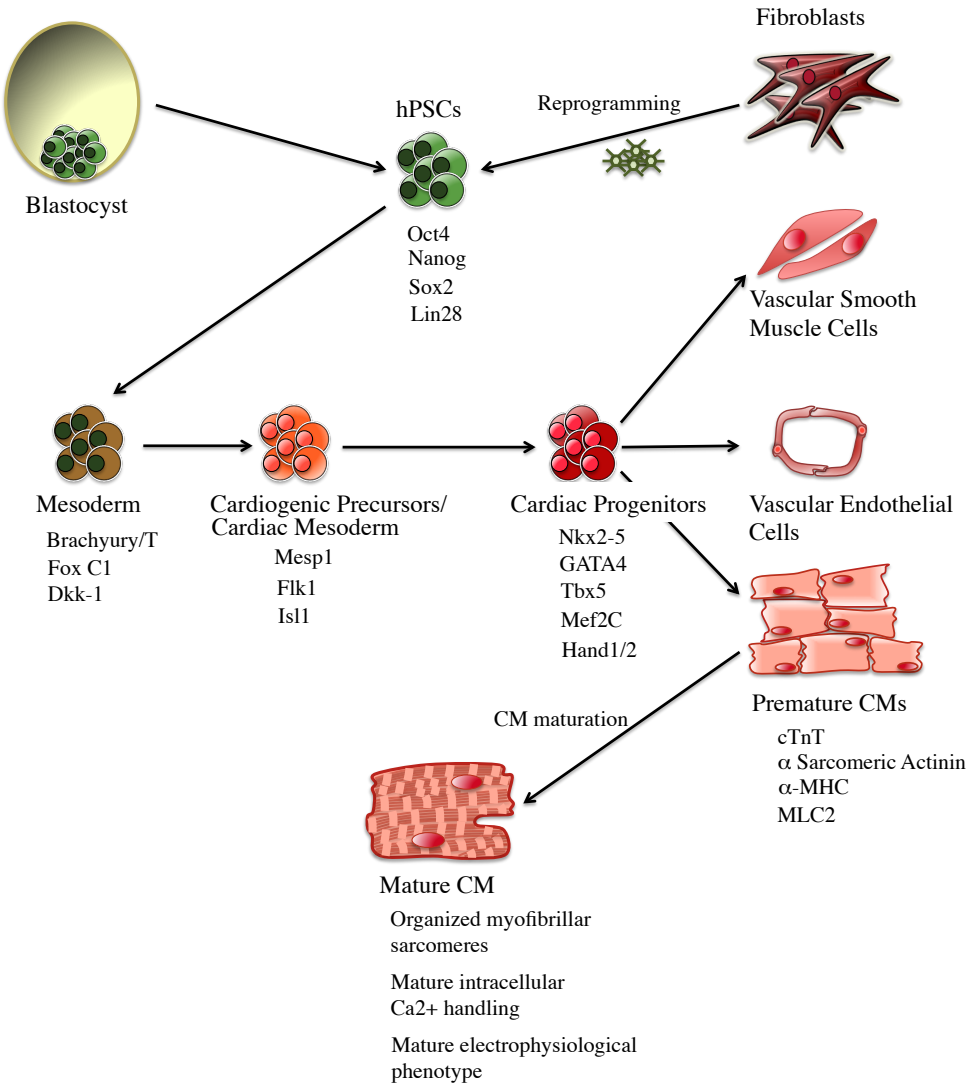

Figure 1. Scheme outlining key events during CM formation and their corresponding markers.

2.3.3 CM maturation

The last step in cardiomyogenesis is the differentiation of the CPCs into premature CMs and their subsequent maturation (Figure 1). This requires inhibiting proliferation of CPCs and stimulating differentiation pathways. Hedgehog signaling, required for proliferation of the SHF progenitors, is inhibited in order for CM formation to proceed (Goddeeris et al., 2008). Similarly, Wnt/β-catenin signaling is downregulated to stop proliferation and stimulate CM progenitor differentiation. However, this pathway directly drives expression of Wnt11, stimulating non-canonical Wnt-signaling, involved in cell polarity formation during the last step of CM formation (Phillips et al., 2005; Zhou et al., 2007). BMP signaling is needed for the terminal differentiation via maintenance of Tbx20 expression (Shi et al., 2000; Walters et al., 2001). Finally, Serum response factor (SRF) regulates the expression of sarcomeric protein genes and genes involved in contractibility. This factor interacts with GATA4 and Nkx2.5 in order to drive the expression of additional cardiac genes. SRF also regulates the expression of microRNAs (miRs), such as miR-1 and miR-133, all essential for heart development (Chen et al., 2006; Zhao et al., 2005). As seen in vitro, neonatal CMs can also still proliferate in order to expand the myocardium. This process is modulated by factors such as IGF1, activating the PI3K/AKT signaling pathway (Evans-Anderson et al., 2008). In general, the molecular identity of the maturation pathway is less well understood compared to the preceding inductive steps.

In conclusion, four main signaling pathways (Nodal/TGF-β, Wnt, BMP, and FGF) control cardiomyogenesis, all of which can impinge on directed differentiation from PSCs toward CMs. Additionally, studying the three main steps in cardiomyogenesis (i.e., induction of cardiac mesoderm, differentiation of cardiac mesoderm into CMs and maturation into mature CMs) can be of great interest in the process of generating efficient cardiac differentiation protocols for regenerative purposes (Willems et al., 2011).

2.4 Pluripotent stem cell differentiation into cardiac myocytes

Deriving CMs from PSCs is of great interest in the translational regenerative medicine field. Understanding embryological heart formation and deciphering cues that guide undifferentiated cells towards CMs have led to many different cardiac differentiation protocols. Most of these protocols are based on timed activation and/or inhibition of the main cardiac pathways described above. However, there is a wide variation in developmental properties between different human PSC (hPSC) lines, and even within the same hPSC line cultured by different labs. It is therefore critical to optimize protocols, which may compromise the standardization of a uniform method for differentiation.

2.4.1 Co-culturing of hPSCs

One of the first methods of hPSC-directed CM differentiation was established through

recapitulation of the in vivo situation in the embryo [28]. Co-culturing hESCs with a visceral endoderm-like cell line (END-2) (Figure 2) consistently induced CM differentiation, accentuating the role of factors secreted by adjacent anterior endoderm in cardiac induction in mesodermal structures (Cao et al., 2008; Synnergren et al., 2008b; Xu et al., 2009). Although this method is relatively inefficient, it stands as a model for optimizing CM differentiation protocols by adding and/or deleting growth factors, morphogenes, cytokines and other signaling factors involved in cardiac differentiation. For example, removing serum from the differentiation medium, adding ascorbic acid (Passier et al., 2005) and removing insulin (Freund et al., 2008) all elevate CM differentiation efficiency. Using a p38 MAPK signaling inhibitor in the differentiation cocktail blocks its neurectoderm-inducing capacity and alleviates its cardiomyogenesis-inhibiting effect (Gaur et al., 2010; Graichen et al., 2008). Another factor identified to enhance cardiac induction in this context was prostaglandin I_2 (PGI2) (Xu et al., 2008b). These modifications have resulted in an increased efficiency in the formation of up to 25% CMs from hESCs. The END-2 co-culturing method works for both ESCs and iPSCs (Fujiwara et al., 2011) and CM differentiation happens within 12 days. Electrophysiological studies have pointed out that most (about 85%) of the CMs generated by this method are ventricular (Mummery, 2003). This is the most relevant subtype needed for restoring heart contractility through cell-based therapy.

2.4.2 Spontaneous cardiac differentiation via embryoid body formation

The first hESC-derived CMs were produced by embryoid body (EB) differentiation (Figure 2), an in vitro 3D multi-lineage cell aggregate formation (Itskovitz-Eldor et al., 2000; Kehat, 2001). EBs recapitulate the early events of embryogenesis (Desbaillets et al., 2000) and CM formation can be witnessed by the spontaneous appearance of rhythmically beating EBs (Doetschman et al., 1985; Kehat, 2001). In these EBs, cell-cell contacts stimulate the expression of mesodermal and early cardiac markers (Tran et al., 2009), and usually after a few days, these structures are cultured on a coated tissue plate, resulting in the outgrowth of cardiac structures. The first described hiPSC-derived CMs were generated via this EB formation technique combined with spontaneous differentiation (Gai et al., 2009; Zhang et al., 2009). Although this spontaneous cardiomyocyte differentiation method is very inefficient (lower than 1% CM formation), all three subtypes of CMs, atrial-, nodal (pacemaker)- and ventricular-like, are formed with this method (He et al., 2003). However, the efficiency of CM differentiation can be increased by factors such as WNT3A (Bu et al., 2009), or ascorbic acid (Cao et al., 2011), both promoting CPC proliferation. IGF1 and IGF2 (McDevitt et al., 2005) or the GSK-3 inhibitor BIO (Tseng et al., 2006) scale up the differentiation process through enhancing CM proliferation.

Another general difficulty with PSC-EB-differentiation is the size variation of cell aggregates formed in suspension (suspension EBs). To control for this heterogeneity, researchers adopted a 'hanging drop' method (Figure 2), in which a defined ESC number (usually between 500 and 5000 cells) is dispensed in a 20 μl drop and hanged from the lid of a Petri

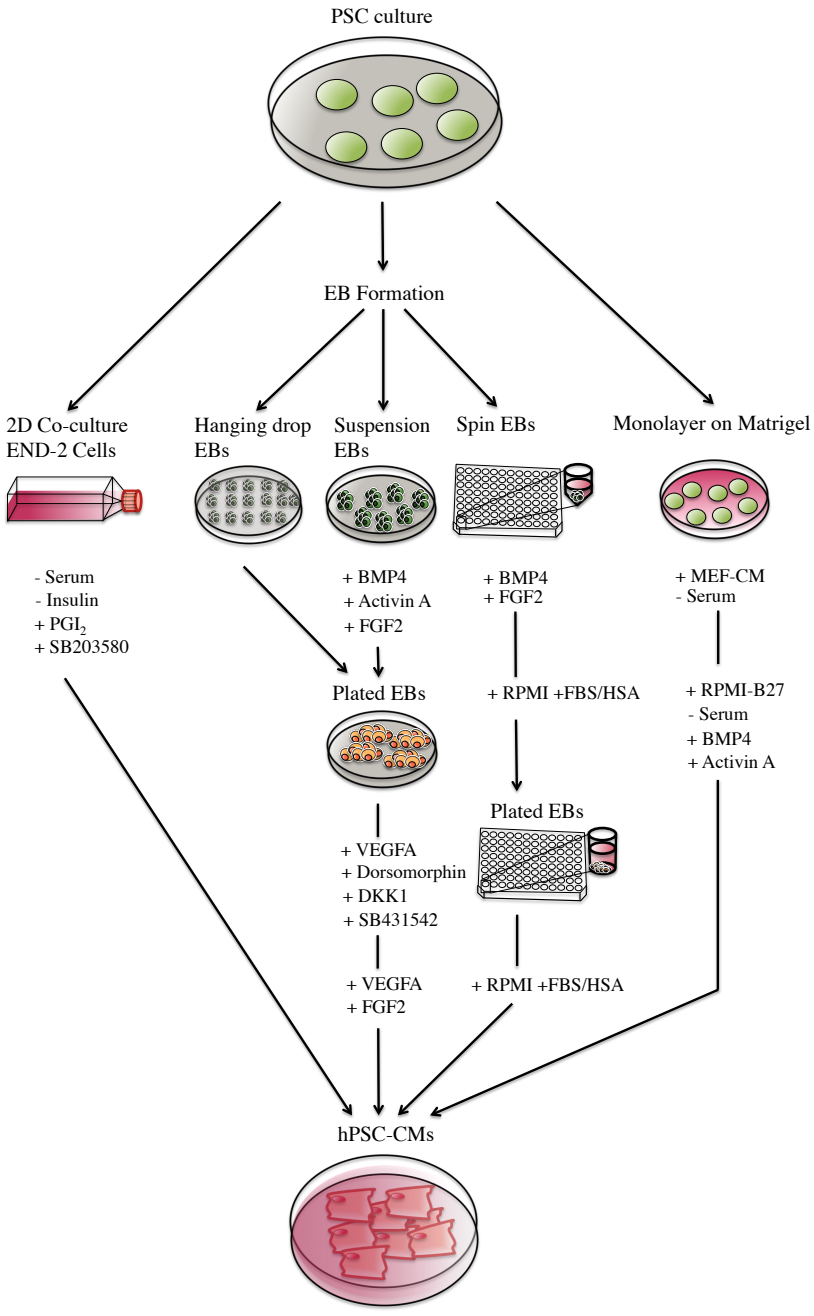

Figure 2. Differentiation of pluripotent stem cells into CMs. Outline of three different and widely used CM differentiation strategies: END-2 co-culture- based CM differentiation (Mummery, 2003), monolayer-based CM differentiation (Laflamme et al., 2007), and EB-based CM differentiation. Three specialized EB-based CM differentiation protocols are depicted, starting from: hanging drops, suspension EBs (Kattman et al., 2011b), and spin EBs (Burridge et al., 2011b).

dish. This method is widely used for differentiating mPSCs. Unfortunately, this method gives varying results when applied to hPSC differentiation. Alternatively, hPSCs can be forced to aggregate via spinning down these cells in a round bottomed or V-shaped, low-attachment 96-well plate, a method termed 'spin EB formation' or 'forced aggregation' (Ng et al., 2005) (Figure 2). Cardiomyocyte yield is generally below 10%. Despite its inefficiency, EB formation is commonly used because of its ease and low cost (Rajala et al., 2011).

2.4.3 Monolayer-based differentiation

An alternative method for deriving CMs is a monolayer-based differentiation protocol. Cardio-inductive activity of the anterior endoderm is partially mediated by the TGF-β family (Sugi and Lough, 1995). Therefore, two of its members, Activin A and BMP4, have been used to drive hESCs towards CM maturation (Laflamme et al., 2005). In the monolayer-based CM differentiation protocol, hESCs are maintained as a high-density monolayer culture without the use of any feeder cell layer. After reaching confluency, hESC medium is replaced with defined serum-free medium and sequential addition of Activin A and BMP4. Differentiating cells are then maintained for another 2-3 weeks without any additional exogenous growth factors. Spontaneously beating cells appear within 10 days after the change in medium. This protocol yields a remarkably higher volume of CMs (30%) compared to spontaneous EB-based differentiation and can also be applied to iPSCs (Elliott et al., 2011; Shiba et al., 2009). Improvements of the monolayer-based method have been reported with the additional use of WNT3A and Dickkopf1 (DKK1), a naturally occurring canonical Wnt-signaling inhibitor (Paige et al., 2010) or the use of a Matrigel overlay, prior to differentiation (Uosaki et al., 2011). Despite the increased efficiency of this method, cell-cell interactions in EBs can stimulate the expression of mesoendodermal and early cardiac markers, which favors the use of 3D structures (EB) for cardiac differentiation.

2.4.4 Directed cardiac differentiation via embryoid body formation

There is a need for greater efficiency of CM differentiation. By merging the EB and monolayer protocols, this new approach incorporates 3D-cell interaction as well as addition of growth factors such as BMPs, FGFs and Wnts (Filipczyk et al., 2007) (Figure 2). Timed administration of Activin A, BMP4 and bFGF (FGF-2) induces mesoderm in a serum-free environment. Subsequent administration of DKK1 in combination with VEGF then leads to cardiac specification (Yang et al., 2008). Replacing DKK1 with bFGF from day eight of differentiation and continued administration of VEGF promote the last step of in vitro cardiomyogenesis. This protocol generates populations consisting of 40-50% CMs (Yang et al., 2008).

Interestingly, analyzing growth factor variables and their timed effects demonstrates that remarkably small changes in BMP and Activin/nodal signaling are needed to determine specification of cardiac mesoderm (Kattman et al., 2011b). This has resulted in an adjusted suspension-EB based protocol with specified concentrations of Activin and BMP4 needed for

mouse and human PSCs, respectively. In this protocol, cardiac mesoderm induction in hESCs can be monitored by the occurrence of the two surface receptors, KDR (Flk1) and PDGFR-α. Cells can be sorted based on the expression of these receptors, reducing contamination with undifferentiated hESCs or other mesodermal cell types. Additionally, the hiPSCs tested with this protocol, needed a defined (Day (D)3-D5) administration of SB-431542 (5.4 μM) (a Nodal signaling inhibitor) and dorsomorphin (a natural inhibitor of BMP4 signaling), probably reflecting different levels of endogenously produced signaling factors (Kattman et al., 2011b). This method yields a differentiated population that contains more than 60% CMs, demonstrating the value of identifying threshold effects and subtle gradients of growth factor concentrations for the differentiation of distinct PSC lines (Willems et al., 2011).

2.4.5 Genetic approaches

In addition to growth factor-based protocols, genetic methods have been developed in which PSCs can be coaxed to the cardiovascular lineage by overexpression of different key transcription factors involved in early cardiogenesis. Mesp1 drives cells towards a cardiac fate by means of timed overexpression (D2-D4) in combination with time-specific Wnt inhibition by Dkk1 (Bondue et al., 2008b; Lindsley et al., 2008) or chemical inhibitors such as IWR-1 or IWP-4 (from D4 until D6) (Hudson et al., 2012). Nkx2.5 also promotes cardiac differentiation in a method described as 'cardiac forward programming (David et al., 2009).

As shown with new pluripotency reprogramming approaches, these integrative techniques for CM differentiation starting from hPSCs will be omitted in the future by delivery of the factors as non integrative viruses, mRNA and/or proteins. These new approaches will avoid the generation of permanently genetically modified cells, allowing them to be used for transplantation purposes in addition to being a tool for *in vitro* studies such as cardiac marker analysis. In order to further boost efficiency and standardize the process of large-scale cardiomyocyte generation, novel strategies must be developed. In the meantime, directed differentiation of hPSCs via EB formation and differentiation of hPSCs as a monolayer remain the most commonly used protocols.

2.4.5.1 Measuring cardiomyocyte differentiation efficiency

Markers of cardiac development are essential for the establishment of CM differentiation protocols, as they are crucial for evaluating the efficiency and quality of the cardiac differentiation process (Figure 1). A common method of measuring CM differentiation efficiency is to count the number of beating EBs that appear during the differentiation process. Although this provides a good indication of CM formation qualitatively, it is not stringent enough to determine the efficiency of CM formation. The beating of an EB does not reflect the number of CMs residing in this 3D aggregate. Non-cardiac cells will not contract, but will still move due to a 'bystander effect', mediated by patches of CMs inside the EB.

Assessing the expression of specific cardiac markers, such as cardiac Troponin-T (cTNNT2) or Troponin I (TnI), may be a better criterion for efficiency, especially if protein expression is assessed rather than only the presence of mRNA. It is also essential to verify the expression pattern of the cardiac structural (in many cases, sarcomeric) proteins, since they contribute to the function of a fully functional CM (see cardiomyocyte characterization) and correct for the possibility of false-positive immunostaining of damaged cells.

Fully differentiated PSC-derived CMs (PSC-CMs) have limited proliferation potential, resulting in the need of an enormous number of CMs for transplantation after myocardial damage. Therefore, cardiac precursor cells or other proliferative intermediate cell types may be a useful alternative for myocardial regeneration. However, cardiac precursors have yet to be definitively identified. Following cardiac differentiation by means of marker expression of intermediate cell types can help identify these precursors, as well as optimize the different steps of directed CM differentiation. PDGFRα+/KDR+ cells appear during cardiac differentiation suggesting that they may be a representative indicator of cardiac mesoderm induction (Kattman et al., 2011b); and Nkx2.5-eGFP marks CPC formation (Elliott et al., 2011). Together, the above examples demonstrate the urgent need for appropriate cardiac markers in order to improve differentiation conditions and production.

The difficulty of sorting for progenitor cells is that they can form multiple cardiac cell types, including vascular endothelial cells, smooth muscle cells and cardiomyocytes. In addition, undifferentiated cells from other lineages can also express some of these markers (Dambrot et al., 2011). Nonetheless, applying these progenitors can be useful for transplantation, since this mixture of cardiovascular cells can enhance engraftment through structural and paracrine properties. The percentage of cells positive for these markers gives a rough indication of CM differentiation efficiency.

2.5 Cardiomyocyte enrichment and purification

The use of PSC-CMs in cell-based therapy has been hampered by the inability to derive and identify a pure population of CMs or CPCs. Eliminating the high degree of heterogeneity in a population of PSCs differentiating/differentiated into cardiac cells is needed to omit unwanted side effects such as tumor (teratoma) formation by contaminating pluripotent cells after transplantation (Behfar et al., 2007; Braam et al., 2009; Kolossov et al., 2006; Miura et al., 2009; Nussbaum et al., 2007). Many cell types have already been used for transplantation post-MI and proven beneficial in transiently ameliorating heart function (Adler et al., 2010; Caspi et al., 2007a; Christoforou et al., 2010; Fernandes et al., 2010; Fujimoto et al., 2009; Laflamme et al., 2007; Lu et al., 2009; Nelson et al., 2009; van Laake et al., 2009).

2.5.1 Enriching CM populations

Depending on the desired cell type different approaches are suitable to enrich a specific cell population. A standard, easy to use and widespread method is manual microdissection of beating patches with a glass knife. This method can deliver a population consisting of 70% CMs (Caspi et al., 2007a; Kehat, 2001; Mummery, 2003; Shiba et al., 2009). However, upscaling this process for therapeutic applications is not feasible, nor is this grade of purity enough to assure the needed safety for cell-based transplantations. In general, cardiac enrichment strategies can be divided into three different types; structural based enrichment, genetically based enrichment and surface protein based enrichment.

2.5.2 Structural based enrichment

CM-purification can be performed based on the cells' physical and structural properties that separate CMs in a Percoll gradient 2(Laflamme et al., 2007; Xu et al., 2002; 2006). This method can enrich CMs five-to 10 fold in a differentiating cell mixture (Murry and Keller, 2008). However, its application on a larger scale is hampered by the low yield of CM purity. A recently described method enriches hPSC-derived CMs with the fluorescent dye tetramethylrhodamine (TMRM), which is specifically retained in mitochondria (Hattori et al., 2009). Since CMs contain a high number of mitochondria, they can be sorted via fluorescence-activated cell sorting (FACS), based on this criterion, with a purity of 99%. The TMRM MitoTracker dye does not affect cell viability and disappears completely after 24 hours. However, it should be noted that only CMs with a high mitochondrial density will produce a high fluorescent signal; immature CMs will be lost by this type of separation (Dubois et al., 2011). Another recent study has reported the selection of hPSC-CM by second harmonic generation (SHG) microscopy, in which the SHG signal is attributable to myosin and dependent on the maturity of the CM, allowing for non-invasive purification of hPSC-CMs (Awasthi et al., 2012).

2.5.3 Genetically-based CM enrichment

Expression of selection markers such as eGFP, DsRed or other fluorescent reporters, under control of a lineage-specific promoter can enhance purification of myocardial precursors (Doevendans et al., 1996). Several papers report fluorescent protein expression based enrichment of cells positive for α-MHC-GFP (Anderson et al., 2007; Ritner et al., 2011), MLC2V-eGFP (Huber et al., 2007), MLC2V-DsRed (Fu et al., 2010), Nkx2.5-GFP (van Laake et al., 2010) Isl-Cre (pCAG-flox-DsRed) (Bu et al., 2009), or ANP-eGFP (Gassanov et al., 2004). Combining these fluorescent reporters with antibiotic resistance such as MYH6-blasticidin (Ma et al., 2011) or α-MHC-neomycin (Xu et al., 2008a) can even increase the level of cardiomyocyte purity to 99%.

A major drawback of this approach is the requirement of prior genetic manipulation by integrating selection markers into the host genome through viral or non-viral transfection/ transduction methods. Random integration of the transgenic constructs can disrupt endogenous genes, causing tumorigenesis and other milder phenotypic defects (Recchia et al., 2006; Tsukahara et al., 2006; Woods et al., 2003). Targeted integration, however, circumvents this last drawback, yet requires selection of the correctly targeted clones and expression analysis (Gropp and Reubinoff, 2006), which is inefficient and time-consuming.

2.5.4 Surface protein-based enrichment

Using antibodies against surface markers specific for CMs or intermediate cell types avoids the need for prior genetic modification. Sorting cells with FACS or magnetic beads can enrich cardiac-specific cell types based on positive or negative selection for one or multiple cell surface markers. This has been shown by isolating a KDR (FLK1/VEGFR2)-positive (Yang et al., 2008), KDR/PDGFRα-double positive (Kattman et al., 2011b), or CXCR4$^+$/FLK1$^+$-double positive cardiac progenitor cell population (Nelson et al., 2008; Yamashita et al., 2005). Another viable marker for CM enrichment is the cellular prion protein (cPp), a CM and CPC surface marker. Sorting for cPp$^+$/ PDGFRα^+ cells also purifies a cardiomyogenic cell population (Hidaka et al., 2010). Recently surface markers present on differentiated cardiomyocytes have been identified, including ALCAM (Rust et al., 2009), EMILIN2 (Van Hoof et al., 2010), SIRPA (Dubois et al., 2011; Elliott et al., 2011) and VCAM (Elliott et al., 2011; Uosaki et al., 2011). Although these markers may also be found on other cell types, immune cells in particular, they appear specific enough for CMs in the relevant *in vitro* cell population that develops with directed CM differentiation protocols. Thus, surface markers may allow the purification of hPSC-CMs *in vitro*.

In conclusion, purifying CMs for clinical purposes has been arduous. Genetic enrichment methods hamper the use of hPSC-CMs for therapeutic purposes due to the retention of a permanent genetic tag. New methods based on CM physical and structural properties, together with the identification of specific surface markers, provide new tools for hPSC-CM purification and enrichment after differentiation.

2.6 Cardiomyocyte characterization

Transplantation of hPSC-CMs requires proper characterization of the purified cells to predict the outcome after implantation and to minimize the risk of complications, such as host rejection, tumor formation or arrhythmias. In general, hPSC-CMs have an immature phenotype resembling human fetal CMs regarding their gene expression profile and structural-, electrophysiological- and pharmacological properties (Cao et al., 2008; Davis et al., 2011; Dolnikov et al., 2005; He et al., 2003; Mummery, 2003; Sartiani et al., 2007; Satin et al., 2004; Snir et al., 2003). HPSC-CMs *in vitro* display various morphologies including spindle-shaped, round, tri- and multi-angular forms, rather than the rod-shaped morphology

characteristic for adult CMs. Few hPSC-CMs show multinucleation (<1%), compared to 20% of their adult counterparts (Snir et al., 2003). Although sarcomeric A, I and Z banding can be identified in hPSC-CMs, their myofibrillar and sarcomeric organization denotes an immature phenotype (Kehat, 2001; Liu et al., 2007; Snir et al., 2003). As hPSC-CMs mature in culture over time, they show more mature electrophysiological properties, however there are still significant differences with adult CMs (Sartiani et al., 2007).

Human PSC-CMs are able to contract *in vitro*, a process triggered by the influx of calcium ions, and show the same calcium transients as mature CMs (Liu et al., 2007; Mummery, 2003; Satin et al., 2004). However, the regulation of this intracellular calcium handling tends to differ between hPSC-CMs and mature CMs, probably due to an immature sarcoplasmic reticulum (Binah et al., 2007; Dolnikov et al., 2005). Human PSC-CMs also display action potentials (APs), which distinguish between atrial, ventricular or nodal identities (He et al., 2003). Yet, these AP parameters tend to be different from those of mature CMs (slower AP upstroke volume, automaticity, depolarized maximum diastolic potential), although some electrophysiological maturation occurs *in vitro* over time (He et al., 2003; Sartiani et al., 2007). Repolarization in hPSC-CMs is generally mediated by the same ion channels (including cardiac sodium channel SCN5A, voltage-dependent L-type calcium channel CACNA1C, and voltage-gated potassium channels KCNH2 and KCNA4 (Sartiani et al., 2007; Satin et al., 2004) as in mature CMs. Another similarity is that hPSC-CMs react as expected to adrenergic and muscarinic hormones, such as isoproterenol, isoprenaline and carbachol, by a modulated contraction pattern. This denotes the presence of membrane receptors able to sense those triggers and signal them through pathway activating ion channels, membrane transporters and structural proteins (Kehat, 2001; Norström et al., 2006; Otsuji et al., 2010; Xu et al., 2002).

In addition to their functional characteristics that are in many respects similar to mature CMs, hPSC-CMs show a characteristic gene expression profile along their differentiation path (Beqqali et al., 2006). QRT-PCR, immunohistochemistry and micro-array data show that the initial phase of the *in vitro* differentiation process is characterized by the upregulation of early mesoderm-like markers like Brachyury, subsequently followed by early cardiac transcription factors, such as GATA4, MEF2C, GATA4, Isl1, TBX5, TBX20 and NKX2.5(Beqqali et al., 2006; Brito-Martins et al., 2008; Cao et al., 2008; Kehat et al., 2004; Synnergren et al., 2008a; Xu et al., 2002). Correspondingly, structural proteins are expressed similar to in vivo cardiogenesis: these include the sarcomeric and myofiber proteins, α-actinin; α- and β-myosin heavy chain (MYH6 and MYH7); atrial and ventricular myosin light chain (MLC-2A and MLC-2V); tropomyosin, cardiac Troponin T (cTnnT) and I (cTnI); and gap junction proteins (Synnergren et al., 2008a). Other cardiac and muscle specific markers such as atrial natriuretic peptide (ANP), creatine kinase-MB (CK-MB) and myoglobin (Kehat et al., 2002; Xu et al., 2002), are also detected during cardiac differentiation of hPSCs. Together, these markers establish a specific cardiac expression profile reminiscent of the developing human heart, thus providing a powerful tool to characterize the derived CMs after directed cardiac

differentiation. Reports on proteomic quantification of the differentiating CMs have been less frequent. However, proteomics are essential in the comparison of *in vitro* derived CMs from hPSCs with in vivo CMs, in order to profile both populations and elucidate more stringent criteria for more relevant CMs obtained from hPSC-CM differentiation protocols. Detailed proteomic analysis also allows the identification of novel surface markers (Rugg-Gunn et al., 2012; Van Hoof et al., 2010) of various intermediate cell types and mature differentiated CMs. This will enhance the accurate identification of new modifiers determining functionality, specialization and maturation characteristics during CM differentiation.

2.7 Conclusions and future developments

Cell-based therapy is a promising new option for the treatment of myocardial damage. PSCs appear to be one of the most appropriate sources for the generation of donor cells, including differentiated CMs or cardiac tissue constructs. Insights into the complex and highly dynamic process of cardiogenesis have supported the establishment of guided CM differentiation *in vitro*. In hPSCs, many of the major signaling pathways involved, including BMP, TGF-β/activin/NODAL, WNT and FGF have been incorporated into specialized cardiac differentiation protocols, which stimulate these factors in different temporal windows. However, despite significant progress in this field, we are still unable to efficiently and safely generate a homogenous mature CM population that effectively contributes to heart tissue regeneration. To date, most iPSCs are still generated by integrative methods based on retroviral- and lentiviral-based transduction of the four reprogramming factors, Sox2, Klf4, Oct4 and c-Myc. These genetically modified cells and their differentiated offspring are not tolerated for cell-based therapy, since they can cause tumors by reactivation of the reprogramming factors or disruption of endogenous genes. Discrepancies between different iPSC lines are common, resulting in varying differentiation efficiencies. Generation of hiPSC-CM for heart regeneration calls for a standardized non-integrative and heterogeneity-reducing method. Culturing hPSCs feeder-free via monolayer growth (Denning et al., 2006) on the basement membrane matrix Geltrex in conditioned medium (Xu et al., 2001) and passaging enzymatically to single cells, combined with plating defined number of cells might resolve this problem (Burridge et al., 2011b), but this method still needs to be replicated.

Many enhancers of cardiac differentiation have been published, yet there is still much unknown about the molecular mechanisms necessary for differentiating PSCs towards the cardiac lineage, and the efficiency often remains lower than 10%. In addition, it is known from transplantation of hESC-CMs that more than 90% of the transplanted cells die within 24 hours post-transplantation (Hodgetts et al., 2000; van Laake et al., 2007), creating the need for larger numbers of cells. Several culturing methods are striving to increase transplantation efficiency, since monolayer-based differentiation produces only a limited amount of cardiac cells per square centimeter. Multilayer cell culture or culturing in larger volumes in suspension, such as bioreactors (Niebruegge et al., 2009), is proving to be promising in overcoming this problem.

Although transplantation of single cell suspensions is the easiest method, grafting a 3D engineered cardiac patch may provide better results with respect to replacing scar tissue in damaged myocardium. Multiple tissue engineering approaches have been reported so far (Caspi et al., 2007b; Gaetani et al., 2015; Stevens et al., 2009a; 2009b; Tulloch et al., 2011; Ye et al., 2011), expertly reviewed previously in this journal (Nunes et al., 2011). All these studies conclude that the key of engraftment and cell-survival after transplantation lays in vascularization of the transplant (Caspi et al., 2007b; van Laake et al., 2008). Vascularization of these tissue constructs can already be primed *in vitro* by seeding a mixture of CMs and vascular cells on a biodegradable matrix, or by adding extra growth factors that enhance neovascularization. Another method could be the modulation and enhancement of exosome content with for example VEGF, stimulating angiogenesis upon reception by the host cell (Vrijsen et al., 2010). Other challenges regarding the progression of hPSC-CMs towards cell-based therapy include determining the optimal window of cell transplantation after heart injury, the best route of administration and the dose of cells needed.

Differentiating hPSCs into CMs is expensive, since large amounts of growth factors are needed to produce enough CMs for transplantation. This could be alleviated by emerging knowledge about the potential of cheap small molecules in cardiac differentiation (Willems et al., 2011). Additionally, small chemical compounds such as triiodothyronine (Lee et al., 2010) can also enhance maturation, akin to inductive signals from endothelial cells (Kim et al., 2010). Currently, most hPSC-CMs retain an immature phenotype compared to their in vivo counterparts, maturing very slowly and incompletely in culture. Because of their immature phenotype, electrophysiological patterns, such as calcium handling and specific ion channel expression, are also underdeveloped and can limit functionality or increase arrhythmogenicity of the engrafted cells due to altered excitability and reactivity. Extensive proteome wide comparisons of CM obtained from hPSCs versus in vivo CMs may help to determine markers for relevant hPSC-CMs, useful for cell-based therapy. Proteomic studies can identify new cell-surface markers that will unequivocally distinguish different subtypes of CMs, which will be useful not only for transplantation of a well-characterized cell population, but also for drug discovery. After all, drug discovery and testing require pure populations of CMs (and their subtypes) in order to generate a specific phenotype, ascribed to an underlying genetic defect. The *in vitro* use of hPSC-CMs has recently been reviewed in a previous edition of this journal (Matsa and Denning, 2012).

In conclusion, human PSC-CMs are a promising cell source that will further our understanding of cardiac development and enable advancements in heart tissue regeneration. The generation of hPSC-CMs has greatly improved and is suitable for many *in vitro* research applications. The field is sensitive to the present considerations necessary for the safe therapeutic application in humans and is forging ahead to develop advances necessary to accomplish this.

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3

Functional implications of circadian networks in human embryonic stem cell-derived cardiomyocytes

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Abstract

Cell-autonomous circadian oscillations strongly influence tissue physiology and pathophysiology of peripheral organs including the heart, in which the circadian clock is known to determine cardiac metabolism and the outcome of for instance ischemic stress. Human pluripotent stem cells represent a powerful tool to study developmental processes in vitro, but the extent to which human embryonic stem (ES) cell-derived cardiomyocytes establish circadian rhythmicity in the absence of a systemic context is unknown. Here we demonstrate that while undifferentiated human ES cells do not possess an intrinsic functional clock, oscillatory expression of known core clock genes emerges spontaneously during directed cardiac differentiation. We identify a set of clock controlled output genes that contains an oscillatory network of stress-related transcripts. Furthermore, we demonstrate that this network results in a time-dependent functional response to doxorubicin, a frequently used anti-cancer drug with known cardiotoxic side effects. Taken together, our data provide a framework from which the effect of oscillatory gene expression on cardiomyocyte physiology can be modeled in vitro, and demonstrate the influence of a functional clock on experimental outcome.

3.1 Introduction

The circadian clock is a conserved time-keeping system that regulates numerous body features such as behavior, metabolism, body temperature, tissue regeneration and organ homeostasis in a diurnal manner (Aschoff, 1983). In the heart, the role of 24-hour rhythmicity is illustrated by oscillations in heart rate, blood pressure and cardiac output (Degaute et al., 1994; Delp et al., 1991; Ohishi et al., 1993; Richards et al., 1986; Shea et al., 2011). The circadian clock comprises a central clock in the brain (the suprachiasmatic nucleus (SCN)) and peripheral clocks that are present in almost all organs. The SCN is mainly entrained by light and subsequently synchronizes the peripheral clocks via neural and humoral factors (Cajochen et al., 2003). Interestingly, peripheral clocks function in a cell-autonomous manner. When ablating the SCN, these clocks remain functional and even synchronized when subjected to a restricted feeding regime (Hara et al., 2001; Pezuk et al., 2010). Autonomous rhythmicity is underscored by the persistence of circadian rhythms in in vitro cultured cells.

The molecular mechanism that underlies the core clock machinery consists of a transcriptional/translational feedback loop in which a heterodimer of BMAL1 and CLOCK drives rhythmic transcription of downstream genes. These include other core clock genes (period 1 (*PER1*), *PER2*, *PER3*, cryptochrome 1 (*CRY1*), *CRY2*, *ROR*α/β, *REV-ER*α/β) as well as clock controlled genes (CCGs) that determine circadian organ physiology in a tissue-specific manner. In the murine heart, ~6-12% of the expressed genes have a circadian expression pattern (Martino et al., 2007; 2004; Storch et al., 2002; Tsimakouridze et al., 2012; Zhang et al., 2014). Rhythmicity is essential for human tissue homeostasis as well, as highlighted by

the fact that genetic or environmental (e.g. shift-work) perturbation of the circadian clock results in a vast array of malignancies such as sleep disorders, inflammation, cancer (Eckel-Mahan and Sassone-Corsi, 2013), impairment of regenerative capacity (Janich et al., 2011; 2013), metabolic disorders (Bray et al., 2013; Kennaway et al., 2013; Rudic et al., 2004) and cardiovascular diseases (Dierickx et al., 2015; Furlan et al., 2000; Martino et al., 2007; 2008; Penev et al., 1998). In addition, the onset of multiple malicious cardiac events is known to follow a diurnal pattern. Myocardial infarction (Mukamal et al., 2000; Muller et al., 1985), arrhythmias (Maron et al., 2009; Tofler et al., 1995) and sudden cardiac death (Arntz et al., 1993; Maron et al., 1994) show a higher incidence in the sleep-to-wake transition in humans. The important role of circadian rhythmicity in cardiac injury and regeneration is further solidified by genetic experiments in mice in which a cardiomyocyte-specific mutation of the *Clock* gene has been shown to blunt the heart's response to induced ischemic damage (Durgan et al., 2010). Accordingly, clinical studies revealed that infarcts were larger and led to increased reduction of cardiac function when occurring in the sleep-to-wake transition (Reiter et al., 2012; Seneviratna et al., 2015; Suarez-Barrientos et al., 2011).

Human pluripotent stem cell-derived cardiomyocytes have emerged as a potential cellular source for replacement therapies. In addition, human ES cell- as well as induced pluripotent stem cell-derived cardiomyocytes are increasingly used for disease modeling and drug testing (Dierickx et al., 2012). While circadian rhythms play an essential role in cardiomyocyte function in vivo, nothing is known about circadian control of gene expression in pluripotent stem cell-derived cardiomyocytes, which are often used to model cardiac function and disease.

Here we analyze temporal clock gene expression networks in human ES cells and ES cell-derived cardiomyocytes. We demonstrate that circadian rhythmicity is absent in human ES cells and is established progressively during directed cardiac differentiation. The identified oscillatory networks are shown to significantly influence the function of human ES cell-derived cardiomyocytes and determine their response to externally applied stressors. Our findings underscore that circadian rhythmicity can affect experimental outcome, which may have important ramifications for processes such as timed cell-based therapy.

3.2 Results

3.2.1 Human embryonic stem cell differentiation towards cardiomyocytes

Circadian rhythmicity was analyzed at 3 stages (day 0 (D0), D15 and D30) during directed differentiation of human embryonic stem (ES) cells toward cardiomyocytes (Figure 1A). To allow for the identification of early cardiac cells, we made use of a *NKX2*.5-eGFP (Homeobox protein Nkx-2.5-eGFP) reporter human ES cell line (Elliott et al., 2011). Cardiac differentiation of human ES cells typically yields significant contribution of cardiomyocytes to the total population of cells (Bunda et al., 2007; Cao et al., 2016; van den Berg et al., 2015), which

was also seen here with ~50% cardiomyocytes at D15 as defined by FACS for cTNNT2 (Supplementary Figure 1A). Different stages were characterized by clear changes in marker gene expression (Figure 1A). At day 0, cells expressed the pluripotency markers *NANOG* and *POU5F1*, both at the RNA and protein level (Figure 1A and B and Supplementary Figure 1B). Upon differentiation, pluripotency factors quickly decreased and the expression of cardiac markers such as *NKX2.5* and *ACTC1 (*Actin alpha cardiac muscle 1) was observed in both (D15 and D30) spontaneously beating cardiac stages by qRT-PCR measurements (Figure 1A). Immunostainings for α-sarcomeric Actin and cTNNT2 confirmed sarcomeric structures at D15 and D30 (Figure 1B). Additionally, staining for MEF2C (Myocyte Enhancer Factor 2C) and GFP, to assess the presence of *NKX2.5*-eGFP positive cells, revealed the abundance of cardiomyocytes at D15 and D30. While the early cardiomyocyte progenitor marker *ISL1* (Insulin gene enhancer protein ISL-1) was highly expressed at D15, maturation markers such as *KCNJ2* (inward rectifier potassium channel 2) and *SERCA2A* (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) (Cao et al., 2016; Liu et al., 2009) were the highest at D30 (Figure 1A). *CASQ2* (Calsequestrin), known to be expressed in adult cardiomyocytes but generally low in human ES cell-derived cardiomyocytes (Liu et al., 2009), was also expressed more at D30 than at D15 (Supplementary Figure 1B). These results validate the in vitro maturation of these cells over time and confirm that the different stages represent distinct cardiac states that can be used to assess the presence of a functional clock across the transition from human ES cells to matured cardiomyocytes.

3.2.2 Human embryonic stem cells express clock genes in a non-oscillatory manner

Nearly all cells in the human body possess a functional clock as indicated by circadian rhythmicity of core clock gene expression. However, whether human ES cells display a functional circadian clock is unknown. Therefore, we compared global expression levels of six core clock genes *ARNTL* (coding for and henceforth referred to as *BMAL1*), *PER2*, *CRY1*, *CRY2*, *CLOCK*, and *NR1D1* between pluripotent human ES cells and differentiated human osteosarcoma U2OS cells, a cell line known to possess a functional clock (Supplementary Figure 2A,B). Transcripts of all genes could be detected in both cell types with 5 out of 6 core clock genes showing higher expression levels in U2OS cells compared to human ES cells (Student's T-Test, $P < 0.05$; Figure 2A). *CRY1* however*,* was more highly expressed in human ES cells (Student's T-Test, $P = 0.506$; Figure 2A), which is in line with previously reported observations in mouse ES cells and NIH3T3 cells (Umemura et al., 2014). Protein levels of BMAL1, CLOCK and CRY1 were detected by western blot (Figure 2B, left), at corresponding levels to their RNA transcripts (Figure 2B, right). From this, we conclude that while core clock genes are expressed and translated into protein in human ES cells, this occurs with a different stoichiometry in comparison to differentiated cells.

The presence of core clock proteins in human ES cells led us to investigate their possible rhythmic expression over time. To this end, human ES cells were synchronized with forskolin (Yagita and Okamura, 2000), and *BMAL1* and *PER2* mRNA levels were measured every 4

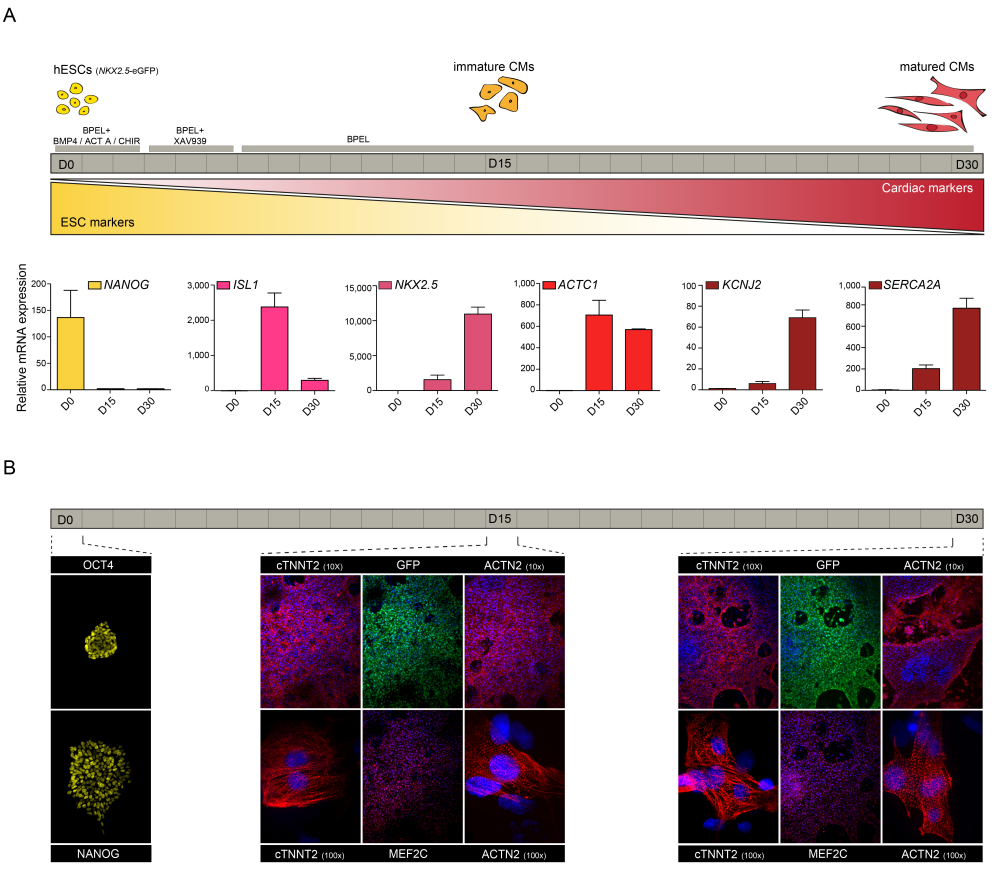

Figure 1. Characterization of distinct stages of cardiac differentiation. (A) Schematic of the directed cardiac differentiation method and the three different stages used in this study. hESCs: human embryonic stem cells. CM: cardiomyocyte. Below are mRNA expression levels of pluripotency and cardiac markers as measured by qRT-PCR. Expression levels were normalized to a non-oscillatory housekeeping gene (*PPIA*). (B) Immunostaining for pluripotency markers OCT4 and NANOG (yellow) in human ES cells. cTNNT2 and Sarcomeric Actin (red) stainings reveal sarcomeric structures at all cardiomyocyte stages. Cardiomyocyte nuclei were stained for MEF2C (red), eGFP-NKX2.5 positive cells were stained with anti-GFP (green) and nuclei with Hoechst (blue).

hours over a period of 48 hours. Significance of 24-hour rhythmicity was assessed using RAIN, a nonparametric method detecting arbitrary wave forms in biological data (Thaben and Westermark, 2014). No apparent oscillatory expression pattern could be identified over the course of two days (RAIN, *BMAL1*, $P > 0.99$ and *PER2*, $P = 0.97$; Figure 2C). Additionally, to assess *BMAL1* and *PER2* transcription over time in the same population of human ES cells, we transduced ES cells with Bmal1- and Per2 promoter-based lentiviral luciferase constructs (Liu et al., 2008; 2007). After synchronization, no rhythmic bioluminescence was observed (Figure 2D). Therefore, clock genes are expressed in human ES cells, but in a non-circadian manner.

3.2.3 Rhythmic expression of clock genes emerges during cardiac differentiation

Multilineage differentiation of human ES cells has proven extremely valuable to understand developmental processes as well as to provide clinically relevant populations for cell-based therapy (Keller, 2005). However, when and whether human ES cells can develop a functional clock upon differentiation, in the absence of systemic cues, is unknown. Since the murine heart possesses a functional circadian clock (Martino et al., 2007; 2004; Storch et al., 2002; Tsimakouridze et al., 2012; Zhang et al., 2014), we sought to determine whether in vitro human ES cell-derived cardiomyocytes can establish rhythmic clock gene expression. To this end, we compared mRNA levels of *BMAL1*, *PER2*, and *CLOCK* in D0 human ES cells and D15 and D30 human ES-cell derived cardiomyocytes. Even though *BMAL1*, *PER2* and *CLOCK* were expressed at all stages, their expression increased significantly from D0 to D15 and D30 human ES cell-derived cardiomyocytes (Student's T-Test, $P < 0.05$; Figure 3A). This indicates that core clock gene expression gradually increases during directed cardiac differentiation. To assess rhythmicity of clock gene expression, D15 and D30 human ES cell-derived cardiomyocytes were synchronized using dexamethasone (Balsalobre et al., 2000).

For each stage, three independent RNA samples were collected every 4 hours across 48 hours (Supplementary Figure 3). *BMAL1* and *PER2* levels were analyzed by qRT-PCR to determine whether their expression oscillated in an anti-phasic manner, a hallmark of a functional molecular circadian clock. Similar to undifferentiated human ES cells, no clear circadian pattern was observed in early cardiomyocytes (D15) (RAIN, $P = 0.095$, $P = 0.68$ for *BMAL1* and *PER2,* respectively; Figure 3B). Matured cardiomyocytes (D30) however, showed significant oscillations for *PER2* but not *BMAL1* (RAIN, $P = 1.4E\text{-}8$ and $P = 0.81$; Fig 3B). To validate that more mature cardiomyocytes (D30) possess a robust clock, human ES cell-derived cardiomyocytes were transduced with *Bmal1*-dLuc and *Per2*-dLuc lentiviral reporters. At D15, after synchronization, a small induction of oscillatory *Per2*-based luciferase signal could be detected (Figure 3C), which is in line with previously described observations of *Per2* as an early oscillator upon retinoic acid-induced differentiation in mouse ES cells (Yagita et al., 2010). In D30 synchronized populations, typical anti-phasic oscillatory *Per2* and *Bmal1*-driven bioluminescence levels were observed, which confirms the presence of a functional clock (Figure 3D and E).

To verify the contribution of cardiomyocytes to the observed oscillatory pattern, *NKX2.5*-eGFP reporter gene expressing cardiomyocytes were selected via FACS. After sorting, strong *Bmal1*-dLuc and *Per2*-dLuc rhythmicity was detected in synchronized cultures (Figure 3F). In addition to these observations in a population of cells, circadian *Per2*-dLuc patterns were also found when recording bioluminescence of single eGFP-positive cardiomyocytes (Figure 3G and H). We thus conclude that human ES cells develop a functional clock upon directed cardiac differentiation and that robust oscillations emerge around D30 during in vitro cardiomyocyte maturation.

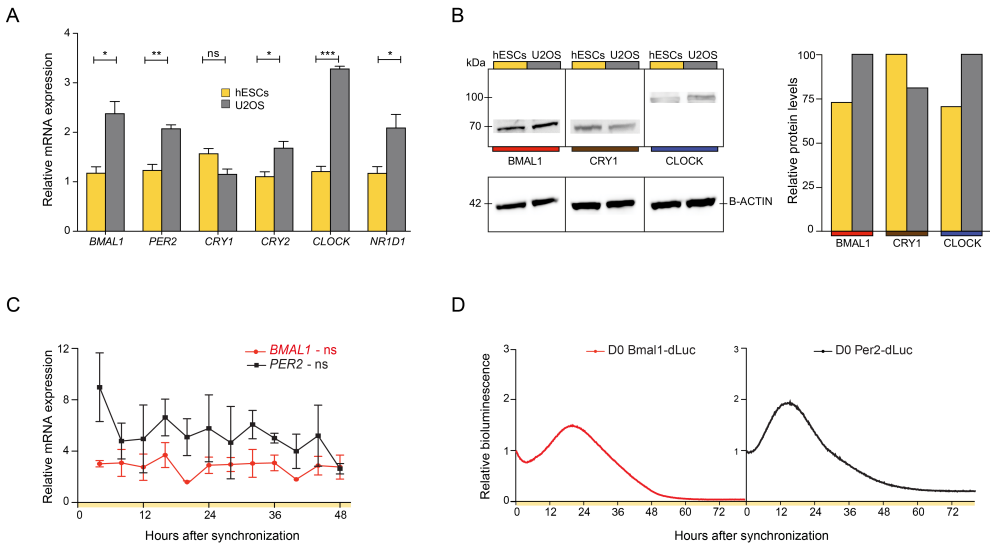

Figure 2. Non-oscillatory expression of clock genes in human ES cells. (A) *BMAL1, PER2*, *CRY1*, *CRY2*, *CLOCK*, and *NR1D1* expression levels in human ES cells compared to U2OS cells as determined by qRT-PCR. Expression levels were normalized to *PPIA* and compared between cell types using an unpaired two-tailed Student's T-Test (ns: not significant; *$P < 0.05$; ** $P < 0.005$). (B) Western blot for BMAL1, CLOCK, and CRY1. Protein levels were quantified through normalization against β-ACTIN. (C) qRT-PCR analysis of *BMAL1* and *PER2* expression over 48 hours at a 4-hour interval in human ES cells. Circadian oscillations were analyzed using the RAIN algorithm and the significance of rhythmicity across 48 hours is indicated (ns: not significant). Throughout, data are represented as mean ± s.e.m. of three independent replicates. (D) Bmal1-Luc and Per2-Luc values in synchronized human ES cells across 76 hours measured by LumiCyle32. Representative tracks are shown.

3.2.4 Human ES cell-derived cardiomyocytes show a network of stress-related clock output genes

A functional circadian clock translates into the oscillatory expression of clock-controlled genes (CCGs). Gene expression profiling in numerous cell types and tissues has shown that around 3% to 16% of the transcriptome exhibits circadian rhythmicity (Zhang et al., 2014). Conform differing physiological demands of organs, oscillating output genes vary per tissue. For the murine heart, ~6-12% of the expressed genes were shown to oscillate in a 24-hour manner (Martino et al., 2007; 2004; Storch et al., 2002; Tsimakouridze et al., 2012; Zhang et al., 2014). To identify CCGs during in vitro cardiomyocyte differentiation, genome-wide mRNA levels were assessed by mRNA sequencing of purified RNA using CEL-Seq, a previously described RNA profiling technique based on sequencing the 3'UTR of mRNAs, generating one read per transcript (Hashimshony et al., 2012). We first compared the overall transcriptional profile of matured cardiomyocytes to that of human ES cells and D15 cardiomyocytes 48 hour post-synchronization (Supplementary Figure 4A and Supplemental Table 1). Lowly expressed genes are typically not picked up robustly when

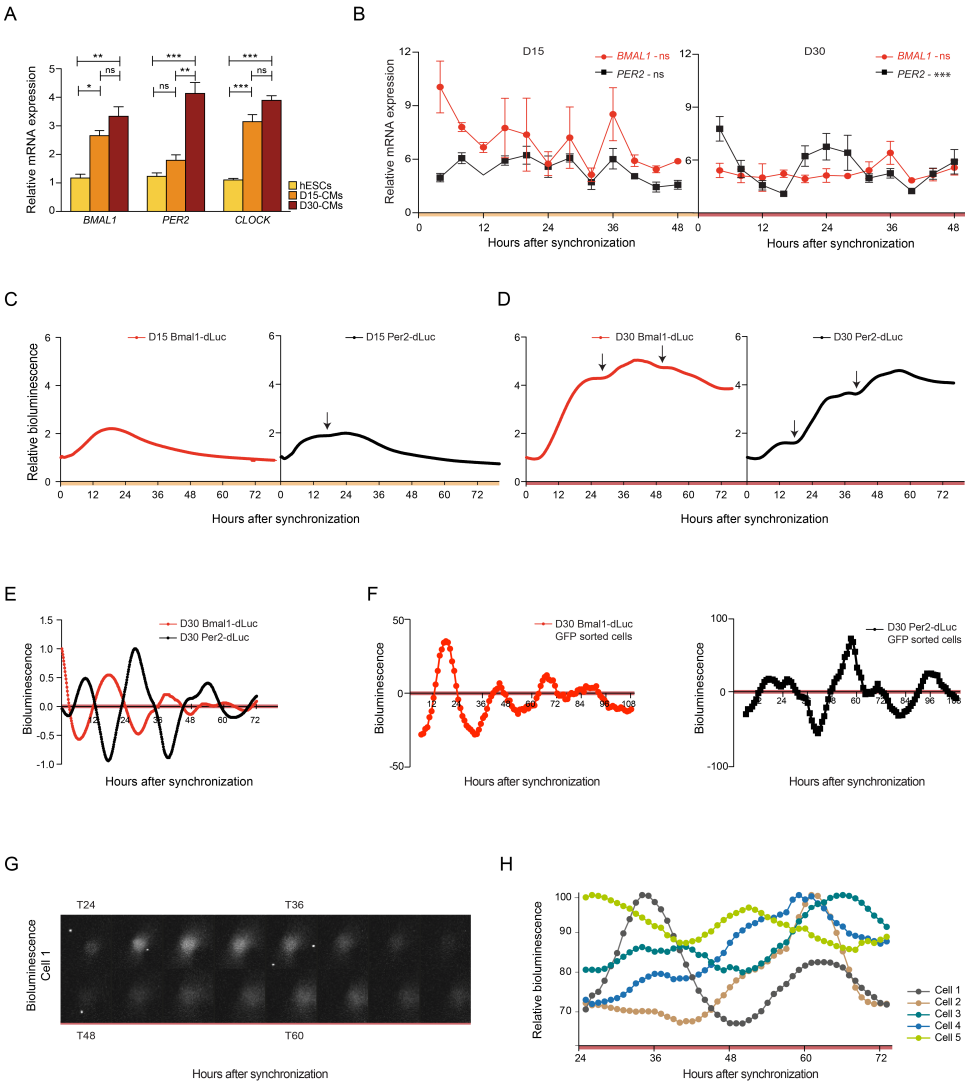

Figure 3. Rhythmic clock gene expression emerges in (matured) cardiomyocytes. (A) *BMAL1, PER2, and CLOCK* expression levels at D0, D15 and D30 during directed cardiomyocyte differentiation as determined using qRT-PCR. Expression levels were normalized to *PPIA*. Data are represented as mean ± s.e.m. of three independent replicates. Significant expression differences were tested by One-Way ANOVA followed by a Bonferroni post test (ns: not significant, ** $P < 0.005$, *** $P < 0.0005$). (B) qRT-PCR analysis of *BMAL1* and *PER2* expression over 48 hours at a 4-hour interval in human ES cell-derived cardiomyocytes at D15. Expression levels were normalized to *PPIA*. Data are represented as mean ± s.e.m. of three independent replicates. Significance of rhythmicity across 48 hours was analyzed using the RAIN algorithm and is indicated (ns: not significant, ** $P < 0.005$, *** $P < 0.0005$). (C) Promoter-based destabilized luciferase (dLuc) reporter assay of the Bmal1 and Per2 promoter in synchronized human ES cell-derived cardiomyocytes at D15. Measurements were performed using a LumiCycle32. (D) Similar analysis as in C) for D30. (E) Detrended Bmal1-dLuc and Per2-dLuc luciferase signal measured in D). (F) Bmal1-dLuc and Per2-dLuc bioluminescence in *NKX2.5*-eGFP$^+$ sorted and synchronized human ES cell-derived cardiomyocytes at D30. (G) Single-cell analysis of Per2-dLuc bioluminescence in sorted eGFP-positive and synchronized D38 human ES cell-derived cardiomyocytes. (H) Representative Per2-dLuc signal in five single D38 human ES cell-derived cardiomyocytes over the course of 48 hours. Measurements in F,G,H were performed with a LV200 microscope.

analyzing highly multiplexed CEL-Seq data at relatively low sequencing depth. To control for this, genes with an average of less than 3 RPM (reads per million) across all time points were not used for further analysis. Based on ~14,000 genes with an expression level of more than 3 RPM at one of the stages, Spearman's rank correlation coefficients (ρ) showed that transcriptional programs were substantially different between D15 and D30 cardiomyocytes (ρ) = 0.53; Supplementary Figure 4A,B). Observed changes between states were consistent between our qRT-PCR and CEL-Seq analyses, as indicated for several marker genes, which highlights the reliability of our sequencing datasets (Figure 1A and Supplementary Figure 4C,D). Indeed, increased *MYH7*/*MYH6* levels and multiple other markers (e.g. *MYL2*, *PLN* and *KCNJ2)* confirm in vitro cardiomyocyte maturation as well as generally higher clock gene expression across differentiation (Supplementary Figure 4C,D). To question whether longer culture would influence circadian rhythmicity and the transcriptional program of human ES cell-derived cardiomyoyctes substantially, D45 cardiomyocytes were analyzed. Using qRT-PCR and real-time reporter-based luciferase measurements, significant *PER2* and *BMAL1* oscillations could also be observed at D45 (Supplementary Figure 5A,B). CEL-Seq showed that these cells have a highly similar gene expression profile to D30 cardiomyocytes (ρ: 0.93, Supplementary Figure 5C), because of which D30 human ES cell-derived cardiomyocytes were selected for further analysis.

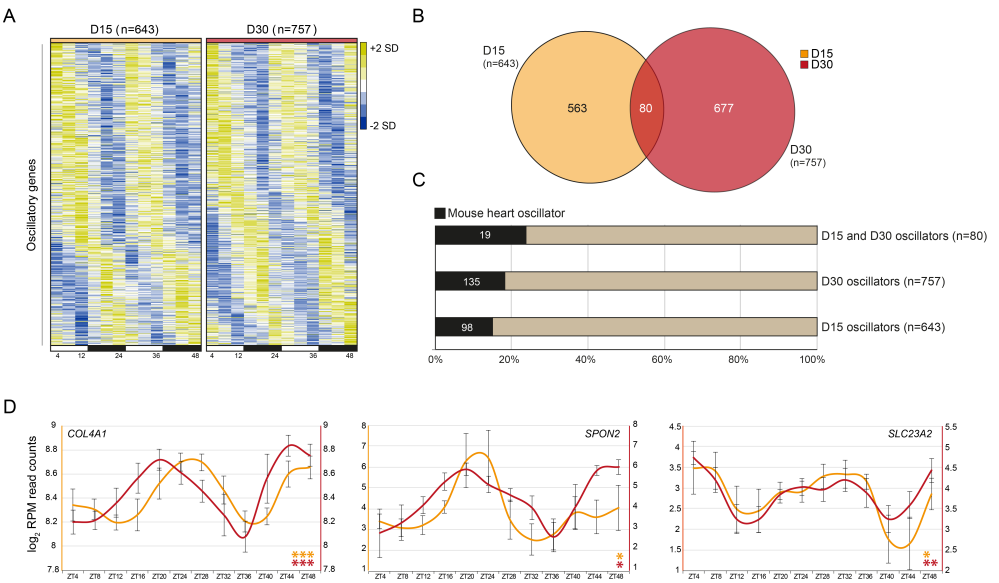

Figure 4. Oscillatory gene expression output in human ES cell-derived cardiomyocytes. (A) 643 and 757 oscillators for D15 and D30 human ES cell-derived cardiomyocytes, as analyzed using JTK-cycle (adj. $P < 0.05$). Heatmaps represent z-normalized RPM values of the average of three independent replicates. Oscillatory genes were ranked by their phase of expression and visualized using Java TreeView. (B) Venn diagram of JTK-cycle detected oscillators for D15 and D30 human ES cell-derived cardiomyocytes. (C) Fraction of JTK-cycle detected oscillators that was previously identified as oscillator in mouse hearts (Zhang et al., 2014). (D) Examples of overlapping oscillators between D15, D30 human ES cell-derived cardiomyocytes and mouse hearts. Log_2 RPM read counts smoothened over two time points ± s.e.m. were plotted (* denotes JTK $P < 0.05$, ** JTK $P < 0.005$, and *** JTK $P < 0.0005$).

To assess the possible presence of oscillatory transcripts at D15 and the identity of CCGs in D30 cardiomyocytes in which a functional clock was found (Figure 3), three independent RNA samples were collected every 4 hours over a period of 48 hours and sequenced using CEL-Seq (Supplementary Figure 3 and Supplemental Table 1). Around 10,000 genes had an average expression of more than 3 RPM in both D15 and D30 (Supplementary Figure 3) and were screened for oscillatory expression over 48 hours as determined by JTK-cycle (Hughes et al., 2010). This revealed 643 and 757 oscillating transcripts ($P < 0.05$) at D15 and D30, respectively (Figure 4A and Supplemental Table 2). The oscillatory transcripts of D15 could result from a starting clock as indicated by small circadian *Per2*-luciferase signals at this time point (Figure 3C), but are mostly distinct from the CCGs that were found at D30 (Figure 4B). As detecting oscillatory transcription of genes has been shown to rely strongly on sequencing depth (Li et al., 2015a), the fraction of overlap might be an underrepresentation. Indeed, in our data for both D15 as well as D30, oscillatory genes had more coverage on average than non-oscillatory transcripts (Supplementary Figure 4E), and shared oscillators between D15 and D30 (n=80) had higher expression levels than stage-specific oscillators (Supplementary Figure 4F). A fraction of the oscillators (D15 only, D30 only and shared) was found to overlap known mouse cardiac CCGs (Zhang et al., 2014) (Figure 4C) including genes (*COL4A1*, *SPON2, SLC23A2*, *AQP1* and *STC1*; Figure 4D) with a known important role in cardiomyocytes (Bray et al., 2008; Li et al., 2015b; Sheikh-Hamad et al., 2003; Yan et al., 2011). These data thus contain common rhythmically expressed clock-controlled genes between mouse hearts and human ES cell-derived cardiomyocytes.

STRING protein-protein analysis (Szklarczyk et al., 2015) on D30 human ES cell-derived cardiomyocyte oscillators with circadian expression in mouse hearts (n=135; Supplemental Table 2), revealed a significant putative interaction network (Figure 5A). D15 circadian transcripts that overlap mouse heart oscillators (n=98), however, did not show such interactions (Figure 5B). Gene ontology (GO) analysis for these oscillators showed enrichment for extracellular matrix formation terms at D15, while D30 human ES cell-derived cardiomyocyte oscillators were enriched for cardiac development and stress response terms (Figure 5A,B). Interestingly, the D30 interaction network was centered around *UBC* (ubiquitin C) (Figure 5A), one of the four genes encoding for ubiquitin in mammals and one of the most abundant proteins in eukaryotic cells (Radici et al., 2013). Although *Ubc* is expressed in multiple tissues in mice (http://biogps.org/) (Wu et al., 2009), it has only been shown to oscillate in the murine heart (Bray et al., 2008) and (skeletal) muscle (Zhang et al., 2014) (CircaDB JTK, $P = 3.32E-6$ and $P = 5.97E-7$ respectively; Figure 5C). This suggests that *Ubc* is a heart- and muscle-specific CCG in vivo, and concurs with our identification of *UBC* as a circadian CCG in in vitro D30 human ES cell-derived cardiomyocytes (JTK $P = 0.0032$; Figure 5D). Amongst the (UBC interacting) CCGs at D30, several genes were known oscillators in the murine heart according to the CircaDB database (http://circadb.hogeneschlab.org/) (Pizarro et al., 2013; Tsimakouridze et al., 2012; Zhang et al., 2014). Interestingly, many of the oscillators in D30 human ES cell-derived cardiomyocytes were involved in cardiac function (*PLN*) (MacLennan and Kranias, 2003), stress response (*BNIP3*,

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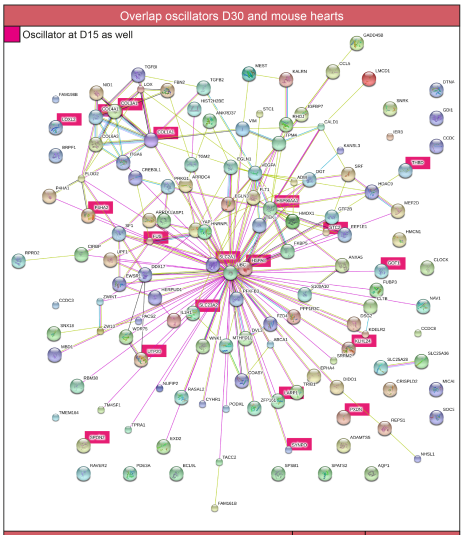
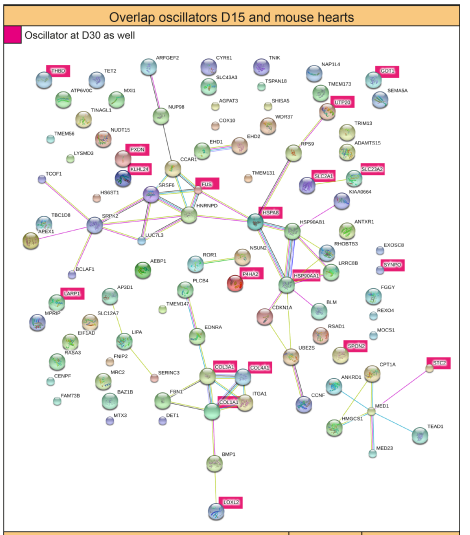


Pathway description - GO biological process	Gene count	FDR
extracellular matrix organization	16	4.60 x 10^{-06}
response to oxygen levels	15	4.60 x 10^{-06}
response to hypoxia	14	5.69 x 10^{-06}
regulation of cell communication	42	2.75 x 10^{-05}
cardiovascular system development	21	2.76 x 10^{-05}
circulatory system development	21	2.76 x 10^{-05}
blood vessel development	16	3.71 x 10^{-05}
peptidyl-proline hydroxylation to 4-hydroxy-L-proline	4	3.71 x 10^{-05}
vasculature development	16	5.89 x 10^{-05}
negative regulation of cellular process	49	9.41 x 10^{-05}

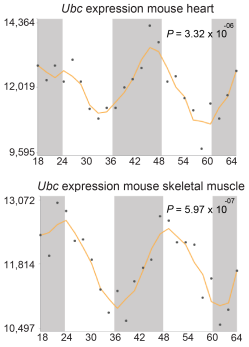
B



Pathway description - GO biological process	Gene count	FDR
regulation of protein import into nucleus, translocation	4	0.0119
extracellular matrix organization	10	0.0262
positive regulation of protein import into nucleus, translocation	3	0.0302

D15
D30

C



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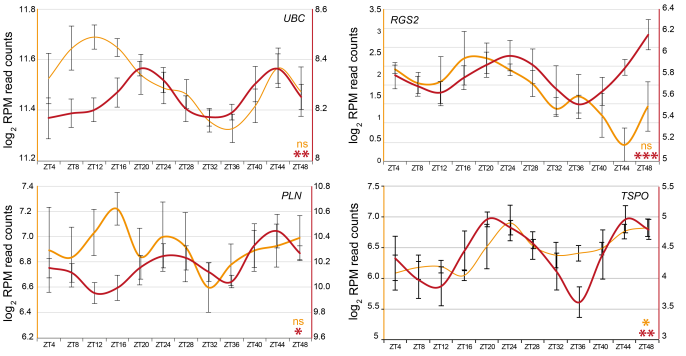

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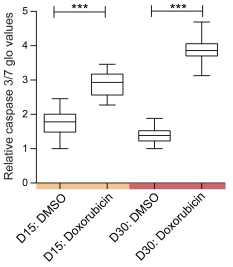

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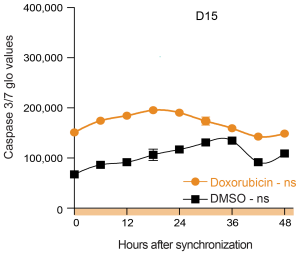

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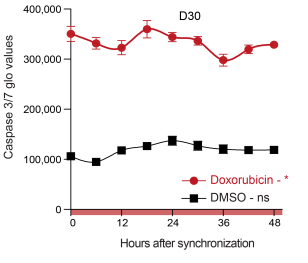

Figure 5. Circadian stress network results in time-dependent apoptotic response of human ES cell-derived cardiomyocytes. (A) STRING interaction network of overlapping oscillators between D30 human ES cell-derived cardiomyocytes and mouse hearts with corresponding GO-analysis. Genes that oscillate in both D15 and D30 human ES cell-derived cardiomyocytes are highlighted in pink. Mouse heart oscillators were deducted from Zhang et al. (Zhang et al., 2014). (B) Same analysis as in A) for D15 human ES cell-derived cardiomyocytes. (C) *Ubc* mRNA oscillation in mouse hearts and skeletal muscle. Data were obtained from CircaDB (http:// circadb.hogeneschlab.org/, deducted from (Miller et al., 2007; Zhang et al., 2014)). Corresponding JTK-cycle calculated p-values are depicted. (D) Expression level of four D30 oscillatory transcripts (*UBC*, *RGS2*, *PLN* and *TSPO*) across 48 hours. Log_2 RPM CEL-Seq counts were plotted for D15 and D30 and smoothened over two time points ± s.e.m. (* denotes JTK $P < 0.05$, ** JTK $P < 0.005$, and ** JTK $P < 0.0005$). (E) Apoptosis, as measured by caspase 3/7 activity, after doxorubicin administration in D15 and D30 human ES cell-derived cardiomyocytes. The effect of doxorubicin versus DMSO was tested using a Mann-Whitney U test (*** denotes $P < 0.0001$). (F) Apoptosis measured with 6-hour intervals in synchronized D15 human ES cell-derived cardiomyocytes after administration of doxorubicin (orange) and DMSO as control (black) for 6 hours. (G) Same as in F) for D30 human ES cell-derived cardiomyocytes with the doxorubicin response depicted in red. Data are represented as the mean ± s.e.m. of three independent replicates. Significance of rhythmicity across 48 hours was analyzed using the RAIN algorithm and is indicated (ns: not significant and * $P < 0.05$).

RRAGA, *DNAJA1* and *HSPH1*) (Chen et al., 2012), hypertrophy (*RGS2*) (Nunn et al., 2010) and the set of rhythmically expressed transcripts even contained therapeutic targets such as *TSPO* (Translocator protein) (Motloch et al., 2015) (Figure 5D). This indicates that the oscillators that were identified here possibly contribute to multiple molecular mechanisms with a circadian clock dependency, but could also suggest a role of circadian processes in pathophysiologies such as ischemic damage after myocardial infarction.

3.2.5 Human ES cell-derived cardiomyocytes show rhythmicity in doxorubicin-induced apoptosis

Mouse hearts show circadian rhythmicity in their tolerance to ischemia and reperfusion after myocardial infarction (Durgan et al., 2010). In humans, a similar time of the day pattern in the onset and severity of myocardial infarction has been described (Muller et al., 1985; 1989; Peckova et al., 1998; Reiter et al., 2012; Seneviratna et al., 2015; Suarez-Barrientos et al., 2011). The combination of time-dependent pathophysiology and the enrichment of oscillating stress-associated genes (around *UBC*) in our CEL-Seq datasets, prompted us to assess whether in vitro human ES cell-derived cardiomyocytes would show a functional circadian reaction to induced stress. The anthracycline doxorubicin is a widely used anti-cancer drug that is often administered in the clinic, but is also known to have severe cardiotoxic side effects (Benjamin et al., 1988; Lefrak et al., 1973). These effects are recapitulated in in vitro human ES cell-derived cardiomyocytes in which doxorubicin is known to induce apoptosis and has proven to be a good model for induced cardiotoxicity (Burridge et al., 2016; Maillet et al., 2016). To determine whether the sensitivity of human ES cell-derived cardiomyocytes to doxorubicin-induced apoptosis displays an oscillatory response, we synchronized D15 and D30 cardiomyocytes and administered doxorubicin (10 μM) every 6 hours over a time frame of 48 hours (see Materials and Methods). A marked induction of apoptosis, as indicated by elevated active caspase 3/7 levels over control DMSO-treated samples, was found at both stages (Mann-Whitney U test, $P < 0.0001$ for D15 and D30; Figure

5E), but D30 human ES cell-derived cardiomyocytes reacted more strongly to doxorubicin (P <0.05; Figure 5E). In addition, the strength of response to doxorubicin showed a significant circadian pattern at D30, but not D15 human ES cell-derived cardiomyocytes (RAIN, P <0.037 and P <0.85 for D30 and D15, respectively; Figure 5 F,G), which reveals the functional consequences of a circadian clock in cardiomyocytes. Therefore, these results highlight the potential of reducing cardiotoxicity in cancer therapy but also indicate that taking diurnal rhythmicity into account could possibly improve other treatment strategies.

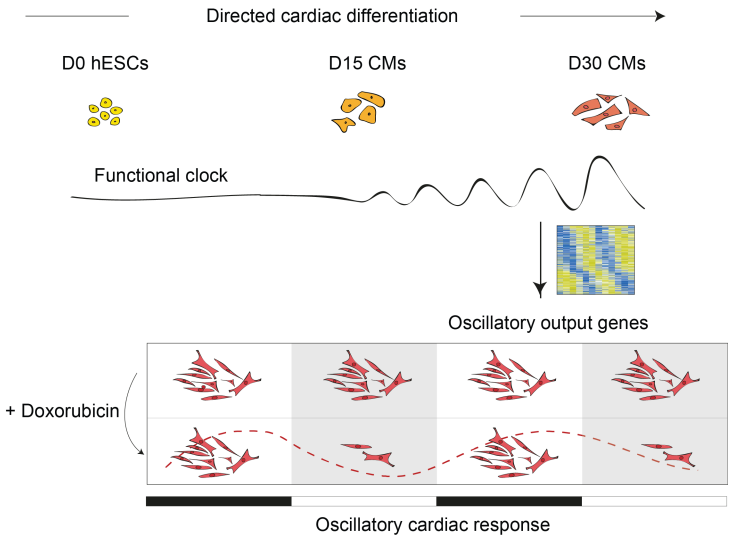

Figure 6. Schematic representation of the emergence and consequences of circadian rhythmicity during directed cardiac differentiation of human ES cells. hESCs: human ES cells. CMs: cardiomyocytes.

3.3 Discussion

Circadian rhythmicity is crucial to heart function, but also influences pathophysiology as indicated by, for instance, diurnal rhythmicity of cardiac damage after infarction (Durgan et al., 2010; Muller et al., 1985; 1989; Peckova et al., 1998; Reiter et al., 2012; Seneviratna et al., 2015; Suarez-Barrientos et al., 2011). As human ES cell-derived cardiomyocytes are emerging as a powerful tool to model developmental and disease processes as well as being a potential cellular source for regenerative therapies, we examined the presence and possible implications of a functional clock in human ES cells and their cardiac derivatives. While human ES cells do express core clock genes, no circadian clock was observed. Upon differentiation towards cardiomyocytes however, a functional core clock pathway was gradually established (Figure 6) as determined by robust anti-phasic oscillations of *BMAL1* and *PER2*. This work is the first demonstration of a functional clock in human ES cell-derived (cardiac) cells and may serve as a paradigm for the emergence of diurnal rhythms in other

pluripotent stem cell-derived cell types. At D30, 757 CCGs were identified, 18% of which are known to oscillate in the murine heart. Importantly, our data uncover additional transcripts with specific oscillatory behavior in human ES cell-derived cardiomyocytes. As some of these newly identified oscillators are known to play an important role in human heart physiology (*PLN*, *KCNE4*, *TSPO*, *CAV1*, *RGS2*), this stresses the importance of using human cells for modeling cardiovascular processes and disease.

Importantly, a defined set of the oscillators could clearly be linked to stress response, which was confirmed by a time-dependent response to doxorubicin administration. This highlights the possible beneficial effects of drug administration at a specific time of the day to decrease cardiotoxic side effects. Notably, next to explicit synchronization steps, simple medium changes can reset the internal clock of cell cultures (Guenthner et al., 2014) and can influence experimental outcome. Therefore, our data not only stress the importance of testing compounds in a time-controlled manner when using in vitro cultured cardiomyocytes, but may also extent to other ES cell-based disease models.

3.4 Materials and methods

3.4.1 ESC culture and cardiomyocyte differentiation

Nkx2.5-eGFP human ES cells (Elliott et al., 2011) (stable reporter line generated from wild type HES3 cells (Reubinoff et al., 2000)) were cultured in Essential 8™ medium (Gibco) on matrigel (BD, Corning) without Penicillin/Streptomycin. Cells were differentiated in a monolayer towards cardiomyocytes as previously described (van den Berg et al., 2015). In short, human ES cells were cultured in E8 until 60% confluent. Cells were then supplemented with 1% DMSO enriched E8 medium for 24 hours. On D0, cells were put on BPEL medium supplemented with Activin A (20 ng/ml, R&D Systems), BMP4 (20 ng/ml, R&D Systems) and Chir99201 (1.5 μM, Axon Medchem). At D3 medium was changed to BPEL with XAV939 (5 μM, Tocris). On D6 BPEL without any supplements was used. BPEL medium: IMDM, no PhenolRed (Gibco) and F12 Ham's F12 nutrient Mix (Gibco) in a 1:1 ratio supplemented with 5% (v/v) PFHM-II (Gibco), 0.25% (w/v) BSA, 1% (v/v) Chemically Defined Lipid Concentrate (Gibco), 0.1% ITS-X (Gibco), 450 μM α-MTG (Sigma), 2 mM GlutaMax, 50 μg/mL L-Ascrobic acid 2-phosphate (Sigma) and 0.25% Penicillin/Streptomycin (10.000 U/ml, Gibco).

3.4.2 Generation of Reporter Cell Lines

Lentiviral plasmids harboring luciferase reporters of the Per2, Bmal1, and Cry1 promoters were described previously and kindly provided by Prof. Dr. Liu (Liu et al., 2007; 2008; Ramanathan et al., 2012). Viral particles were concentrated via ultracentrifugation after 3 harvests in HEK293T cells. Human ES cells were then transduced with concentrated Bmal1-dLuc, Per2-dLuc or Cry1-dLuc lentivirus and selected with 2 μg/ml blasticidin (Invivogen) for at least 6 days. Stable polyclonal lines were propagated.

3.4.3 Bioluminescent recording and data analysis

Human ES cell-derived cardiomyocytes were differentiated for up to 30 days and transduced with Lentiviral reporters at described time points after synchronization with 100 nM dexamethasone (Balsalobre et al., 2000) for 2 hours. Subsequently, medium was changed to recording medium (BPEL, 15 mM HEPES, 100 μM D-Luciferin Potassium Salt (Promega)). Culture dishes were sealed with high vacuum grease (Dow Corning) and monitored via the use of a LumiCycle32 device (Actimetrics) at 37°C. Bioluminescence from each dish was continuously recorded (integrated signal of 70 seconds with intervals of 10 minutes). Raw data (counts/seconds) were baseline subtracted (polynomial order 3).

3.4.4 Microscopic real-time bioluminescence analysis

Stable reporter human ES cell lines were differentiated for up to 30 days and bioluminescence was recorded with an LV200 microscope (Olympus) in a humidified chamber under 5% CO_2, at 37°C. Bioluminescence was detected for multiple consecutive days, using an EM CCD camera (Hamamatsu), with exposure times of 2 hours. Image series were analyzed in ImageJ. Cells were synchronized with 100 nM dexamethasone for 2 hours and changed to normal BPEL medium, containing 100 μM D-Luciferin Potassium Salt (Promega). For pure cardiomyocyte population experiments, D26 and D34 human ES cell-derived cardiomyocyte populations were sorted with a FACS ARIA flow cytometer (BD Biosciences) based on GFP positivity.

3.4.5 Immunostaining

Cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes, blocked for 1 hour in blocking buffer (5% FBS, 0.25% Triton-X100 in PBS) and stained for OCT4 (SantaCruz, #5279), NANOG (Cell Signaling, #3580S), TNNT2 (ThermoFisher Scientific, #MA5-12960), ACTN2 (Sigma, #A7811), MEF2C (Cell Signaling, #sc-13266), GFP (Abcam, #ab6556) in staining buffer (1% BSA, 0.25% Triton-X100 in PBS). Nuclei were stained with Hoechst for 15 minutes. Pictures were taken with a spinning disk microscope (PerkinElmer).

3.4.6 RNA isolation and CEL-Seq

Human ES cell-derived cardiomyocytes were differentiated in 48-well plates. Biological triplicates (independent wells) were collected every 4 hours over the course of 48 hours (ZT4-ZT48) (Supplementary Figure 3). RNA was extracted using the standard TRIzol (Invitrogen) protocol and 10 ng of total RNA per sample was used for library preparation and sequencing. RNA was processed as described previously (Hashimshony et al., 2012; Simmini et al., 2014) and paired-end sequencing was performed on the Illumina Nextseq platform with a read length of 75 base pairs. Read 1 was used to identify the sample barcode and library index, while read 2 was aligned to the hg19 human RefSeq transcriptome (downloaded from the

UCSC genome browser) using BWA (Li and Durbin, 2010). CEL-Seq only sequences the most 3'end of a transcript, generating one read per transcript. Reads that mapped equally well to multiple locations were discarded. Around 500,000 reads per million (RPM) were sequenced per sample. Samples were reads per million (RPM) normalized.

3.4.7 Quantitative RT-PCR

Purified RNA was treated with DNAse (Promega) and reversibly transcribed with Superscript III reverse transcriptase (ThermoFisher Scientific). qRT-PCR on biological triplicate samples was carried out in triplicate (technical replicates) in CFX-384 Touch™ Real-time PCR detection system (Biorad). *PPIA* was used as housekeeping gene and fold changes were calculated to the lowest values among all replicates. Primer sequences: *PPIA* (fw): ttctgctgtctttgggacct, *PPIA* (rv): caccgtgttcttcgacattg, *NANOG* (fw): cagccctgattcttc, *NANOG (rv):* tgcatctgctggaggctgag, *POU5F1* (fw): ctgaagcagaagaggat, *POU5F1* (rv): gggccgcagcttacacat, *ISL1* (fw): ctgcttttcagcaactggtca, *ISL1* (rv): ggactggctaccatgctgtt, *NKX2.5* (fw): caagtgtgcgtctgcctttc, *NKX2.5* (rv): ctttcttttcggctctagggtcct, *ACTC1* (fw): atgccatcatgcgtctggat, *ACTC1 (rv):* acgttcagcagtggtgacaa, *KCNJ2* (fw): tgggtcttgggaattctggttt, *KCNJ2* (rv): gaacatgtcctgttgctggcg, *SERCA2A* (fw): cgaacccttgccactcatct, *SERCA2A (rv):* ccagtattgcaggttccaggt, *CASQ2* (fw): acgactttcctctgctcgtt, *CASQ2* (rv): tgtgacattcaccaccccaa, *BMAL1* (fw): ggctcatagatgcaaaaactgg, *BMAL1* (rev): ctccagaacataatcgagatgg, *PER2* (fw): ggccatccacaaaaagatcctgc, *PER2* (rv): gaaaccgaatgggagaatagtcg, *CRY1* (fw): ctccatgggcactggtctcagtg, *CRY1* (rv): tccccaccaatttcagctgcaac, *CRY2* (fw): ccaagagggaagggcagggtagag, *CRY2* (rv): aggatttgaggcactgttccgagg, *CLOCK* (fw): aagttagggctgaaagacgacg, *CLOCK* (rv): gaactccgagaagaggcagaag, *NR1D1* (fw): acagctgacaccacccagatc, *NR1D1* (rv): catgggcataggtgaagatttct.

3.4.8 Western blotting

Cells were lysed in RIPA buffer and protein concentration was measured using a BCA assay (ThermoFisher Scientific). 12.5ug protein lysate was loaded, separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk powder (Nestlé) in T-BST and probed with anti-BMAL1 (1:1000, #ab3350, Abcam), anti-CRY1 (1:1000, #13474-1-A, Proteintech), anti-CLOCK, #PA1-520, ThermoFisher Scientific) antibodies, followed by a peroxidase-conjugated antibody (1:5000, #sc-2004, Santa Cruz). ECL Plus Western blotting substrate (#32132, ThermoFisher Scientific) was used for chemiluminescence detection with an ImageQuant™ LAS 4000 imager (GE Healthcare). HRP-coupled anti-β-ACTIN (1:5000, #5125S, Cell Signaling) was used as a loading control. Band intensities were calculated with ImageJ.

3.4.9 Apoptosis measurements

Human ES cells were differentiated in 96-well white walled plates for the course of 30 days. Cardiomyocytes were synchronized with 100 nM dexamethasone for 2 hours and 10 μM doxorubicin HCl (Sigma D1515) was put on the cells at 6-hour intervals for a total time of 6 hours. Apoptosis levels of 3 replicate wells (per condition), represented by active caspase 3 and 7, using a CaspaseGlo kit (Promega) were measured following manifacturer's instructions. Bioluminescence was read out with a Centro microplate luminometer (Berthold Technologies).

3.4.10 JTK-cycle analysis

RPM normalized read counts were obtained for each sample. As lowly expressed genes are typically not picked up robustly using CEL-Seq, genes with an average of >3 RPM over all time points and replicates were selected for JTK-cycle analysis. Around 10,000 genes reached this threshold in both D15 and D30, and these represent the list on which JTK-cycle was run (Supplemental Table 2). The following settings were used: jtkdist (12,3), periods (6:6), jtk. init (periods, 4). Significant oscillators with an adjusted *P*-value of < 0.05 were selected for further analyses. To identify mouse heart oscillators, JTK was run with similar settings on normalized GC-RMA intensity values of 24 samples (CT18-CT62, sampled every 2 hours) for 35,556 genes downloaded from the GEO-database (GSE54652) (Zhang et al., 2014).

3.4.11 STRING and Gene Ontology Analysis

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (www.string-db.org) was used to investigate the relationship between the overlap of known murine heart oscillators and identified D15 and D30 human ES cell-derived cardiomyocytes oscillators (Szklarczyk et al., 2015). Gene ontology terms were retrieved via www.string-db.org.

3.4.12 CircaDB gene expression website

The circadian expression database (CircaDB, http:// circadb.hogeneschlab.org/) is an open access online platform (Pizarro et al., 2013) compiling circadian gene expression profiles from microarrays and RNA sequencing experiments (Andrews et al., 2010; Hoogerwerf et al., 2008; Hughes et al., 2009; Keller et al., 2009; Miller et al., 2007; Panda et al., 2002; Rudic et al., 2004; 2005; Tsimakouridze et al., 2012; Zhang et al., 2014). The embedded JTK-cycle algorithm plots expression levels and calculates period, phase, amplitude and p-value.

3.4.13 Statistics

All data were shown as means ± s.e.m. One-tailed T-Tests were carried out to assess differences between qRT-PCR mean values within the same experiments. A difference of *P* <

0.05 was considered significant. To calculate general induction of apoptosis upon doxorubicin a Mann-Whitney U test was used. Differences in doxorubicin-effect sizes between D15 and D30 CMs were assessed via non-overlapping 95% effect interval sizes. Statistical analyses to detect circadian oscillations in RNA (qRT-PCR) and doxorubicin-induced apoptosis levels were performed by RAIN (Thaben and Westermark, 2014).

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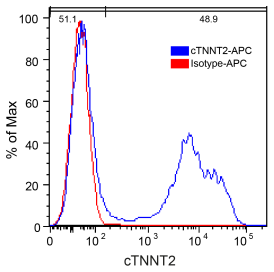
3.6 Supplementary Figures

A



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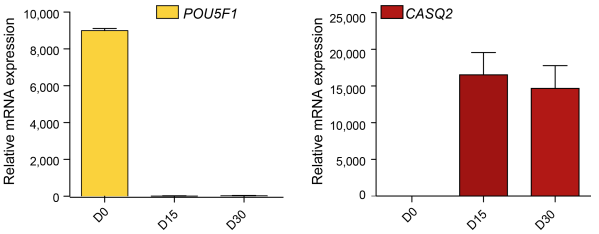
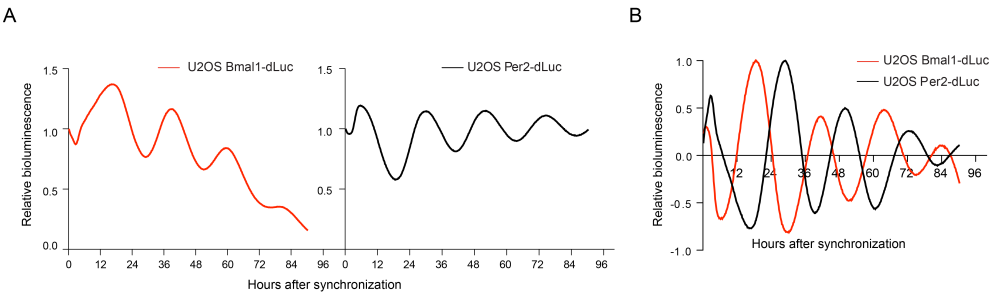
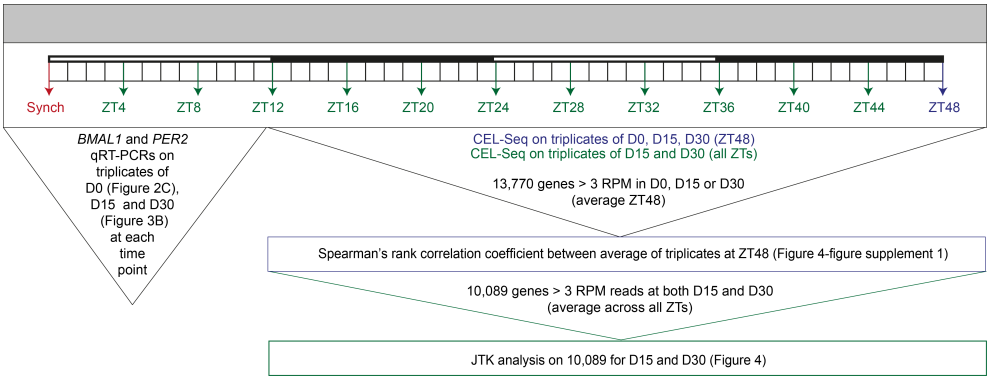


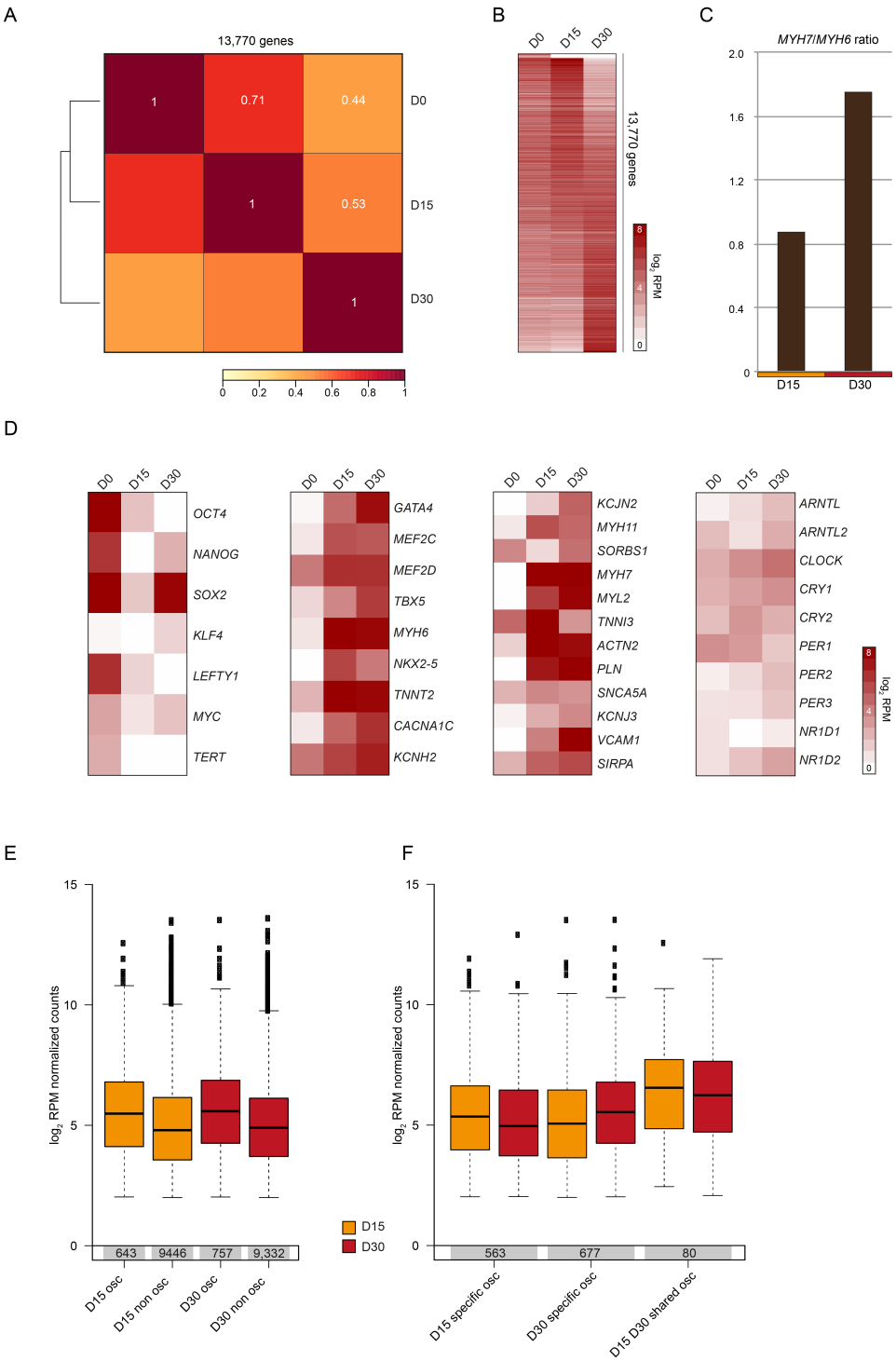
Supplementary Figure 1. Cardiomyocyte quantification and characterization by FACS and qRT-PCR. (A) FACs staining for cTNNT2 in D15 human ES cell-derived cardiomyocytes. IgG_1 isotype was used as a control. (B) *POU5F1* and *CASQ2* mRNA expression levels in human ES cells and human ES cell-derived cardiomyocytes measured by qRT-PCR. Expression levels were normalized to a non-oscillatory housekeeping gene (*PPIA*).



Supplementary Figure 2. Circadian oscillation of Bmal1- and Per2-dLuc bioluminescence in U2OS cells. (A) Raw lentiviral promoter-based luciferase reporter bioluminescence in U2OS cells after dexamethasone synchronization. Bioluminescence was measured with a LumiCycle32. (B) Detrended bioluminescent signals measured in (A).



Supplementary Figure 3. Setup of CEL-Seq experiment. Schematic of RNA samples that were processed for qRT-PCR and/or CEL-Seq. ZT: Zeitgeber and RPM: reads per million.


A
13,770 genes
1
0.71
0.44
1
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D0
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0.6
0.8
1
B
D0
D15
D30
13,770 genes
log2 RPM
8
4
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C
MYH7/MYH6 ratio
2.0
1.6
1.2
0.8
0.4
0
D15
D30
D
D0
D15
D30
OCT4
NANOG
SOX2
KLF4
LEFTY1
MYC
TERT
GATA4
MEF2C
MEF2D
TBX5
MYH6
NKX2-5
TNNT2
CACNA1C
KCNH2
KCJN2
MYH11
SORBS1
MYH7
MYL2
TNNI3
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PLN
SNCA5A
KCNJ3
VCAM1
SIRPA
ARNTL
ARNTL2
CLOCK
CRY1
CRY2
PER1
PER2
PER3
NR1D1
NR1D2
log2 RPM
8
4
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E
log2 RPM normalized counts
15
10
5
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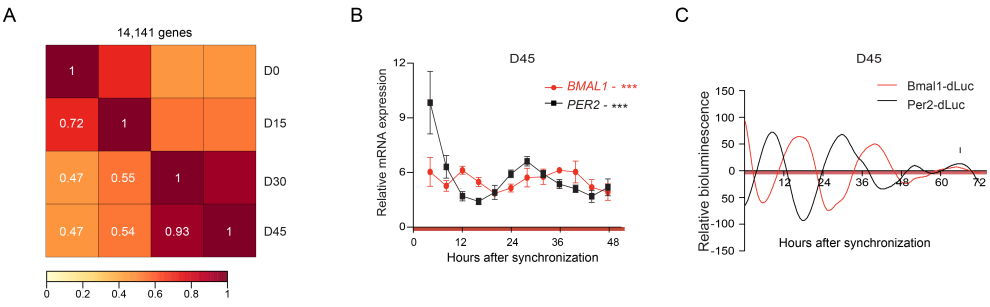
643
9446
757
9,332
D15
D30
D15 osc
D15 non osc
D30 osc
D30 non osc
F
log2 RPM normalized counts
15
10
5
0
563
677
80
D15 specific osc
D30 specific osc
D15 D30 shared osc

Supplementary Figure 4. CEL-Seq based characterization and validation of transcriptional profiles of human ES cells and human ES cell-derived cardiomyocytes. (A) Spearman correlation with average linkage based on average RPM normalized CEL-Seq read counts of three ZT48 replicates. Genes (n=13,770) with an average ZT48 expression level of more than 3 RPM (reads per million) for at least one stage (D0, D15 or D30) were used for analysis. Spearman's rank correlation coefficients are indicated in white. (B) Heatmap of average ZT48 CEL-Seq read counts for 13,770 genes used in (A). Genes were sorted according to human ES cell expression from high (top) to low (bottom). Log2 RPM values were plotted. (C) MYH7/MYH6 CEL-Seq read count ratio in D15 and D30 human ES cell-derived cardiomyocytes (average of three ZT48 replicates) as a measure of maturation. (D) Heatmaps of average ZT48 CEL-Seq read counts in human ES cells (D0) and human ES cell-derived cardiomyocytes (D15 and D30). The left heatmap shows pluripotency markers, the two middle heatmaps depict cardiomyocyte and cardiomyocyte maturation markers, and the right panel comprises core circadian clock genes. Log2 RPM values were plotted. (E) Boxplots depict average log2 RPM normalized CEL-Seq counts across all time points for D15 and D30 human ES cell-derived cardiomyocyte oscillating and non-oscillating genes. 10,089 genes with average RPM values above 3 across all time points (ZT4-ZT48) on which JTK analysis was done in both D15 and D30 were used. (F) Boxplots depict average log2 RPM normalized CEL-Seq counts across all time points (ZT4-ZT48) for D15-specific, D30-specific and shared human ES cell-derived cardiomyocyte oscillators.



Supplementary Figure 5. D45 human ES cell-derived cardiomyocytes are transcriptionally highly similar to D30 cardiomyocytes and possess a functional core clock. (A) qRT-PCR analysis of *BMAL1* and *PER2* mRNA expression over 48 hours at a 4-hour interval in human ES cell-derived cardiomyocytes at D45. Expression levels were normalized to *PPIA*. Data are represented as mean ± s.e.m. of three independent replicates. Significance of rhythmicity across 48 hours was analyzed using the RAIN algorithm and the significance is indicated (ns: not significant, *** $P < 0.0005$). (B) Relative detrended Bmal1-dLuc and Per2-dLuc luciferase signal in synchronized D45 human ES cell-derived cardiomyocytes. Representative tracks were shown. (C) Spearman correlation with average linkage based on average RPM normalized CEL-Seq read counts of three ZT48 replicates. Genes (n=14,141) with an average ZT48 mRNA expression level of more than 3 RPM (reads per million) at either one stage (D0, D15, D30 or D45) were used for analysis. Spearman's rank correlation coefficients are indicated in white.

4

Neonatal rat cardiomyocytes as an in vitro model for circadian rhythms in the heart

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Abstract

Circadian rhythms are biorhythms with a 24-hour period that are regulated by molecular clocks. Several clinical and animal models have been developed to analyze the role of these rhythms in cardiovascular physiology and disease, but a convenient in vitro model that replicates both molecular and functional circadian effects of the heart is not available. Therefore, we established a neonatal rat cardiomyocyte model that recapitulates in vivo circadian rhythmicity and that shows functional dependence on the clock as indicated by an oscillating response to induced apoptosis. In addition, perturbation of the cardiac clock by the use of several compounds was found to result in loss of functional rhythmicity. This indicates that neonatal rat cardiomyocytes are a good model to investigate the cardiac clock as well as a system that allows for fast and easy preclinical testing of the influence of compounds on circadian rhythmicity.

4.1 Introduction

Circadian rhythms allow the body to anticipate diurnal environmental changes (Dierickx et al., 2015; Pré et al., 2014) and in humans, these rhythms are regulated by multiple clocks. One central master clock is located in the suprachiasmatic nucleus of the brain, and distinct peripheral clocks are present in almost every organ and/or cell. The clock pathway relies on the oscillatory expression of core clock genes such as *CLOCK*, *ARNTL, PER* and *CRY* (Takahashi, 2015), which results in the rhythmic expression of clock-controlled output genes (CCGs). CCGs vary per organ and have important functional implications. For instance, circadian rhythms in the heart influence various cardiovascular features such as metabolism, electrophysiological characteristics, (cardiac) hormone receptor functionality and coagulation (Durgan and Young, 2010; Jeyaraj et al., 2012; Scheer et al., 2011; Witte et al., 1995). In addition, the clock plays an important role in a number of cardiovascular disorders. The incidence of several diseases, such as myocardial infarction and sudden cardiac death, follows a diurnal pattern (Muller et al., 1989; 1985; Peckova et al., 1998; Portaluppi and Hermida, 2007), and disruption of rhythmicity by genetic defects, genetic manipulation or sleep disturbance, is involved in cardiac pathophysiology (Martino and Young, 2015; Vyas et al., 2012; Woon et al., 2007).

As the role of the circadian clock in proper organ functioning is becoming increasingly clear, many studies have investigated circadian rhythmicity in the heart (Beesley et al., 2016; Martino and Young, 2015; Young, 2016). The interest for the use of circadian rhythms in (pharmacological) treatment is rising (Hermida et al., 2011; Ohdo, 2010; Ortiz-Tudela et al., 2013) and at preclinical level, several animal models are used to uncover the contribution of circadian rhythmicity to (cardiac) physiology or disease (Bray et al., 2008; Durgan et al., 2011; Martino and Young, 2015). An easy to use *in vitro* system to analyze cardiac circadian rhythmicity on a transcriptional, protein and functional level, however, is currently lacking. A handful of experimental studies used adult cardiomyocytes to mimic circadian

rhythmicity in the heart (Peliciari-Garcia et al., 2011), but isolation and culture of ventricular cardiomyocytes is difficult and time-consuming. In the current study, we propose neonatal rat cardiomyocytes as an easy *in vitro* system to study and analyze molecular and functional circadian rhythmicity in the heart and proof that it can serve as good model for clock interference compound testing.

4.2 Results

4.2.1 Neonatal rat cardiomyocytes show a functional molecular clock

Previously described results demonstrate that the rodent heart has a functional molecular circadian clock that drives rhythmic cardiac physiology. Interestingly, *in vitro* cultured rodent ventricular cardiomyocytes have shown to retain their rhythms even without systemic input (Peliciari-Garcia et al., 2011). While these studies have provided important insights, it takes time to breed animals to adulthood, and derivation and culture of these cells is time-consuming. To this end, we investigated the use of neonatal rat cardiomyocytes (nrCMs), which are easy to derive and allow for the recovery of high cell numbers. To investigate the presence of a circadian clock in nrCMS, we synchronized nrCMs via serum shock (Balsalobre et al., 1998) and mRNA was sampled every 6 hours for 42 hours, starting 9 hours after synchronization. *BMAL1* expression, as measured by qRT-PCR, was observed to significantly oscillate in a diurnal manner (RAIN, $P < 0.05$; Figure 1A). 24 hour period significance was assessed by the nonparametric algorithm RAIN (Thaben and Westermark, 2014). To further validate the presence of a functional clock, anti-phasic oscillatory expression of *Bmal1* and *Per2*, a hallmark of a functional circadian clock, was investigated by the use of lentiviral promoter based *Bmal1*-and *Per2*-dLuc (destabilized luciferase) reporter constructs. nrCMs were transduced with both constructs, synchronized and bioluminescence was measured using a LumiCycle32. Anti-phasic bioluminescent oscillations for *Bmal1* and *Per2* were detected over the course of 4.5 days (Figure 1B). In addition to mRNA oscillations, western blot analysis revealed rhythmic protein presence in nrCMs (RAIN, $P < 0.0001$; Figure 1C) with peaks that followed gene expression with a delay of approximately 6 hours (Figure 1A). From these data, we conclude that neonatal rat cardiomyocytes contain a functional molecular circadian clock at the mRNA as well as protein level.

4.2.2 Neonatal rat cardiomyocytes show 24-hour rhythmicity in beating frequency and induced damage response

A functional circadian clock drives rhythmic output of clock-controlled genes, which allows for tissue-specific oscillations. To test functional rhythmicity in nrCMs, we measured beating frequency in a temporal manner, as this known property of the heart is proven to be controlled by the cardiac clock and external neurohumoral influences (Yaniv and G Lakatta, 2015). After serum shock based synchronization, circadian rhythmicity was observed when counting the number of beats per minute over the course of two days (RAIN, $P < 0.01$; Figure

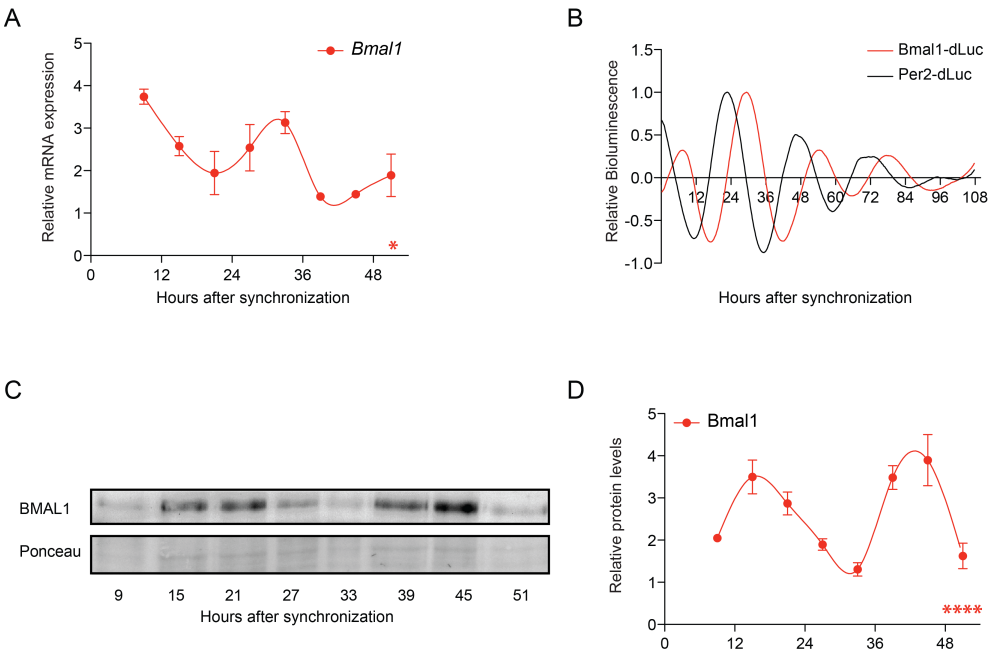

Figure 1. Neonatal rat cardiomyocytes possess a functional circadian clock. (A) Bmal1 mRNA expression levels in synchronized neonatal rat cardiomyocytes (nrCMs) as determined by qRT-PCR. Lowest value was set to 1. Expression levels were normalized to the non-oscillatory housekeeping gene PPIA. Significance of rhythmicity across 48 was analyzed using the RAIN algorithm and is indicated (* $P < 0.05$). (B) Bmal1-Luc and Per2-Luc bioluminescence in synchronized nrCMs across 108 hours. Data was detrended and normalized to the highest value. Measurements were done using a LumiCyle32. Representative tracks are shown. (C) BMAL1 protein expression as determined through western blot across 48 hours. (D) Relative levels protein levels that were deducted from western blots (n=3). Lowest value was set to 1. Ponceau staining was used as a loading control. Significance of rhythmicity across 48 was analyzed using the RAIN algorithm and is indicated (**** $P < 0.0005$).

2A). In contrast, cells that only underwent a medium change 24 hours before recording did not show this (RAIN, $P = 0.99$; Figure 2A). These results highlight the functional consequences of a circadian clock in nrCMs.

Both for the murine heart and for human embryonic stem (ES) cell-derived cardiomyocytes, *UBC* was shown to oscillate in a diurnal manner (Zhang et al., 2014). In human ES cell-derived cardiomyocytes, this was shown to result in a strongly connected oscillatory network of stress-associated transcripts. Interestingly, this circadian transcriptional program controlled a time-dependent differential response of differentiated cardiomyocytes to doxorubicin, an anti-cancer drug with known cardiotoxic side effects. To test whether nrCMs showed a comparable stress-response behavior, we investigated whether synchronized nrCMs respond to doxorubicin-induced apoptosis in a time-dependent manner. General apoptosis levels, as measured by a Caspase-Glo 3/7 assay, after serum shock were not higher compared to cells that did not receive a serum shock (two-tailed Student T-Test, $P = 0.95$; Figure 2B), which

indicates that the synchronization step itself does not induce cell death. We then compared active Caspase 3/7 levels between synchronized and non-synchronized nrCMs and observed that, when normalized to background values (Methods), a significant circadian doxorubicin-induced response was found in synchronized nrCMs (RAIN, $P < 1E-07$; Figure 2C) but not in non-synchronized cultures (RAIN, $P = 0.58$; Supplementary Figure 1B). Additional TUNEL staining on doxorubicin treated nrCMs showed a similar oscillating output (RAIN $P < 0.01$ and $P < 0.0001$ for non-treated and doxorubicin-treated samples; Supplementary Figure 1C), which confirms that there is a time-dependent vulnerability of nrCMs to doxorubicin.

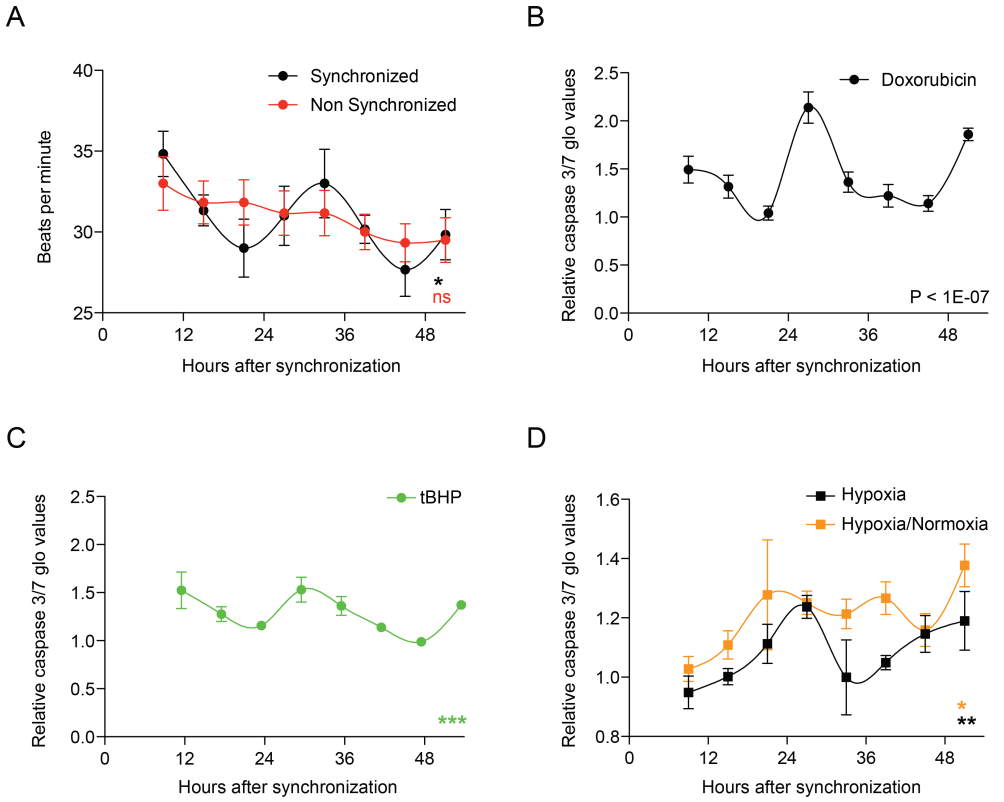

Figure 2. Neonatal rat cardiomyocytes show functional oscillations. (A) Counts of spontaneous contraction in nrCMs, measured as beats per minute and monitored across 48 hrs (n=6). (B) Relative apoptotic levels in doxorubicin treated synchronized nrCMs measured by Caspase-Glo 3/7 values across 48 hours. Values are normalized to non-treated cardiomyocytes. (C) Similar analysis as in b) for tBHP. (D) Similar analysis as B) for hypoxia or hypoxia followed by normoxia treatment across 48 hours. Significance of rhythmicity in (A-D) across 48 was analyzed using the RAIN algorithm and is indicated (ns: not significant, * P < 0.05, ** P < 0.005, *** P < 0.0005).

To test whether similar patterns could be observed for other stress or apoptosis-inducing agent, we analyzed whether tert-butyl hydroperoxide (tBHP) would lead to equal results. Indeed, a circadian apoptotic response pattern was observed for tBHP as well (RAIN, $P < 0.0001$; Figure 2C). Next, we investigated the in vitro equivalent of ischemia and ischemia/reperfusion, which is known to follow a diurnal pattern in in vivo hearts (Durgan et al., 2010). After synchronization, we induced ischemia via hypoxic conditions (1% O_2) during 3 hours at a 6-hour interval. To mimic ischemia/reperfusion, 3-hour hypoxia conditions were followed by 2 hours of normoxia (5% CO_2 and 20% O_2). The apoptotic response of nrCMs after hypoxia treatment followed a significant 24-hour pattern (RAIN $P < 0.001$; Figure 2D). However, a less significant response was noted in the hypoxia/normoxia situation (RAIN $P = 0.043$; Figure 2D). This indicates that, like in vivo hearts, nrCMs respond to ischemia in a circadian manner, which further underscores the relevance of using nrCMs when aiming to assess the role of rhythmicity in response to stressors.

4.2.3 Neonatal rat cardiomyocytes function as a good model for molecular cardiac clock interference in vitro

Many widely used compounds affect the circadian clock. Nonetheless, researchers are often not aware of this, and therefore do not take into account possible consequences. Nevertheless, perturbed cardiac clocks have shown to influence cardiovascular physiology and have a profound effect on heart pathophysiology. To this end, we investigated whether nrCMs can be used to model the consequences of cardiac clock interference in vitro and analyzed the effect of resveratrol, a compound known for its beneficial effects against cardiopathologies such as atherosclerosis, hypertension and ischemia/reperfusion (Petrovski et al., 2011), on Per2-dLuc bioluminescence rhythmicity. A dose-dependent decrease of amplitude was observed (Figure 3A), which indicates dampening of the clock upon compound administration. To analyze whether other compounds would have comparable consequences, Ex-527 was administered. This compound is known to have a cardioprotective role during stress in cardiomyocytes (Gertz et al., 2013), but also inhibits the sirtuin SIRT1 (Zheng et al., 2013) that has an essential role in linking metabolism to the core clock system. Indeed, similar to resveratrol, a dose-dependent dampening of oscillations was observed after Ex-527 addition to nrCMs (Figure 3B). Low concentrations of both compounds (resveratrol and Ex-527) had moderate effects, but importantly, high dosage significantly decreased the amplitude of Per2-dLuc levels (Student's T-Test: resveratrol, $P < 0.05$, Ex-527, $P < 0.005$; Figure 3C). Additionally, both compounds were found to result in period lengthening at high dose (Student's T-Test $P < 0.05$ for resveratrol, and $P < 0.05$ for Ex-527 respectively). Dampened circadian amplitudes have been correlated with premature aging (Chang and Guarente, 2013) and clock disturbance has been observed in numerous pathologies (hypertension, diabetes, sleep disturbance and cancer (Dierickx et al., 2015; Woon et al., 2007)). Gaining insight on the consequences of compounds on the cardiac clock is thus essential considering the pros and cons of using a specific compound. As illustrated here, nrCMs would be an excellent system to model how compounds affect clock dynamics.

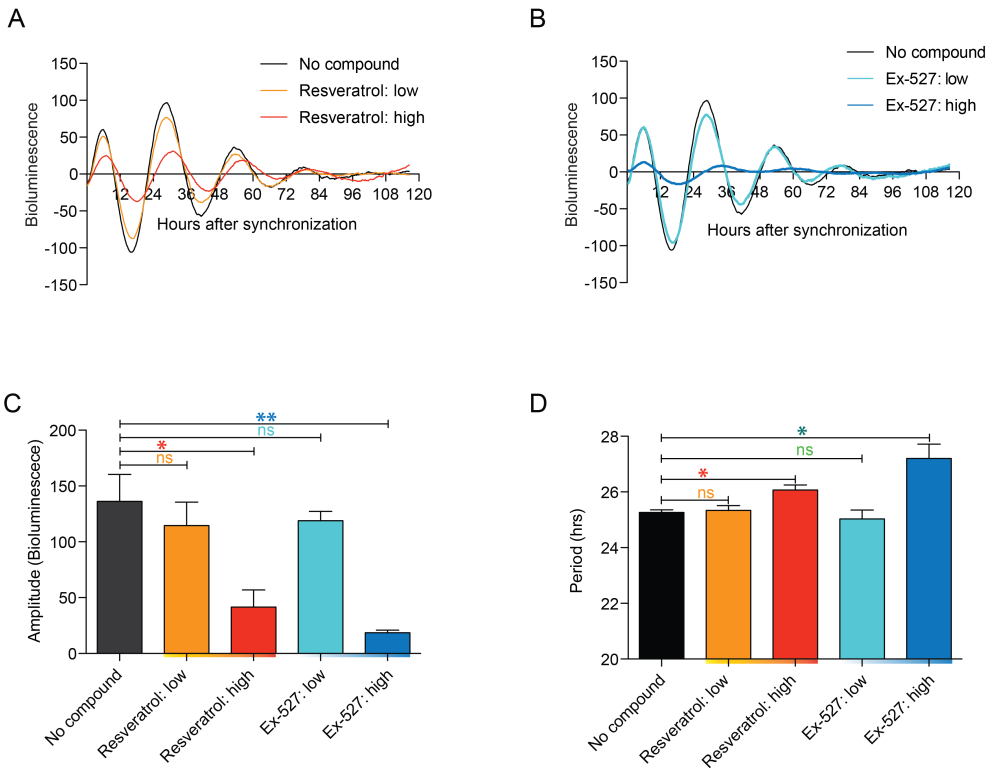

Figure 3. Resveratrol and Ex-527 affect the molecular clock of neonatal rat cardiomyocytes. (A) Per2-Luc bioluminescence in synchronized neonatal rat cardiomyocytes (nrCMs) treated with low or high doses of resveratrol. Bioluminescence was measured by a LumiCyle32 across 116 hours. Means of 3 independent replicates are shown. (B) Similar analysis as in a) for nrCMs treated with low or high doses of Ex-527. (C) Per2-dLuc amplitudes in nrCMs treated with resveratrol or Ex-527. (D) Period of circadian waves in nrCMs treated with resveratrol or Ex-527. Difference between conditions in C) and D) were analyzed using an unpaired Student's T-Test and significance levels are indicated (ns: not significant, * P <0.05, ** P < 0.005).

4.2.4 Neonatal rat cardiomyocytes lose functional rhythmicity upon clock disturbance

As both resveratrol and Ex-527 were found to dampen the clock in nrCMs (Figure 3A-D), we questioned whether this would have consequences for the previously observed time-dependent response to doxorubicin. In general, resveratrol had a clear protective effect on nrCMs, as indicated by lower baseline apoptotic levels (Student's T-Test $P < 0.05$; Figure 4A). Nevertheless, the strong apoptotic effect of doxorubicin (Student's T-Test $P < 0.05$) could not be reversed by resveratrol (Figure 4A). On the contrary, when our second compound Ex-527 was added, basal apoptosis levels significantly increased (Student's T-Test $P < 0.005$; Figure 4B). Administration of Ex-527 in combination with doxorubicin had an additive apoptotic effect when compared to doxorubicin only (Student's T-Test, $P < 0.0001$; Figure 4B).

To analyze the effect of both compounds on the oscillating response to doxorubicin (Figure 2C), we assessed rhythmicity of stress-response in the presence of both compounds and noted that both resveratrol and Ex-527 abolished the rhythmic response to induced apoptosis (RAIN, resveratrol, P = 0.29, Ex-527, P <0.001; Figure 4C,D). This highlights that disturbing the molecular clock of nrCMs through the use of compounds can lead to impaired circadian functionality.

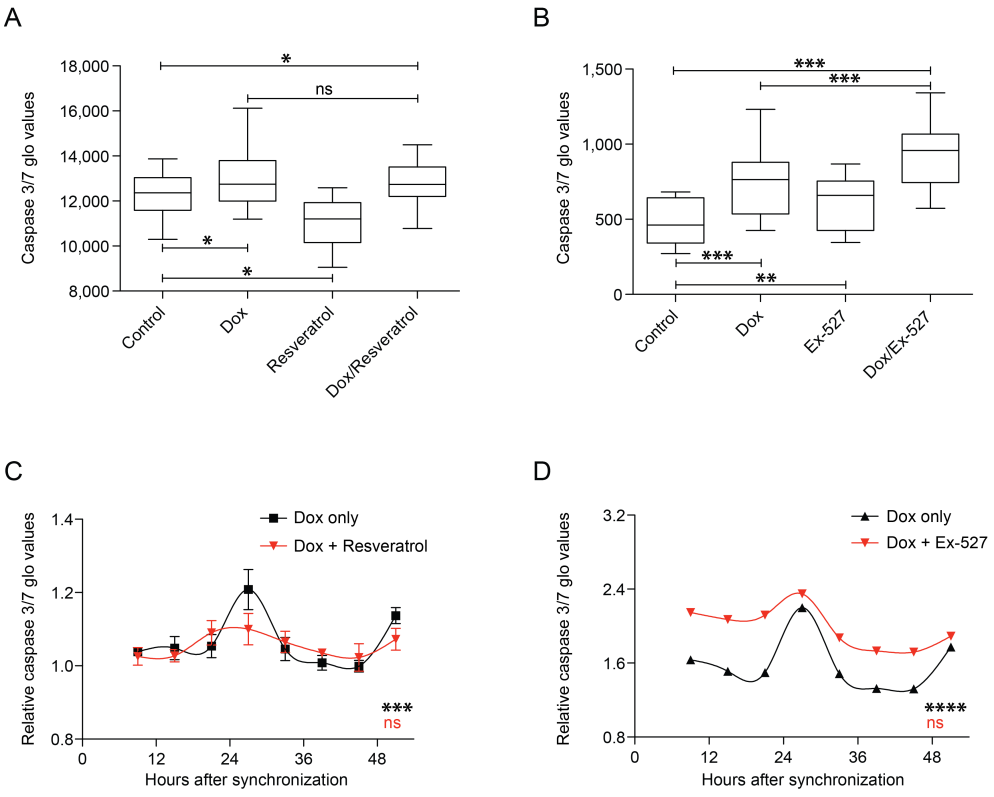

Figure 4. Resveratrol and Ex-527 impair time-dependent apoptotic response to doxorubicin in neonatal rat cardiomyocytes. (A) Apoptotic levels in neonatal rat cardiomyocytes (nrCMs) treated with doxorubicin (dox), resveratrol or a combination compared to non-treated cells (control). (B) Similar analysis as in A) for Ex-527 treatment. (C) Relative doxorubicin-induced apoptosis levels in nrCMs treated with resveratrol across 48 hours. Apoptotic levels were normalized to baseline apoptosis levels of non-treated cells. (D) Similar analysis as in C) for Ex-527 treatment across 48 hours. Effect between groups in A) and B) were analyzed using unpaired Student's T-test (ns: not significant, * P < 0.05, ** P < 0.005, *** P < 0.0005). Significance of rhythmicity in C) and D) were analyzed using RAIN and indicated (** P < 0.005, ****:P < 0.00005).

4.3 Discussion

The role of circadian rhythmicity in physiological and biochemical processes as well as behavioral patterns is becoming increasingly clear. Over the last years, pharmacological

studies show increased interest in circadian rhythms (Ohdo, 2010) to investigate for instance time-of-day dependent drug efficacy. However, to study the consequences of circadian oscillations, functional results have to be analyzed at various time-points. This often leads to an undesired, but necessary large, number of animals, which is why alternative assays are required.

In the current study, we present neonatal rat cardiomyocytes (nrCMs) as a representative system to model the circadian clock of the heart. While Circadian rhythms were previously shown to be present in adult cardiomyocytes (Durgan, 2005; Peliciari-Garcia et al., 2011; THARP and FOLK, 1965), the use of neonatal cells has important advantages, such as high cell number to obtain, easy to derive, easy to culture, and omit the need to breed rats to adulthood.

We show that nrCMs have a functional clock and observe 24-hour rhythmicity in spontaneous contraction as well as induced apoptosis resistance. Importantly, we show that different compounds, used for their beneficial cardiac effects, influence and disrupt the cardiac clock. All this highlights the parallels between our in vitro nrCM clock model and physiological circadian behavior. Rather then using expensive and long-lasting animal or clinical studies, nrCMs would thus be a good and easy-to-use alternative to study (the effect of compounds on) circadian rhythmicity in the heart.

4.4 Materials and Methods

4.4.1 Isolation of neonatal rat cardiomyocytes

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, with prior approval by the Animal Experimentation Ethics Committee, Utrecht University, The Netherlands.

Ventricular cardiomyocytes were isolated from 1-day-old neonatal Wistar rats (Charles River). After sacrifice, hearts were excised and flushed with Solution A (NaCl 8 mg/L, KCl 0.4 mg/L, glucose 1g/L, $Na_2HPO_4 \cdot 2H_2O$ 60 mg/L, KH_2PO_4 60 mg/L, phenol red 20 mg/L and HEPES 4.77mg/L in MilliQ pH 7.2-7.4) to get rid of any remaining blood. Atria and large vessels were removed and the ventricles were cut in 1 mm^3 pieces. Tissue pieces were put in a glass flask containing 14 ml Solution A supplemented with 750 μL trypsin (2,5%; #15090046, GIBCO) and shaken for 15 minutes at 37°C. The tissue/suspension mix was subsequently pipetted up and down several (+- 20) times using a glass pipet to detach cells from the tissue pieces. Supernatant was transferred to a new tube, pelleted (3 min at 1100 RPM without brake) and re-suspended in 5 ml culture medium (Ham's F10 without Ca^{2+} and Mg^{2+} (#31550-023, Gibco) supplemented with 1% penicillin-streptomycin (#DE17-602E, Lonza), 1% L-glutamine (BE17-605E, Lonza) and 5% fetal bovine serum (#F7524, Sigma)). New Solution A and trypsin were added to the remaining tissue pieces and the same procedures were followed until no

tissue pieces were left (approximately 5 cycles). Cell suspensions were combined, filtered using a sterile non-woven compress (#45847, Cutisoft), and plated on uncoated culture dishes (#430167, Corning). After 2 hours, non-adhering cells were collected, counted and plated as a confluent monolayer on laminin-coated (10mg/L in Solution A, #11243217001, Roche) culture dishes (35mm, #353001, Falcon). After 20 hours, medium was replaced to remove dead cells.

4.4.2 Bioluminescence reporter recordings

Neonatal rat cardiomyocytes were transduced with *Bmal1*- and *Per2*- destabilized luciferase lentiviruses. Lentiviral plasmids, harbouring luciferase reporters of the *Per2*- and *Bmal1*-promoters, were previously described and kindly provided by Prof. Dr. Liu (Liu et al., 2008; 2007; Ramanathan et al., 2012). 1.5 days after transduction, cells were synchronized with 100 nM Dexamethasone for 2 hours (Balsalobre et al., 2000) and put on recording medium (Phenol Red-free DMEM, 10% FCS, 10 mM HEPES, 0.035% Bicarbonate, 4.5 g/L glucose and Pen/Strep +100 μM D-Luciferin (Promega). Culture dishes were sealed with high vacuum grease (Dow Corning) and analyzed in a 37°C incubator using a LumiCycle32 (Actimetrics). Bioluminescence from each dish was continuously recorded (integrated signal of 70 seconds with intervals of 10 minutes). Raw data (counts/seconds) were baseline subtracted (polynomial order 3) and smoothened over 1 hr.

4.4.3 Synchronization of nrCMs

nrCMs were synchronized by a 2 hr serum shock (SS, 50% culture medium / 50% horse serum (#16050-122, Gibco), forskolin (10 μM, #F6886, Sigma) or dexamethasone (100 nM, #D1756, Sigma) for 30 minutes (Balsalobre et al., 1998; 2000; Yagita and Okamura, 1999). Non-synchronized cardiomyocytes, that had only a medium change more than 1 day before the start of the experiments, served as controls.

4.4.4 RNA extraction and qRT-PCR

RNA was isolated using phenol-chloroform (Merck) extraction. Purified RNA was treated with DNAse (Promega) and reversibly transcribed with Superscript III reverse transcriptase (ThermoFisher Scientific). mRNA expression was measured using a SYBR Green (Biorad) qRT-PCR. The following primer sequences were used: *Bmal1* (fw): GGCTCATAGATGCAAAAACTGG; *Bmal1* (rv): CTCCAGAACATAATCGAGATGG). *PPIA* (fw): TTCTGCTGTCTTTGGGACT; *PPIA* (rv): CACCGTGTTCTTCGACATTG.

4.4.5 Western Blotting

For protein analysis, nrCMs were washed with PBS and lysed using RIPA-buffer as described previously (van Veen et al., 2002). Lysate concentrations were measured using a BCA kit

(ThermoFisher Scientific), separated by 10% SDS-PAGE, and transferred to a nitrocellulose membrane. Reverse Ponceau staining was used to quantify protein loading. Membranes were blocked with 5% Protifar (Nutricia), probed with anti-BMAL1 (1:2000, #ab3350, Abcam) antibody, followed by a peroxidase-conjugated antibody (1:7000, #170-6515, Biorad) and ECL chemiluminescence (#sc-2048, SantaCruz) for detection. Ponceau-corrected BMAL1 protein levels were quantified with Image Lab (Version 5.1, Biorad).

4.4.6 Cell death assay

Induced cell death was quantified using a Caspase-Glo 3/7 (#G8091, Promega) assay and a TUNEL (#11684795910, Roche) assay according to the manufacturer's instructions. nrCMs were isolated, plated (for the Caspase-Glo 3/7 assay in a white clear 96-well plate (#3610, Corning), for the TUNEL assay, on 12 mm glass coverslips in a 24 well plate (#3524, Corning)) and synchronized. Between 9-51 hours (with 6-hour intervals), cells were exposed to several stressors: doxorubicin (10 μM during 6 hours, #D1515, Sigma), tert-butyl hydroperoxide (10 μM during 1 hour, Sigma) or placing cells in an incubator with 1% O_2 for 3 hours (hypoxia) or 3 hours followed by 2 hours in regular incubator (hypoxia/normoxia). Non-stressed and/or non-synchronized nrCMs served as controls.

4.4.7 Spontaneous beating

nrCMs were cultured in laminin-coated culture dishes and synchronized. Between 9-51 hours (with 6-hour intervals), spontaneous beating frequency was measured by manual counting of cardiomyocyte contractions on 6 locations during 1 minute (Nixon light microscope, 10x magnification).

4.4.8 Compounds

The following concentrations of compounds were used for high and low dose, respectively. Ex-527 (#E7034, Sigma): 250 μM and 25 μM. Resveratrol (#R5010, Sigma): 2.5 μM and 250 nM.

4.4.9 Statistical analysis

Data are presented as mean ± standard error of mean. Circadian rhythmicity was assessed via RAIN, a non-parametric method detecting arbitrary wave-forms in biological data (Thaben and Westermark, 2014). Student's T-test was used to compare non-circadian differences between groups. *P*-values < 0.05 were considered statistically significant.

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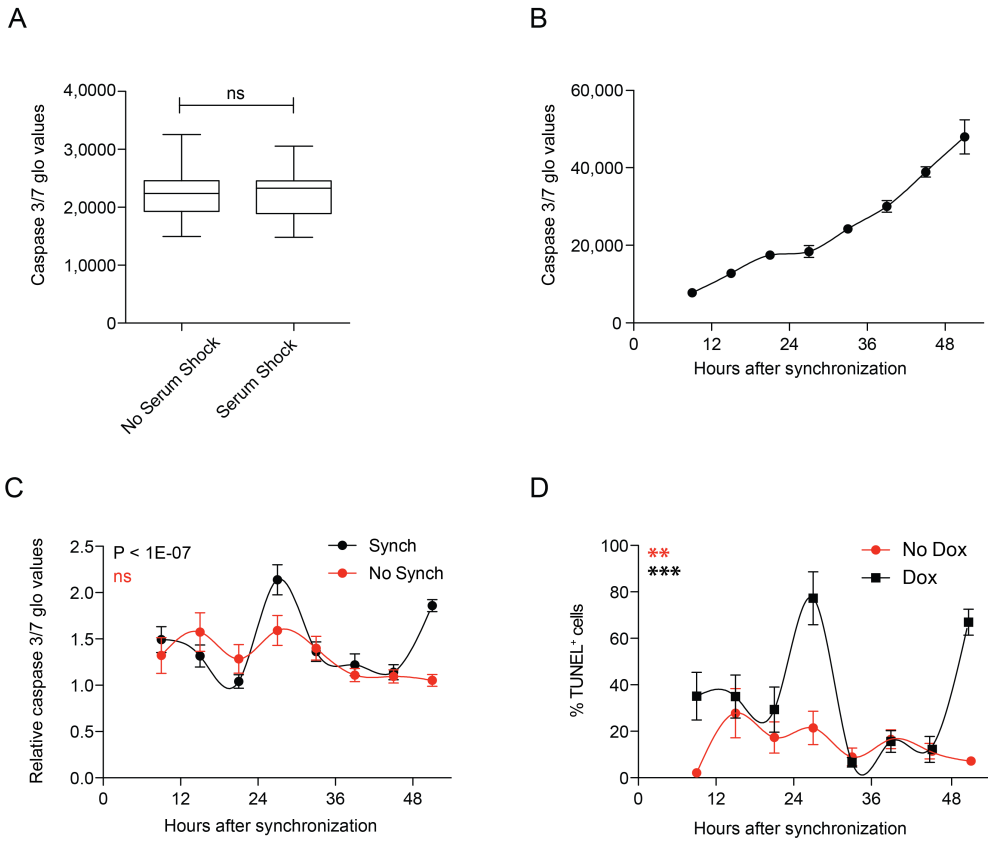
4.6 Supplementary Figures



Supplementary Figure 1. Apoptosis in neonatal rat cardiomyocytes. (A) Apoptotic levels in nrCMs > 1 day after medium change (no serum shock) or after a synchronizing serum shock as measured by a Caspase-Glo 3/7 assay. Two conditions were compared via unpaired Student's T-Test (ns = non significant). (B) Basal apoptosis levels in synchronized neonatal rat cardiomyocytes (nrCMs) measured across 2 days. (C) Apoptotic levels in synchronized (horse serum, Synch) or non-synchronized (medium change 24 hrs before measurement, No Synch) doxorubicin treated nrCMs. (D) TUNEL assay on synchronized nrCMs treated with (Dox) or without doxorubicin (No Dox). Significance of rhythmicity was analysed via RAIN and indicated (** $P < 0.005$, *** $P < 0.0005$).

5

SR9009 drives a metabolic shift and activates the amino acid response pathway in mouse embryonic stem cells

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Abstract

Circadian rhythms are essential to proper organ physiology, and clock disruption has been shown to have profound effects on mammalian cell, tissue and organ systems. The nuclear receptors REV-ERBα and REV-ERBβ regulate both circadian rhythmicity and metabolism, and mediate the interplay between these fundamental processes. While mouse embryonic stem cells (mESCs) do not possess a functional circadian network, REV-ERBs are expressed. To explore their (metabolic) role in mESCs, we stimulated REV-ERB activity through the use of a synthetic agonist SR9009. In mice, REV-ERB activation results in increased energy expenditure, which underlies the potential use of SR9009 as anti-obesity drug. In addition to these metabolic effects, SR9009 has been shown to decrease somatic cell proliferation rates, suggesting that REV-ERB plays a broader role integrating metabolic and proliferative pathways. Here, we demonstrate that the REV-ERB agonist reprograms mESC metabolism towards a glycolytic profile and reduces mESC proliferation. Transcriptome analysis revealed that SR9009 stimulation of mESCs results in profound changes in amino acid metabolism and activation of the amino acid starvation pathway. This indicates that REV-ERB modulation may have profound clock-independent effects on defined stem cell populations, which could result in unexpected consequences of in vivo SR9009 administration.

5.1 Introduction

Circadian rhythms follow a near 24-hour period, and influence many physiological processes in the human body, such as behaviour, sleep, metabolism, tissue regeneration and organ homeostasis (Dierickx et al., 2015). A central clock system in the brain and peripheral clocks in almost every organ regulate these diurnal rhythms, and the molecular mechanism that underlies these oscillations consists of a transcriptional/translational feedback loop based on BMAL1 and CLOCK heterodimerization, which drives rhythmic expression of clock-controlled genes (CCGs). CCGs include other core clock genes such as periods (*Per1*, Per2, Per3), cryptochromes (Cry1, Cry2), *Rorα/β*, and *Rev-erbα/β*, as well as tissue specific genes that determine organ functionality in a circadian manner. REV-ERBα and β, hereafter referred to as REV-ERB, are nuclear receptors that drive the negative branch of the circadian feedback loop through repression of core clock genes. This is mediated through binding of E-box motifs in the *Bmal1* promoter and subsequent recruitment of nuclear receptor co-repressor-histone deacetylase 3 (NCoR-HDAC3), which represses *Bmal1* expression. Combined with the activating branch of the core clock pathway, REV-ERB-mediated repression ultimately results in *Bmal1* mRNA oscillations and therefore ensures clock functionality.

In addition to this role in the core circadian pathway, REV-ERBs are known to affect metabolism through recruitment of HDAC3 to specific binding sites in a tissue-dependent manner (Bugge et al., 2012; Zhang et al., 2015). Recently, two synthetic ligands of REV-ERB, SR9009 and SR9011, have been developed and were proven to regulate circadian rhythms

as well as metabolism in vivo and in vitro (Solt et al., 2012a). Both ligands were found to activate REV-ERBs and abolished circadian rhythms through REV-ERB-mediated repression of *Bmal1* transcription (Solt et al., 2012a). In addition, murine studies revealed that these compounds altered the rhythmic expression of multiple metabolic genes in distinct tissues, thereby increasing energy expenditure. Obesity decreased in mice that were treated with the REV-ERB agonists and fat mass reduction as well as dyslipidaemia and hyperglycaemia improvements were observed (Solt et al., 2012a). Interestingly, studies in other systems or cell types have revealed additional effects of these compounds. For instance, in multipotent leukemia stem cells that are highly proliferative and have the capacity to self-renew, SR9011 decreased *Bmal1* and *Per2* mRNA expression, downregulated numerous key factors in the regulation of leukemia stem cell renewal and reduced cell viability (Puram et al., 2016). This highlights the possible consequences of REV-ERB agonists on proliferative cell types.

Pluripotent mESCs are highly proliferative, able to self-renew and can form the three germ layers of the embryo proper. In contrast to leukemia stem cells, mESCs do not contain a classical functional clock pathway, but even though clock genes do not oscillate, several core factors are expressed (Kowalska et al., 2010; Paulose et al., 2012; Yagita et al., 2010). The function they may exert in mESC however, remains largely unknown. Here, we investigate the role of REV-ERBs in mESCs to uncover a possible function in expression of other clock genes and/or metabolism, and discovered that the REV-ERB agonist SR9009 drives mESCs towards the glycolytic pathway at the expense of oxidative phosphorylation. In addition, transcriptional programs of mESCs changed significantly after SR9009 administration with clear upregulation of multiple genes involved in the amino acid stress sensing GCN2-ATF4 pathway. This resulted in decreased proliferation rates, which suggests that REV-ERB agonists may have severe effects on stem cell populations in vivo.

5.2 Results

5.2.1 REV-ERB modulation slows down ES cell proliferation

The REV-ERB agonist SR9009 has been shown to influence proliferation rate and viability of leukemic stem cells, a cell type known to possess a clock system (Puram et al., 2016). To assess whether similar effects were obtained in mESCs that do not possess a functional clock, the compound was added to mESC cultures for 3 days. Cellular ATP levels, as a proxy for cell number, were measured, and a dose-dependent reduction was observed after SR9009 administration (Figure 1A). In line with this, cell counts and protein content decreased (cell counts, $P < 0.005$, protein content, $P < 0.0005$ Figure 1B,C). Decreased proliferation rates were even more pronounced when culturing mESCs in 2i (2 inhibitors) conditions, a medium known to enhance pluripotency of ES cells through the inhibition of two key pathways (MEK/ERK and GSK3β) (Ying et al., 2008) (Supplementary Figure 1A,B), which may link pluripotency to REV-ERB functionality.

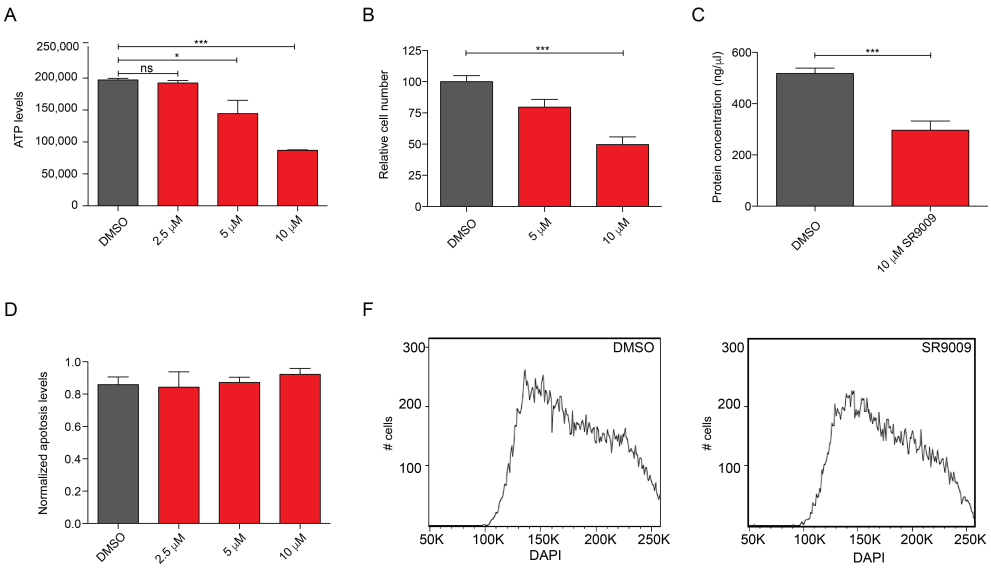


Figure 1. SR9009 slows down proliferation in mESCs. (A) Cellular ATP levels in mESCs treated with different concentrations of SR9009 for 3 days (n=8). (B) mESC counts and (C) protein content after 3 days of SR9009 treatment (n=3). (D) Apoptosis in mESCs treated with different concentrations of SR9009 for 3 days, measured via Caspase 3/7 glo (n=8). (F) DAPI based cell cycle profile of mESCs treated with SR9009 versus DMSO (control) for 1 day. Representative profiles were shown (n=3). Differences between DMSO (control) and SR9009 treatment were analyzed by Student's T-Test (ns: not significant; *$P < 0.05$; *** $P < 0.0005$).

We next questioned whether the reduced cell proliferation was a consequence of increased apoptosis. Analysis of Caspase 3/7 activation demonstrated SR9009 application did not increase apoptosis in mESCs (Figure 1D). In addition, SR9009 administration did not result in a change in cell cycle profile for mESCs, ruling out cell cycle arrest as a potential cause of the reduced cell numbers (Figure 1F). From this, we conclude that the REV-ERB agonist slows down the overall proliferation rate of mESCs, but does not induce cell death or cause a cell cycle block.

5.2.2 mESCs do not possess a core clock, but do express core clock factors

Since the REV-ERB ligand influenced the proliferation rate of mESCs, we wondered whether this could be due to its known effect on other clock genes. REV-ERB is known to decrease *BMAL1* expression in differentiated cells, and REV-ERB agonists have previously been shown to disrupt circadian rhythmicity in leukemia stem cells (Puram et al., 2016; Solt et al., 2012a). Using a luciferase reporter, we confirmed these findings on U2OS cells and noted complete abolishment of *Bmal1*-reporter rhythmicity after SR9009 administration (Supplementary Figure 2). This confirms the profound effect of REV-ERB activation on the oscillatory expression of clock genes in a cell system that harbors a functional clock. mESCs however, have been shown to express some clock genes, but in a non-oscillatory manner (Kowalska et al., 2010; Paulose et al., 2012; Yagita et al., 2010). This was confirmed by two mESC reporter

lines with luciferase in the *Bmal1* locus to investigate endogenous *Bmal1* transcription (Bmal1-2A-dfLuc) and translation (Bmal1::fLuc) (Supplementary Figure 3). After horse serum-mediated synchronization (Balsalobre et al., 1998) of mESCs, no oscillations were observed for either of the reporters (Figure 2A), while after random differentiation across 24 days steady oscillations were observed in both the transcriptional and the translational reporter. This verifies the functionality of our reporter constructs and confirms the establishment of rhythmicity during ESC differentiation (Figure 2B) that was observed here and was previously described for neuronal differentiation as well (Yagita et al., 2010).

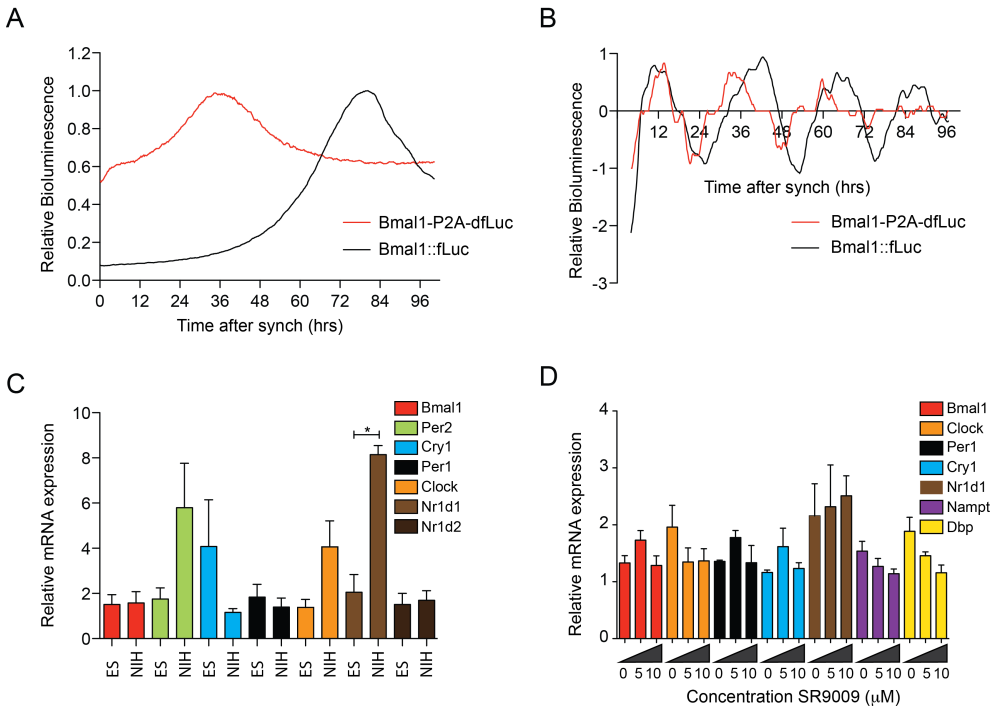


Figure 2. Endogenous *Bmal1* expression starts to oscillate during differentiation. (A) Relative *Bmal1*-fLuc values in synchronized Bmal1-P2A-dfLuc and Bmal1::fLuc mESCs across 100 hours measured by LumiCyle32. Representative tracks were shown. (B) Relative detrended *Bmal1*-fLuc values in 24-day old, synchronized, randomly differentiated Bmal1-P2A-dfLuc and Bmal1::fLuc cells. (C) Relative mRNA levels of core clock factors in mESCs (ES) compared to expression levels in NIH3T3 cells (NIH). (D) Relative mRNA levels of core factors in mESCs treated with different concentrations of SR9009 for 3 days. Expression levels were normalized to the housekeeping gene *PPIA*. Differences between groups were assessed via a Student's T-Test (*$P < 0.05$).

To further solidify the absence of a clock in mESC, core clock genes were assessed using qRT-PCR across 48 hours after serum shock-based synchronization (Balsalobre et al., 1998). Indeed, no *Bmal1, Per2* or *Gm129* oscillations were observed (Supplementary Figure 4). While clock gene expression levels were lower compared to somatic NIH3T3 cells, most clock genes could still be picked up reliably in mESCs using qRT-PCR (Figure 2C). To interrogate

the possible effect of SR9009 on clock genes, expression levels were compared with and without this REV-ERB ligand. In striking contrast to clock compatible cell types that show dose-dependent downregulation of *BMAL1* and *PER2* (Puram et al., 2016; Solt et al., 2012a), no significant changes were observed after 3 days in mESCs (Figure 2D). This indicates that clock factor regulation through REV-ERB is different in mESCs compared to somatic cells. Therefore, we conclude that the effect of SR9009 on stem cell proliferation is most likely not linked to the role of REV-ERB in the circadian core clock pathway.

5.2.3 REV-ERB agonist shifts mESC metabolism towards a glycolytic profile

The stemness of pluripotent stem cells is strongly influenced by their metabolic state. Unlike human ES cells, which predominantly depend on a glycolytic metabolism, mESCs are metabolically bivalent which means they can switch between glycolysis and oxidative phosphorylation depending on their energetic need (Zhou et al., 2012). Next to its role as negative component of the circadian clock, REV-ERB has an important role in metabolic regulation and SR9009 treatment has been shown to alter the expression of a set of metabolic transcripts in murine tissues (Solt et al., 2012b). As the clock-based function of REV-ERB was unlikely to contribute to the observed effect of SR9009 on mESC proliferation (Figure 2B,C), we examined whether metabolic changes could contribute to the observed phenotype of decreased proliferation in mESCs. To this end, we probed the metabolic flux of mESCs treated with SR9009 via the use of an XF96 Extracellular Flux Analyzer (Seahorse bioscience). We assessed their mitochondrial respiration via measuring the oxygen consumption rate (OCR), and their glycolytic activity via extracellular acidification rate (ECAR) using a mitochondrial and glycolysis stress test. Since oxygen is consumed in the electron transport chain in mitochondria, OCR reflects global amount of mitochondrial respiration. ECAR assesses the glycolytic flux via measuring medium acidification, caused by lactate formation upon recycling of NADH to NAD^+ during glycolysis.

For the mitochondrial stress test, the following compounds were serially added to measure ATP production, maximal respiration, and non-mitochondrial respiration; respectively: oligomycin (OM), Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP) and antimycin (AM) mixed with rotenone (Rot). From this, spare capacity (difference between maximal OCR and basal OCR) and coupling efficiency (ATP-linked OCR/basal OCR) can be deducted. For the glycolytic stress test, cells were incubated in non-glucose containing medium. Glucose, OM and 2-deoxy-glucose (2-DG) were sequentially added to assess glycolysis, glycolytic capacity and to calculate the glycolytic reserve (maximal ECAR-basal ECAR) and non-glycolytic acidification (ECAR after 2-DG addition).

We noted a significant metabolic shift towards glycolysis (Figure 3A,B). Basal respiration rate declined, while the glycolytic flux increased (Student's T-Test $P < 0.0001$; Figure 3C). The loss of basal respiration was accompanied by increased spare respiratory capacity (Student's T-Test $P < 0.0001$; Figure 3D) and decreased coupling efficiency (Student's T-Test $P < 0.005$;

Figure 3D). Accordingly, more glycolytic flux was characterized by higher glycolytic capacity (Student's T-Test $P < 0.0001$; Figure 3E) and an increase in glycolytic reserve (Student's T-Test $P = 0.112$; Figure 3E). This indicates that REV-ERB ligand administration results in a metabolic shift from oxidative phosphorylation towards glycolysis in mESCs, and suggests that this may contribute to decreased proliferation rates.

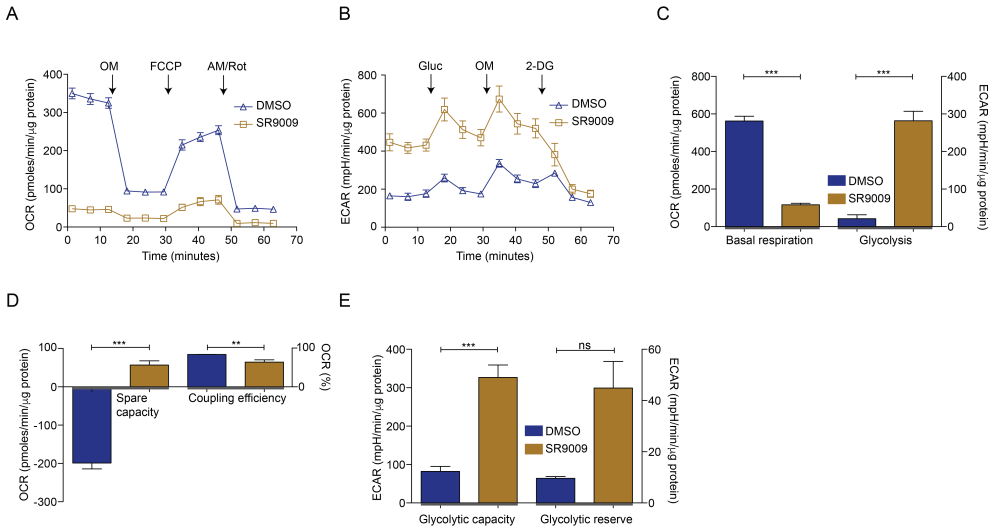


Figure 3. SR9009 induces a metabolic shift towards a highly glycolytic profile in mESCs. (A) Protein normalized OCR of mESCs treated with 10 μM SR9009 or DMSO (control) for 3 days in a mitochondrial stress test. (B) Protein normalized ECAR values of mESCs treated with 10 μM SR9009 or DMSO (control) for 3 days in a glycolysis stress test. OM: oligomycin, FCCP: Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone, AM: antimycin, Rot: rotenone, Gluc: glucose, 2-DG: 2-deoxy-glucose. (C) Basal respiration and glycolysis, (D) spare capacity and coupling efficiency, and (E) glycolytic capacity and glycolytic reserve in mESCs treated with SR9009 vs DMSO. Parameters in (C-D) were deducted from (A-B). Differences between SR9009 and DMSO were assessed via a Student-s T-Test (ns: not significant; $^{**}P < 0.005$; $^{***}P < 0.0005$).

5

5.2.4 REV-ERB activation remodels the mESC transcriptome

To uncover the molecular basis of the observed metabolic reprogramming and slowdown of stem cell proliferation, we sought to determine the transcriptional program that might underlie these phenotypes. To this end, we treated ES cells with increasing concentrations of SR9009 (from 1 μM to 10 μM) for three days and assessed genome-wide expression levels by mRNA sequencing using CEL-Seq, a previously described RNA profiling technique based on sequencing the 3'UTR of mRNAs (Hashimshony et al., 2012). Since lowly expressed genes are typically not picked up robustly when analyzing highly multiplexed CEL-Seq data, we selected genes with more than 5 RPM (reads per million normalized) read counts in one of the samples. From those (~10,000 genes), 1,137 were 2-fold upregulated (10 μM vs 1 μM), and a subset of 220 showed a gradual response to increased doses of SR9009. A total of 1,194 genes were 2-fold downregulated, 105 of which gradually decreased with increasing SR9009 concentration.

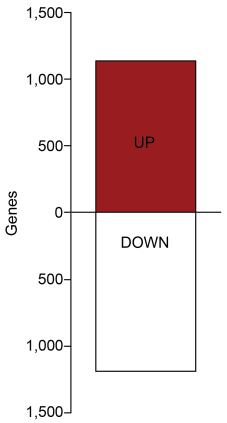
Gene ontology (GO) analysis on the gradually upregulated SR9009 response genes (n=220) revealed that these transcripts were linked to translation and tRNA loading (Figure 4A). In addition, STRING interaction analysis (Szklarczyk et al., 2015) (Supplementary Figure 5) revealed three interesting interaction clusters all linked to the amino acid starvation response (AAR) (Gcn2-Eif2α-Atf4) pathway (Figure 4B) through *Atf4* (Activating transcription factor 4), a central player in this evolutionary conserved pathway (D'Aniello et al., 2015). In mESCs, this mechanism is known to safeguard stem cell identity through a regulatory loop that actively creates an intrinsic shortage of L-Proline, thereby restricting proliferation of stem cell colonies (D'Aniello et al., 2015). Next to *Atf4* as a hub gene, we noted a number of other upregulated AAR/Atf4-related genes with a role in transcriptional regulation (second cluster) as well as tRNA charging and amino acid metabolism (third cluster) (Figure 4C).

To further explore the role and dynamics of *Atf4*, we generated an *Atf4* promoter-based luciferase reporter to follow RNA levels over time after SR9009 administration. Real-time bioluminescence with a Lumicycle32 revealed a concentration dependent upregulation of *Atf4* transcription, and largest differences were found between 24 and 48 hour of SR9009 treatment (Figure 5A). To confirm this, the early *Atf4* response was measured through qRT-PCR, and a rapid increase in mRNA was noticed (Figure 5B). This was verified by Western blot, and indicates that both *Atf4* mRNA and protein significantly increase after addition of a REV-ERB agonist with high protein levels already as fast as 4 hours after SR9009 addition (Figure 5C,D). Since ES cells activate this pathway endogenously through the creation of an intrinsic L-Proline shortage to maintain stem cell integrity (D'Aniello et al., 2015), we explored whether addition of L-Proline to the culture medium could reverse the proliferation block caused by SR9009. ATP levels and protein content analyses revealed that L-Proline could not rescue the inhibitory effect of SR9009 on proliferation (Figure 5E,F), indicating that the observed effects are independent of L-Proline shortage, and could therefore hint towards a reduced tRNA loading. All this highlights the quick induction of *Atf4* after compound administration, which drives a profound transcriptional response. However whether this is a consequence or cause of decreased proliferation remains to be determined. In conclusion, the transcriptional profile of mESCs is significantly altered upon the synthetic REV-ERB ligand administration, and changes include the activation of the AAR stress pathway.

5.2.5 SR9009 induces additional transcriptomic changes in mESCs

In addition to *Atf4*-related transcriptomic alterations, we noted additional changes in other key factors that might contribute to the observed phenotype of mESCs after SR9009 treatment. For instance, *Lefty1* (Left-right determination factor 1), an inhibitor of TGF-β1-mediated epithelial-mesenchymal transition (EMT) is strongly downregulated, and in accordance with this *Twist1* and *Zeb2*, two EMT inducers, are upregulated. This suggests that REV-ERB modulation may induce a mesenchymal state in mESCs. A number of WNT-signaling pathway genes (*Wnt4*, *Wls*, *Tcf12*) were also found upregulated. As WNT-signaling plays an important role in stem cell proliferation, self-renewal and differentiation capacity

A



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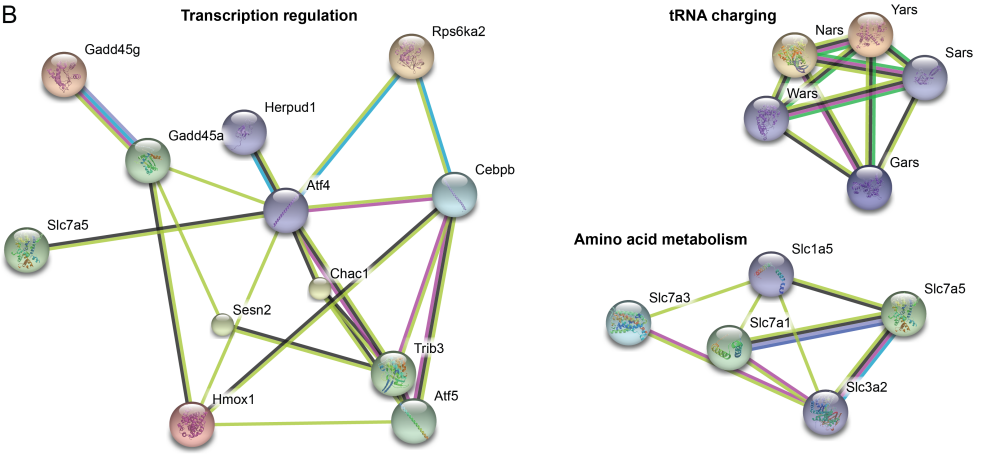

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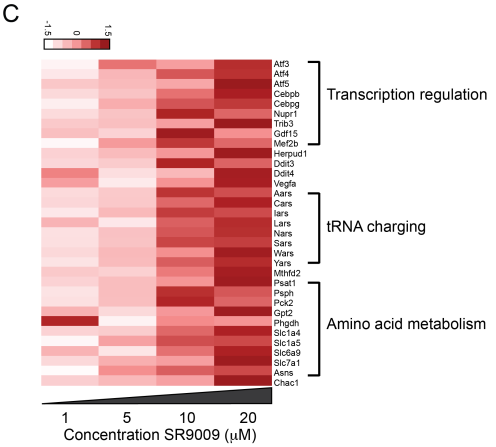

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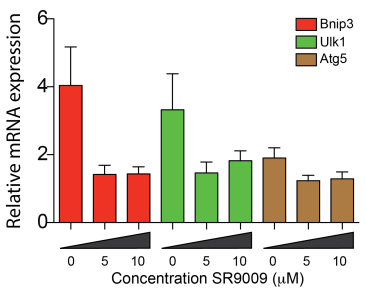

Figure 4. SR9009 induces large transcriptional changes in mESCs. (A) Number of 2-fold up- and down regulated genes in mESCs treated with 10 μM SR9009 for 3 days (left) and GO-analysis analysis (right) on gradually upregulated genes upon increasing SR9009 concentrations. (B) STRING interaction network of three clusters linked to the Gcn2-Eif2a-Atf4 stress response pathway. (C) Heatmaps represent z-normalized RPM values of Gcn2-Eif2a-Atf4 stress response pathway-related genes in mESCs treated with increasing levels of SR9009. Genes were grouped into 3 defined clusters. Heatmaps were visualized using Java TreeView. FDR: False discovery rate. (D) qRT-PCR analysis for 3 core autophagy markers, on mESCs treated with varying concentrations of SR9009. Expressions levels were normalized to the housekeeping gene *PPIA*.

(Merrill, 2012), SR9009 may activate WNT signaling in mESC, which could interfere with proliferative capacity. Interestingly, Heme Oxygenase 1 (*Hmox1*) was also upregulated under SR9009 conditions in mESCs. HMOX1 mediates the first step in heme catabolism and has a protective effect in avoiding excessive amounts of heme, which sensitizes the cells to undergo apoptosis. Heme is a natural ligand of REV-ERB (Raghuram et al., 2007; Yin et al., 2007) , and SR9009 binds the same binding domain of REV-ERB as heme. This suggests that *Hmox1* upregulation could be part of a protective mechanism to compensate for overactivation of the nuclear receptor. Amongst the down-regulated genes, we noticed a

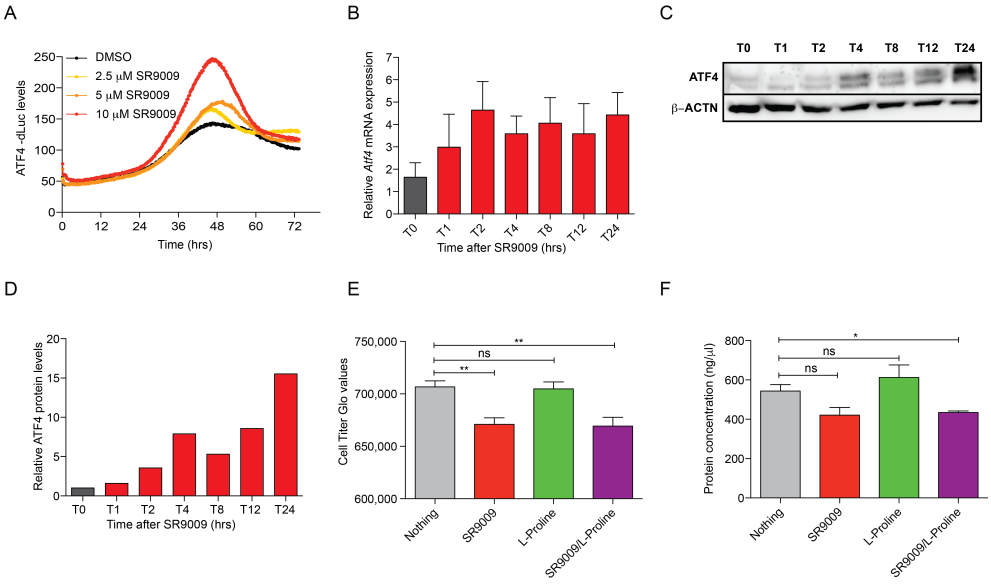

Figure 5. *ATF4* is rapidly upregulated in mESCs upon REV-ERB agonist treatment. (A) Bioluminescence levels in *ATF4*-dLuc mESCs treated with varying concentrations of SR9009, measured with a Lumicycle32 (Actimetrics) across 72 hours. (B) Relative *Atf4* mRNA levels of mESCs treated with SR9009 for the indicated incubation times. Expression levels were normalized to the housekeeping gene *PPIA* (n=3). (C) ATF4 western blot of mESCs treated with SR9009 for the indicated incubation times. β-ACTN was used as a housekeeping gene. (D) Quantification of ATF4 levels deducted from C) normalized to total amount of protein loaded (β–ACTN). (E) CellTiter-Glo assay, measuring ATP levels on mESCs that were untreated, treated with 10 μM SR9009, 500 μM L-Proline or both. (F) Protein levels for the same conditions as in E). Differences between conditions were assessed using a Student's T-Test (ns: not significant; $*P < 0.05$; $**P < 0.005$; $*** P < 0.0005$).

number of cell cycle/proliferation-related genes (*Gmnn*, *Ndrg2*, *Pole2*, *RPA*, *CD320*), which is in line with the slow down of mESC proliferation rate after SR9009 administration. From the observed transcriptional changes, we conclude that REV-ERB ligand significantly remodels the transcriptome of mESCs, and that different mechanisms might contribute to slower stem cell growth.

5.3 Discussion

While almost all cells possess an intrinsic circadian clock system, pluripotent stem cells seem to be an exception, as they do not demonstrate oscillations of core clock genes. Yet, clock genes are expressed, which suggests a functional role for these factors in ES cells. In somatic cells, REV-ERB regulates circadian rhythmicity via recruitment of NcoR-HDAC3 to the promoters of target genes and thereby inhibits their transcription. This also affects metabolism related transcripts, and underlies REV-ERB dependent metabolic programs that are often tissue-specific. While these mechanisms have been clarified in differentiated cells, the role of REV-ERB expression in stem cells remains largely unknown.

We discover that the synthetic REV-ERB agonist SR9009, a compound known to have both metabolic and circadian effects in mice as well as differentiated clock-containing cell types (Puram et al., 2016; Solt et al., 2012a), also has severe consequences when administered to mESCs. We show that mESCs, despite the absence of a functional circadian clock, slow down their proliferation and switch their metabolism to glycolysis upon SR9009 treatment. No significant clock pathway-related changes were observed upon compound treatment, which is why we suggest that the consequences of REV-ERB activation in mESCs are most likely clock-independent. Nevertheless, these results highlight the importance of proper clock factor regulation even when a functional oscillatory clock is absent.

Severe reprogramming of mESC energy metabolism occurred upon REV-ERB ligand administration, with a drastic downregulation of oxidative phosphorylation that was countered by a strong increase in glycolysis. We speculate that this could imply that naïve pluripotent mESCs may be pushed towards a primed state upon treatment, which facilitates differentiation towards epiblast stem cells (EpiSCs). This cell-state more resembles human ESCs, and both of these cell types are known to be highly glycolytic (Zhou et al., 2012). The switch from mESCs towards EpiSCs is modulated by Hypoxia-inducible factor 1-alpha (HIF1α). Interestingly, we noted an increase in *Hif1*α expression upon SR9009 treatment, which could explain the concomitant switch to a glycolytic metabolism (Zhou et al., 2012). However, known direct HIF1α target genes such *PDK1*, *LDHA* and *PYGL* were downregulated, which rules out that the synthetic REV-ERB agonist induces a fast differentiation switch in mESCs towards EpiSCs. Nonetheless, REV-ERB activation could act to prime mESCs metabolically for a slower process of differentiation.

In addition to metabolic changes, we demonstrate that SR9009 reprograms mESCs

transcriptionally, with *Atf4* as a central factor. *Atf4* is a key player in the Gcn2-Eif2a-Atf4 amino acid starvation response (AAR) pathway, and has been shown to be upregulated to safeguard stem cell identity upon an intrinsic shortage of L-Proline (D'Aniello et al., 2015). Related to this pathway, we find three clusters of genes that are upregulated upon SR9009 treatment and that were linked to the following processes: transcriptional regulation, tRNA charging and amino acid metabolism. All this corroborates that in ES cells, SR9009 activates the AAR pathway. However, in our system *Atf4* upregulation could not be reversed by L-Proline, which suggests that the REV-ERB agonist results in a tRNA loading defect, rather than enhancing the active shortage of L-Proline.

In addition to AAR factors, other genes were differentially expressed after REV-ERB agonist treatment and included EMT modulators, WNT-signaling players and cell cycle-related transcripts. This highlights the broad effects of the REV-ERB agonist and stresses that multiple mechanisms will most likely contribute to changes in metabolism and cell proliferation. Nevertheless, whether these alterations are a direct consequence of the REV-ERB agonist, or whether they are part of a slower remodeling process remains to be determined. For instance, we noted a significant increase in *Hmox1*, which is a factor that catabolizes heme to protect cells against excessive amounts of heme. As heme is the natural ligand of REV-ERB, mESCs may also sense an artificially high amount of heme, and may respond to this environmental change by upregulating *Hmox1*. Since heme is an important factor in the electron transport chain in mitochondria as well, we hypothesize that compound administration may results in abberant mitochondrial respiration, thereby favoring glycolysis over oxidative phosphorylation as their main source for energy generation.

In conclusion, we report the anti-proliferative and stress-causing effect of a REV-ERB agonist in mESCs. This indicates that while no circadian clock is present, the proper regulation of clock factors may be indispensable for mouse ES cell physiology. Investigation of early transcriptomic alterations will be needed to uncover which event initiates the cascade that ultimately results in metabolic and proliferative changes in mESCs, but the data obtained here indicate that synthetic REV-ERB agonists have a profound impact on stem cells populations, which may have consequences for in vivo tissue regeneration and homeostasis.

5.4 Materials and methods

5.4.1 Cell culture

mESCs were maintained in mESC medium (DMEM, 15% fetal bovine serum, 1 μM β-mercaptoethanol, non-essential amino acids, Penicillin/Streptomycin and LIF containing conditioned medium (1:1000)) on a feeder layer of irradiated mouse embryonic fibroblasts (MEFs). Additionally, mESCs were cultured in 2i medium (DMEM/F12, Neurobasal, N2/B27 supplements, non-essential amino acids (0.1μM) β-mercaptoethanol, Penicillin/Streptomycin and LIF containing conditioned medium (1:1000), 1 μM PD0325901 and 3

μM CHIR99021 on gelatin). mESCs were randomly differentiated for 24 days, as described previously (Umemura et al., 2013). In short, mESCs grown on MEFs were trypsinized, and 2,000 cells were seeded onto low-attachment 96-well plates in differentiation medium without LIF (high glucose DMEM, 10% FBS, 0.1 μM non-essential amino acids, 100 μM β-mercaptoethanol and 100 U/ml penicillin-streptomycin) to form embryoid bodies (EBs). Two days later, EBs were plated on 35 mm dishes and cultured for an additional 22 days. For analytical experiments mESCs were transferred to gelatin coated dishes without MEFs. NIH3T3 and U2OS cells were maintained in MEF medium (DMEM, 10% fetal bovine serum, Penicillin/Streptomycin).

5.4.2 Protein content, cell viability, and apoptosis measurements

Protein content was measured via a BCA analysis (ThermoFisher Scientific) and the use of a spectrophotometer. Cell viability was assessed through a CellTiter-Glo assay (Promega), and apoptosis levels, represented by active Caspase 3 and 7, were measured using a CaspaseGlo kit (Promega) according to manifacturer's instructions. Bioluminescence was read out using a Centro microplate luminometer (Berthold Technologies). For both CellTiter-Glo and Caspase-Glo 3/7 assays mESCs were cultured on white-walled 96-well plates.

5.4.3 Cell cycle analysis

mESCs were resuspended and fixed in phosflow Fix buffer I (BD Biosciences) for 10 minutes at 37°C. Fixative was quenched with PBS/1%fetal bovine serum and cells were resuspended in staining buffer (PBS, DAPI and RNAseA). Stained mESCs were analyzed on a FACs Canto (BD Bioscience).

5.4.4 Generation of Bmal1^{luc} knockin mESCs

Two targeting vectors were constructed consisting of two arms of homology overlapping a 300bp region upstream of mouse *Arntl* STOP codon and a 600 bp region downstream of the *Arntl* STOP codon. In between these arms a P2A (for Bmal1-P2A-fLuc) or a Glycine linker (for Bmal1::fLuc) was pasted, followed by a destabilized firefly luciferase (dfLuc2 for Bmal1-P2A-fLuc) or a normal firefly luciferase (fLuc2 for Bmal1::fLuc) and a neomycin selection cassette.

10 μg of the targeting plasmids together with TALENs (1,5 μg of each plasmid), designed to cut the STOP-codon region, were co-transfected in mESCs. mESCs were selected for 6-8 days with Neomycin (G418, Gibco) and resistant clones were picked and genomic DNA was isolated. Surviving clones were screened by Southern blot to detect homologous recombinants. A PCR probe upstream of the 5'-arm of homology of the genomic *mArntl* locus was amplified and labeled with [α-^{32}P]dCTP. Correct recombinant alleles could be distinguished from WT alleles via the presence of a 7.6kb instead of 8.6kb band.

5.4.5 OCR and ECAR measurements using XF96 Extracellular Flux analyzer

mESCs were cultured in mESC medium on Seahorse 96-well plates (Agilent Technologies) pre-coated with 0.1% gelatin. Culture medium was switched to basal medium (unbuffered DMEM, supplemented with 2 mM glutamine) 30 minutes before the start of the assay and used for the total duration of the measurements. For the mitochondrial stress test OM, FCCP and AM/Rot were serially injected. For the glycolysis stress test, glucose, OM and 2-DG were sequentially injected. OCR and ECAR values of 8 replicates per condition were normalized to the total amount of protein per well, as a proxy for cell number, via BCA analysis. In general, the average of 3 baseline OCR and ECAR levels were calculated, as well as the average of 3 measurements after each compound injection. Basal respiration was calculated as the basal OCR – non-mitochondrial OCR and basal glycolysis as ECAR after glucose – ECAR before glucose. Spare capacity: OCR after FCCP – baseline OCR, coupling efficiency: ATP-linked OCR/basal OCR, glycolytic capacity: ECAR after OM – ACAR after 2-DG, glycolytic reserve: glycolytic capacity – glycolysis.

5.4.6 Generation of Reporter Cell Lines

Lentiviral plasmids harboring luciferase reporters of the murine *Per2* and *Bmal1* promoters were described previously and kindly provided by Prof. Dr. Liu (Liu et al., 2008; 2007; Ramanathan et al., 2012). Viral particles were concentrated via ultracentrifugation after 3 harvests in HEK293T cells. NIH3T3 and U2OS cells were transduced with concentrated *Bmal1*-dLuc or *Per2*-dLuc lentivirus, and selected with Blasticidin to obtain stable cell lines. mESCs were transfected (Lipofectamine 3000) with an *ATF4*-dLuc reporter plasmid and selected with puromycin to obtain a polyclonal mESC line.

5.4.7 Bioluminescence recording and data analysis

mESCs, NIH3T3 and U2OS cells were switched to recording medium (mESC medium/MEF medium, 10 mM HEPES, 100 μM D-Luciferin Potassium Salt (Promega)). Culture dishes were sealed with high vacuum grease (Dow Corning) and monitored via the use of a LumiCycle32 (Actimetrics) at 37°C. Bioluminescence from each dish was continuously recorded (integrated signal of 70 seconds with intervals of 10 minutes). Raw data (counts/seconds) were baseline subtracted (polynomial order 3).

5.4.8 RNA isolation and CEL-Seq

RNA from mESCs and NIH3T3 cells was extracted using the standard TRIzol (Invitrogen) protocol and 10 ng of total RNA per sample was used for library preparation and sequencing. RNA was processed as described previously (Hashimshony et al., 2012; Simmini et al., 2014) and paired-end sequencing was performed on the Illumina Nextseq platform with a read length of 75 base pairs. Read 1 was used to identify the sample barcode and library index,

while read 2 was aligned to the mm9 mouse RefSeq transcriptome (downloaded from the UCSC genome browser) using BWA (Li and Durbin, 2010). Reads that mapped equally well to multiple locations were discarded. Around 500,000 reads per million (RPM) were sequenced per sample. Samples were reads per million (RPM) normalized.

5.4.9 Quantitative RT-PCR

Purified RNA was treated with DNAse (Promega) and reversibly transcribed with Superscript III reverse transcriptase (ThermoFisher Scientific). qRT-PCR on biological triplicate samples was carried out in triplicate (technical replicates) in CFX-384 Touch™ Real-time PCR detection system (Biorad) using SYBR Green (Biorad). *PPIA* was used as housekeeping gene and fold changes were calculated to the lowest values among all replicates. Primer sequences: *PPIA* (fw): ctgatggcgagcccttg, *PPIA* (rv): tctgctgtctttggaactttgtc, *Bmal1* (fw): tcgttgcaatcgggcg, *Bmal1* (rev): ccgtatttccccgttcgc, *Gm129* (fw): actcaagatgggtcgctttg, *Gm129* (rv): gggcagctatgtgaggaaac, *Per1 (fw):* tcgaaaccaggacaccttctct*, Per1 (rv):* gggcaccccgaaacaca*, PER2* (fw): cccgagctagagacggtaga, *PER2* (rv): tgctctcagaagagtcccga, *Cry1* (fw): tcgccggctcttccaa, *Cry1* (rv): tcaagacactgaagcaaaaatcg, *Clock* (fw): aaagacggcgagaacttgg, *Clock* (rv): ggaggcagaaggagttggg, *Nr1d1* (fw): ggatgcccgtctgccc, *Nr1d1* (rv): ccctggactccaataacaacaca, *Nr1d2* (fw): tcatgaggatgaacaggaacc, *Nr1d2* (rv): gaattcggccaaatcgaac. *Dbp* (fw): gacaccgtggaggtgctaat, *Dbp* (rv): acctccggctccagtacttc, *Nampt* (fw): ggtcatctcccgattgaagt, *Nampt* (rv): tcaatccaattggtaagcca, *Bnip3* (fw): gctcctgggtagaactgcac, *Bnip3* (rv): gctgggcatccaacagtatt, *Ulk1* (fw): gagccgagagtggggctttgc, *Ulk1* (rv): gccctggcaggataccacgc, *Atg5* (fw): agcagctctggatgggactgc, *Atg5* (rv): gccgctccgtcgtggtctga.

5.4.10 Western blotting

Cells were lysed in RIPA buffer and protein lysate was loaded, separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk powder (Nestlé) in T-BST and probed with anti-ATF4 (1:1000, #11815, Cell Signaling Technologies) antibody, followed by a peroxidase-conjugated antibody (1:5000, #sc-2004, Santa Cruz). ECL Plus Western blotting substrate (#32132, ThermoFisher Scientific) was used for chemiluminescence detection with an ImageQuant™ LAS 4000 imager (GE Healthcare). HRP-coupled anti-β-ACTIN (1:5000, #5125S, Cell Signaling) was used as a loading control. Band intensities were calculated with ImageJ.

5.4.11 STRING and Gene Ontology Analysis

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (www.string-db.org) was used to identify putative interaction networks amongst the genes gradually upregulated in mESCs treated with varying concentrations of SR9009 (Szklarczyk et al., 2015). Gene ontology terms were retrieved via www.string-db.org.

5.4.12 Statistical analysis

All data were shown as means ± s.e.m. One-tailed T-Tests were carried out to assess differences between qRT-PCR mean values within the same experiments. A difference of $P < 0.05$ was considered significant.

5.5 References

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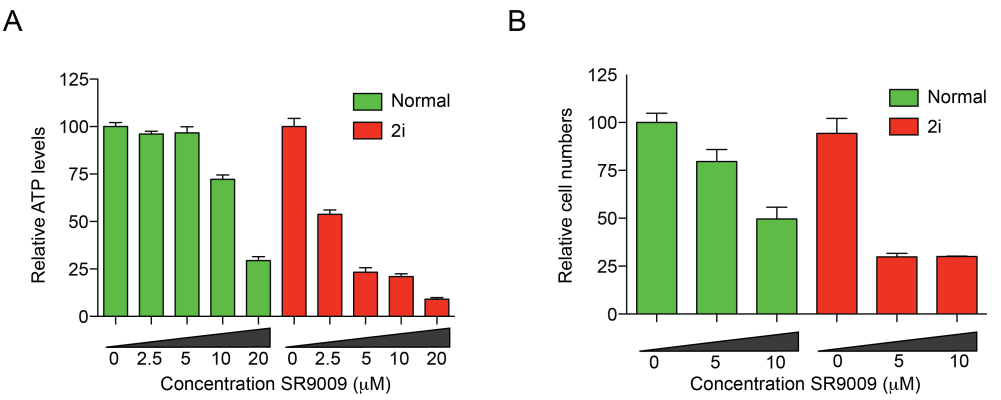
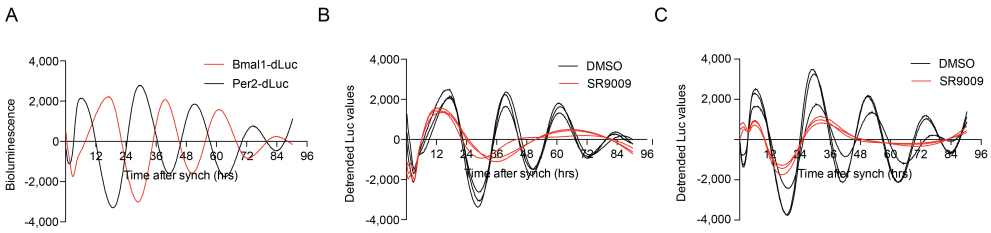
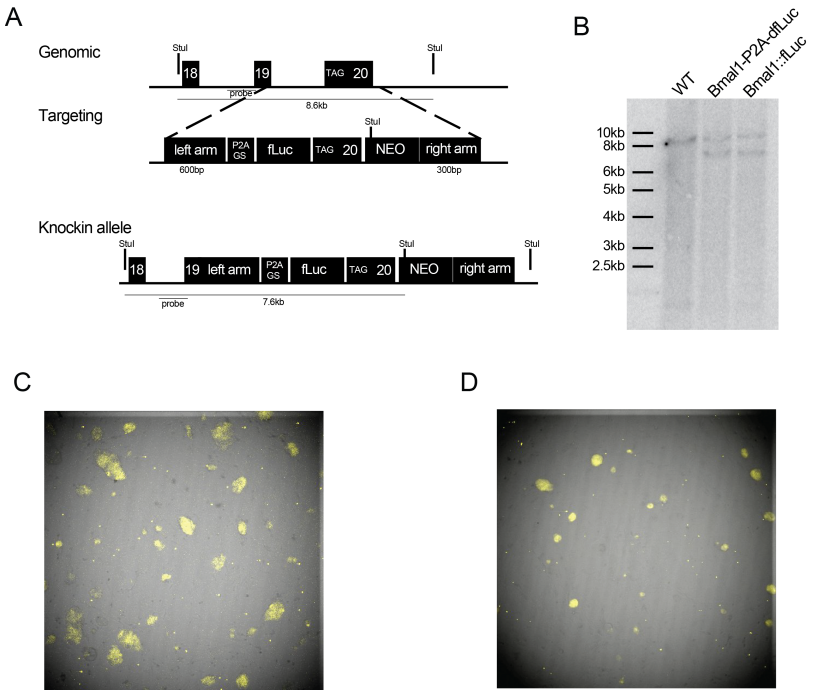
5.6 Supplementary Figures



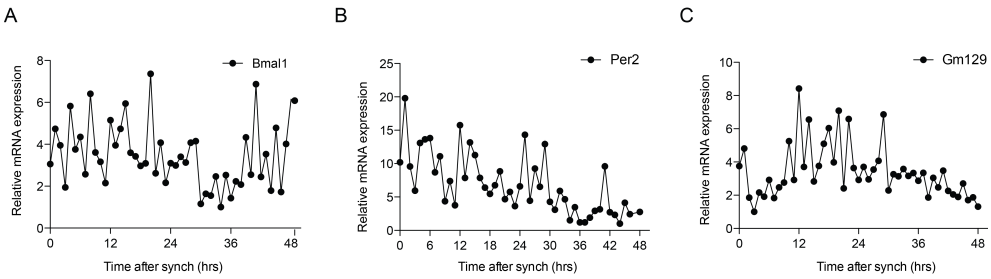
Supplementary Figure 1. mESC cultures in 2i are more sensitive to SR9009. (A) Relative ATP levels and (B) cell numbers for mESCs cultured in either mESC medium (normal) or N2B27-based 2i medium (2i) after treatment with different concentrations of SR9009.



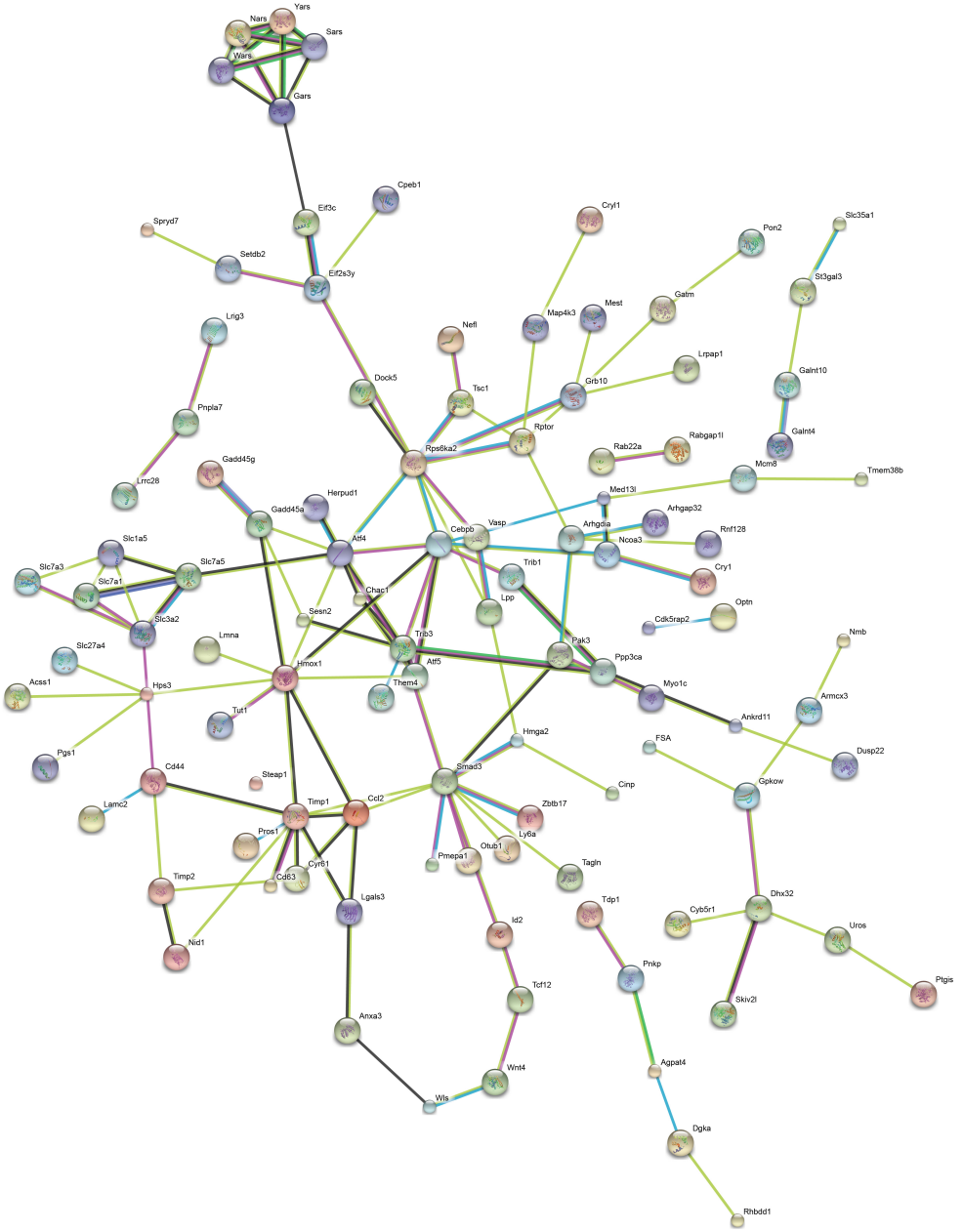
Supplementary Figure 2. SR9009 abolishes circadian rhythmicity of *Bmal1* and *Per2* reporter expression. (A) *Bmal1*- and *Per2*-dLuc bioluminescence in synchronized U2OS reporter cells. Representative tracks were shown. (B) Bioluminescence in synchronized *Bmal1*-dLuc and (C) *Per2*-dLuc U2OS cells treated with 10 μM SR9009. Bioluminescence was measured across 96 hours using a Lumicycle32 (Actimetrics) and values were detrended.



Supplementary Figure 3. Construction and confirmation of Bmal1-P2A-dfLuc and Bmal1::fLuc reporters. (A) Targeting strategy with a schematic of the *mArntl* locus, targeting vector and knockin allele. Exons are indicated as numbered black blocks. fLuc: firefly luciferase, NEO: neomycin cassette. (B) Southern blot of DNA from targeted clones after StuI digestion. The radioactive probe detects a 8.6 kb WT fragment and a 7.6 kb targeted fragment. WT: wild type. (C) Confirmation of luciferase expression (yellow) in Bmal1-P2A-dfLuc and (D) Bmal1::dLuc mESCs. Images were obtained using a LV200 microscope (Olympos).



Supplementary Figure 4. mESCs do not possess a functional transcriptional core clock. (A) Relative *Bmal1*, (B) *Per2* and (C) *Gm129* mRNA expression in synchronized mESCs, followed over the course of 48 hours. mRNA levels were normalized to the housekeeping gene *PPIA*.



Supplementary Figure 5. STRING-interaction analysis on upregulated genes in SR9009 treated mESCs.

6

General discussion

6.1 Introduction

The circadian clock is a conserved time-keeping system that adapts body physiology to light/dark cycles of approximately 24 hours. Cell-autonomous circadian oscillations dominate many processes such as cell cycle, differentiation, metabolism, aging, regeneration, and, of particular interest for this thesis, cardiovascular function. The latter is illustrated by the occurrence of distinct cardiac disorders upon clock impairment (Chang and Guarente, 2013; Dierickx et al., 2015; Eckel-Mahan and Sassone-Corsi, 2013; Feillet et al., 2014; Furlan et al., 2000; Janich et al., 2014; 2013; Martino et al., 2007).

In this chapter we elaborate on the importance of choosing the right cardiac system when wanting to study or model (a certain aspect of) circadian rhythmicity, and speculate on the potential of exploiting the clock for regenerative, cell-based cardiac therapy. We explain the pros and cons of the different techniques that are used to investigate the circadian clock, and discuss how our results on REV-ERB are in line with the current knowledge on this core clock factor.

6.2 Circadian networks in different cardiac cell types, which model to choose

6.2.1 Murine versus human systems

A number of cell types of distinct origin can be used to model circadian rhythmicity of the heart. In **chapter 1**, we describe multiple studies in both mouse and human that show how the circadian clock impacts cardiovascular function. For instance, a striking resemblance between human and mice is observed for circadian oscillations of infarct size that significantly depend on the time of the day at which the infarct takes place. As mice are nocturnal, biggest infarcts are noted in the early evening, while cardiac damage is reported to be largest in the sleep-to-wake transition in humans (Durgan and Young, 2012; Seneviratna et al., 2015).

Controlled settings are indispensable to study the circadian clock, and are relatively easy to obtain for mouse experiments. Because of the possibility to keep certain variables such as age, background and light-dark conditions constant, murine models are often of choice. In addition, in vivo oscillatory amplitudes are large (in comparison to those seen in in vitro systems) and several possibilities exist to manipulate the clock through genetic interference. Nevertheless, murine body physiology is significantly distinct when compared to humans, which is illustrated for the heart by a difference in beating frequency (10x higher in mouse), and a differential expression of ion channels such as Kv7.1 and hERG (Redfern et al., 2003; van Meer et al., 2016). As severe clock manipulation is infeasible in humans for obvious reasons, murine studies should be complemented with human model systems to study and explore the role and consequences of a functional clock in human cardiomyocytes.

6.2.2 In vitro differentiated cardiomyocytes

Human stem cell-derived cardiomyocytes hold great potential for the investigation of development as well as for cardiac disease modeling, and provide a platform for high-throughput screening of cardiac drugs in vitro. In **chapter** 2, we describe different cardiac differentiation and purification strategies. Nevertheless, in vitro differentiated cardiomyocytes have one important disadvantage, which is their "embryonic-like" character. This means that most mature cardiac markers are expressed at significantly lower levels in differentiated cells compared to observed levels in the heart. Indeed, RNA profiling techniques have revealed that in vitro derived cardiomyocytes tend to cluster with embryonic hearts rather than adult hearts (van den Berg et al., 2015). In addition, several functional parameters such as contractility and response to compounds are limited. Importantly, a number of recent studies have reported that multiple compounds can induce the maturation of stem cell-derived cardiomyocytes in vitro (Birket et al., 2015; Ribeiro et al., 2015), which opens up new opportunities and may result in cell type states that resemble the in vivo heart much better.

Next to the previously described progress on in vitro maturation of cardiomyocytes, multidimensional systems are emerging and are found to represent organ physiology better than monolayer cultures. To this end, co-culture systems, such as human engineered heart muscle (EHM) that consists of a mixture of human ES cell-derived cardiomyocytes and human fibroblasts embedded in an extracellular matrix, may offer new opportunities (Soong et al., 2012; Tiburcy et al., 2014; Zimmermann et al., 2002). These cardiac mixtures are cultured as ring-like structures in between two upstanding poles that put physical constraint on the rings. This results in maturation and a higher contractile force than would be generated in the absence of any physical constraint, and highlights that electrical pacing and mechanical stimulation of such heart constructs can promote maturation and structural parameters such as force generation (Ruan et al., 2016). Therefore, these types of systems are well suited to model the human heart.

6.2.3 Circadian networks in human stem cell-derived cardiomyocytes

Although circadian rhythmicity has been described in the heart of mice and humans (Reitz and Martino, 2015; Tsimakouridze et al., 2015), it has never been interrogated in human ES cell-derived cardiomyocytes. In **chapter 3,** we show the absence of a functional clock system in human ES cells, and demonstrate the gradual establishment of a clock during differentiation with robust rhythms in day 30 human ES cell-derived cardiomyocytes. At day 15, immature cardiomyocytes were found to show weak *Per2* oscillations, which is in line with previously observed *Per2* rhythms in mouse ES cells upon random differentiation (Umemura et al., 2013; Yagita et al., 2010). While these low-amplitude oscillations are probably indicative of a starting clock, we detect a robust clock system in human EHMs at day 15 of differentiation (unpublished data), which hints towards a maturation dependent

clock ontology. Since EHMs consist out of fibroblasts for ~50%, it cannot be ruled out that the rhythms that are picked up in EHMs at these time points are mainly stemming from fibroblasts. In addition, fibroblasts may stimulate rhythmicity in cardiomyocytes when cultured together as EHM. Nonetheless, transcripts that are measured through bulk RNA sequencing stem from both cell types, which is why uncoupling signal from multiple cell types remains challenging. Using fibroblasts and stem cells from a different and known genetic background, (common) variants may however be used to uncover the source of oscillatory transcripts.

Amongst the human cardiac clock-controlled transcripts that we identified in human ES cell-derived cardiomyocytes, a stress-response network, centered on *UBC*, was observed. This circadian network underlay a time-dependent response to doxorubicin, a widely used anti-cancer agent with severe cardio-toxic effects. Our dataset presents an excellent resource to mine for oscillatory transcripts that may also be expressed in a rhythmic way in the human heart. Indeed, next to genes that are known oscillators in mouse, we find some key cardiac genes that were newly identified as clock-dependent in human ES cell-derived cardiomyocytes. The obtained data could thus be used to explore the role of rhythmic expression in for instance the engraftment of derived cardiomyocytes when used for regenerative purposes. In conclusion, these results highlight that in vitro human stem cell based models will be of additional value when integrated with known or new insights that are gained from murine studies.

6.2.4 Testing circadian rhythmicity in systems that are relatively easy to use

While in vivo mouse studies as well as human ES cell-derived cardiomyocytes have their own advantages when modeling circadian oscillations of the heart, both systems are subject to practical limitations. Therefore, the need for a straightforward cardiac clock model, to study for instance the possible cardiac side effects of compounds on the clock, remains to be established. In **chapter 4,** we propose the use of neonatal rat cardiomyocytes to study the molecular clock of the heart, and show that these cells that are relatively easy to derive share oscillatory phenotypes with humans. A rhythmic response to multiple stressors, such as hypoxia, peroxide as well as doxorubicin was reported. In addition, compounds that interfere with the activity of SIRT1 (Gertz et al., 2013; Petrovski et al., 2011), a beneficial transcript for heart function (Zheng et al., 2013), affected the amplitude of observed oscillations. Since decreased clock amplitude is correlated with aging (Chang and Guarente, 2013), administration of different compounds might be tested to reverse the loss of clock amplitude. These results demonstrate that our in vitro neonatal rat cardiomyocyte model can be used to study a variety of clock-related mechanisms including the effect of widely used drugs on rhythmicity.

6.3 Different techniques provide different information on circadian rhythmicity

6.3.1 Reporter-based systems

A wide variety of techniques have been applied to study circadian clocks and the dynamics of gene expression in a plethora of different organs and cell types. Clock gene expression oscillates with a mere 24-hour period, and since most fluorescent proteins have a slow protein folding time and long half-life, clock reporter systems are mainly firefly luciferase-based. In **chapter 3, 4 and 5** we make use of lentiviral promoter-based systems to report clock gene expression and note clear anti-phasic oscillations of the *Bmal1* and *Per2* reporter constructs in a variety of cell types. The advantage of such reporters is that they are easy to introduce in different cell systems, that they report high luciferase levels and that they are a good measure of general promoter activity. However, since most of these reporters make use of a destabilized luciferase gene that is cloned downstream of a small promoter region, they do not always represent true protein levels and some monoclonal lines do not show (perfect) oscillatory patterns as a result of this. In addition, since these viral systems integrate randomly, they are not under the control of endogenous epigenetic processes and thus not regulated by putative enhancer elements within their genomic locus.

In **chapter 5** we constructed two novel *Bmal1*-luciferase reporter ES cell lines, for which destabilized luciferase (containing two degradation domains and preceded by a P2A signal) was knocked in, into the endogenous murine *Bmal1* locus through the use of TALEN technology. Additionally, we created another mouse ES cell line in which a Bmal1::fLuc fusion was generated. These lines were shown to report transcriptional and translational dynamics of *Bmal1* expression and translation after random differentiation of mouse ES cells. As gene-editing has become straightforward through the CRISPR-CAS9 technology (Cong et al., 2013; Mali et al., 2013), we stress that endogenous reporter strategies, rather than standard promoter-based lentiviral vectors, should be used when possible, to obtain results that also depend on *cis*-regulatory elements that may fine-tune oscillatory waves of gene expression.

6.3.2 Bioluminescence in cell population or single cells

The golden standard apparatus that is used to read out bioluminescence as a measure of clock gene dynamics is a Lumicycle device (Actimetrics). This luminometer can record the bioluminescent signal of 32 to 96 samples (35 mm-dish or 24-well format) in real time at 37 degrees. This medium-throughput format allows for a fast assessment of multiple conditions and/or a large number of replicates. Yet, this device consists of 4 different photon multiplier tubes with different sensitivity, which makes a quantitative comparison between samples (measured with different detectors) rather difficult. Additionally, the system cannot be used in humid environments, forcing samples to be sealed because of which medium needs to be buffered with HEPES or MOPS. These considerations highlights

some of the pros and the cons of the Lumicycle, a system that allows for a relatively easy readout of bioluminescence but is insufficient for specialized measurements of vulnerable cell types that do not survive in buffered recording medium.

The LV200 microscope provides an alternative and measures bioluminescence within one field of view in a single dish, at the single cell resolution. As a result, spatial information on reporter gene expression can be gained. This microscope allows for combined fluorescence detection and contains a small incubator, because of which the need for buffering supplements in the medium is omitted. In **chapter 3**, we demonstrate that circadian rhythms are present on a single cell level in day 30 human ES cell-derived cardiomyocytes using the LV200, but notice that not all cells are oscillating in phase. This highlights the added value of single-cell measurements, which allow for the identification of non-synchronized rhythms in a population of cells as well as the discovery of pioneer cells that may establish rhythms.

6.3.3 RNA sequencing as a powerful technique to uncover novel oscillators

Another important readout of circadian rhythmicity is to measure the oscillatory expression of clock-controlled output genes (CCGs). The detection of oscillatory transcripts has mostly relied on micro-arrays in the past, but recent technological advancements have made RNA sequencing the new standard. Nonetheless, standard clock experiments contain many samples and/or replicates, which makes these assays extremely expensive. In **chapter 3**, we apply CEL-Seq (Hashimshony et al., 2012), an mRNA-profiling technique based on sequencing the 3'UTR of mRNAs, for the identification of CCGs in human ES cell-derived cardiomyocytes. The tremendous advantage of CEL-Seq is the possibility to multiplex. This means that different samples, which are labeled with specific barcodes, can be pooled for library preparation, which significantly decreases the financial burden of these types of experiments. The downside of this technique however, is that lowly expressed genes are typically not picked up because of which clock genes often have low read counts. In addition, as it is based on 3' UTR sequencing, it does not allow for the assessment of different transcript variants. Nevertheless, as deep mRNA sequencing is often unrealistic, CEL-Seq does present as a cost-affective approach for the detection of novel oscillators.

The use of RNA sequencing for the detection of CCGs has been shown to depend strongly on sampling frequency and sequencing depth. The proposed sampling frequency is every 2 hours across 2 consecutive days, with a depth of around 20-25 million reads per sample (Li et al., 2015), and will result in the optimal detection of real oscillators, with a minimum of false-positives. In line with this, it has been postulated that highly expressed genes are more likely to be identified as oscillator. In **chapter 3**, we clearly observed this phenomenon (Supplemental Figure S4) with highly expressed genes more often called as significant oscillators compared to lowly expressed (non-oscillating) transcripts. Importantly, since we did not sequence at high depth, we need to take into account that our lists of oscillators may contain some false-positives, and that other rhythmic genes possibly remained undetected

(false-negatives). In conclusion, RNA sequencing (in the form of CEL-Seq) represents a powerful tool to detect oscillatory gene expression, and will allow for a better identification of CCGs in many distinct cell types and tissues.

6.4 Clock-dependency of circadian oscillations

6.4.1 Clock perturbation and (loss of) rhythmicity

Several knockout animals have been engineered to study the role of single core clock transcription factors. However, the clock pathway is highly conserved and extremely robust because of several homologous genes, which often results in the need of double or triple knockouts when trying to shut down the circadian clock completely (Dierickx et al., 2015). Previously, it was thought that circadian oscillations were solely controlled by a master clock in the brain, but recent findings indicate that many genetic and environmental factors are able to influence and drive rhythmic gene expression of CCGs, that are often found to be tissue-specific (Patel et al., 2014). Combined for different organs, ~68% of the protein coding transcripts have now been shown to be expressed in an oscillatory manner in at least one organ (Patel et al., 2015). How tissue-specific output is driven remains largely unknown, but an important notion is that certain clock knockout animals still show a set of genes with oscillatory expression (Yang et al., 2016). This underscores that clock perturbation does not necessarily result in a complete absence of rhythmic transcripts, and raises the question on how many of the identified (tissue-specific) CCGs are actually regulated by classical rhythmic BMAL1:CLOCK binding.

In **chapter 3** and **5**, we show that human and mouse ES cells do not possess a functional clock. Therefore, ES cells present as a good model to study clock-independent oscillations, and others and us have indeed detected rhythmic transcripts in the absence of a circadian clock. For instance, *Slc2a1* as well as *Slc2a8* (encoding the glucose transporters GLUT1 and GLUT8; respectively) were found to be expressed in a time-dependent manner (Paulose et al., 2012), and in accordance with this mouse ES cells showed rhythmic uptake of the glucose analog 2-deoxy-glucose (Paulose et al., 2012). This suggests that ES cells show clock machinery independent metabolic oscillations and highlights that it may be interesting to profile the metabolism and gene expression profile of stem cells over time to uncover the (non-canonical clock) factors that may couple transcription to metabolic rhythmicity.

6.4.2 The non-circadian role of clock factors in ES cells

While metabolic oscillations are independent of a functional clock in mouse ES cells, we speculate that clock factors may still have a non-circadian role in these pluripotent cells. In **chapter 3** and **5**, we show that ES cells have a different stoichiometry for a number of core clock genes compared to differentiated cells. Nonetheless, mRNA of all clock genes that were assessed could be detected in mouse ES cells and in **chapter 5**, we present the

consequences of REV-ERB modulation in mESCs through the administration of the synthetic agonist SR9009. In both mice and leukemia stem cells, this compound has been shown to abolish the circadian clock, and concomitantly drive metabolism alterations in mice (Puram et al., 2016; Solt et al., 2012). We find that SR9009 induces a metabolic shift in mESCs too, which is accompanied by extensive changes on the transcriptomic level and ultimately results in a slow down of proliferation. Amongst the upregulated genes upon REV-ERB agonist administration, we find a set of genes that is involved in the amino acid starvation response pathway, but many more genes were affected. Genome-wide assessment of REV-ERB binding to the DNA via Chromatin Immunoprecipitation (ChIP) could clarify whether the observed effects can be attributed directly to REV-ERB presence at their promoter or maybe even *cis*-regulatory elements in their proximity. Overexpression as well as knockout experiments for REV-VERB could help to solidify these clock-independent effects of REV-ERB stimulation. We speculate that differences in cofactors could underlie the distinct effect of REV-ERBs agonists in differentiated versus pluripotent stem cell populations. While REV-ERB inhibits gene expression via the recruitment of NCoR-HDAC3 complexes in somatic cells, different rules may apply for stem cells. Nonetheless, these data indicate that the core clock gene REV-ERB most likely has a function in ES cells and that addition of a REV-ERB agonist has severe consequences on stem cells (in vitro).

6.5 Concluding remarks

Many of the mechanisms that underlie the clock pathway have been uncovered, and circadian rhythmicity is mainly established through a transcriptional/translational feedback loop of core clock genes (Takahashi, 2015). This knowledge, in combination with the time-dependent occurrence of cardiac events, has driven multiple scientific groups to study the link between the time at which cardiac stress occurs and the magnitude of resulting damage in both humans and mice (Bray et al., 2008; Durgan and Young, 2012; Durgan et al., 2010; 2011; Ibanez et al., 2012; Reiter et al., 2012; Seneviratna et al., 2015; Suarez-Barrientos et al., 2011). While this link has been proven to be present, the exact molecular mechanisms that contribute to this phenomenon in the heart remain largely unknown. Next to in vivo experiments, (straightforward) cardiac in vitro systems will have to shed light on the pathways that play a role in this. Nevertheless, a limited number of efforts have focused on how the circadian clock influences the function and potential of cells in tissue culture.
In this thesis, we demonstrate the profound role of a functional clock in cultured cells, and show that circadian rhythmicity influences the outcome of tissue culture experiments. In addition, we developed a model system to study the cardiovascular clock in an in vitro setting, and show that clock factors can even play a role in stem cells that do not have a functional clock system.

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Addendum

Nederlandstalige samenvatting

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Nederlandstalige samenvatting

Cardiovasculaire ziektes zijn nog steeds de grootste doodsoorzaak in de westerse wereld, en worden beïnvloed door de circadiane klok. Deze zorgt dat bepaalde lichaamsprocessen volgens een vast 24-uurs ritme verlopen, en is ervoor verantwoordelijk dat sommige genen een oscillerend expressiepatroon hebben in het hart. Niet alleen voor transcriptie, maar ook op functioneel vlak spelen deze ritmes een bijzonder grote rol. Dit wordt geïllustreerd door een aantal metabole en slaapgerelateerde ziektes, die het gevolg zijn van een verstoorde circadiane klok.

Uitgaande van pluripotente embryonale stamcellen, is het inmiddels vrij gemakkelijk om in vitro via een goed gekarakteriseerd proces hartspiercellen (cardiomyocyten) te maken. Dit zorgt voor een onuitputtelijke bron aan materiaal voor onderzoek naar de ontwikkeling en functie van het hart. Daarenboven kunnen stamcel-afgeleide hartcellen gebruikt worden om bepaalde medicijnen te testen, en hebben ze potentie om ingezet te worden voor transplantatie bij patiënten die lijden aan hartfalen en waarbij dus een groot aantal van de hartspiercellen is afgestorven (bv. na een hartinfarct).

Of en hoe we circadiane ritmes kunnen exploiteren om het gebruik van stamcel-afgeleide hartspiercellen te optimaliseren, is tot op heden grotendeels onbekend. Daarom hebben we in dit proefschrift uitgebreid de rol onderzocht van klokfactoren en circadiane netwerken in zowel embryonale stamcellen als daarvan afgeleide cardiale celtypes. In **hoofdstuk 1** geven we een gedetailleerd overzicht van de rol van 24-uurs ritmes in cardiovasculaire fysiologie. We beschrijven hoe het verstoren van de circadiane klok de werking van het hart schaadt, en lichten toe hoe kennis van de klok kan worden geïmplementeerd in de context van regeneratieve harttherapie. In **hoofdstuk 2** beschrijven we verschillende strategieën waarmee hartspiercellen kunnen worden afgeleid van pluripotente stamcellen. We leggen in detail uit hoe deze cardiomyocyten kunnen worden gekarakteriseerd, en hoe ze kunnen gebruikt worden voor regeneratieve doeleinden.

Aangezien hiervoor nog niets bekend was over circadiane ritmes in humane stamcel-gedifferentieerde hartspiercellen, hebben we in **hoofdstuk 3** onderzocht of ook dit celtype een klok bezit en welke implicaties dit heeft. Het is namelijk aangetoond dat de biologische klok cel-autonoom kan zijn, wat betekent dat er niet noodzakelijk prikkels van buitenaf nodig zijn om ritmes te induceren. We laten zien dat zowel op genexpressie-, als op eiwitniveau, humane embryonale stamcellen geen functioneel kloksysteem bezitten, wat waarschijnlijk komt doordat zij minder klokfactoren bevatten dan gedifferentieerde celtypes. Dit wordt bevestigd in onze verdere analyse, want tijdens het differentiëren van pluripotente cellen naar hartspiercellen hebben we een gradueel opkomende klok geobserveerd, die robuust blijkt te oscilleren na 30 dagen. Met behulp van bioluminescentie-gebaseerde microscopie hebben we ook laten zien dat deze klok in afzonderlijke hartspiercellen aanwezig is. Naast de oscillatie van enkele bekende klokfactoren, laten we zien dat de expressie van zo'n 600

andere genen ook een 24-uurs ritme volgt. Deze genen lijken grotendeels betrokken in een netwerk van stress-geassocieerde transcripten, hetgeen eerder ook werd waargenomen in muizenharten. We tonen aan dat dit stressnetwerk in stamcel-afgeleide hartspiercellen een ritmische respons op schade tot gevolg heeft door middel van toediening van het anti-kankermiddel doxorubicin dat cardiotoxische bijwerkingen heeft. We laten zien dat de hoeveelheid schade correleert met het tijdstip waarop het medicijn wordt toegediend, wat aantoont dat het toedienen van medicijnen op een bepaald moment van de dag tot meer of minder neveneffecten kan leiden. Dit is niet alleen relevant voor het hart, maar kan ook van groot belang kan zijn voor andere op medicijnen gebaseerde therapieën.

In **hoofdstuk 4** beschrijven we het gebruik van neonatale hartspiercellen van de rat als een goed en eenvoudig model om circadiane ritmes in het hart te bestuderen. We hebben gevonden dat deze cellen in vitro nog steeds een functionele klok bezitten, die een invloed heeft op het ritmische gedrag van deze cellen, zoals bijvoorbeeld de oscillerende klopfrequentie. Aangezien hartinfarctgrootte bij de mens effectief grotendeels tijdsafhankelijk is, hebben we gekeken of dit fenomeen ook voorkomt in neonatale rathartspiercellen. Door de cellen op meerdere tijdstippen onderhevig te maken aan verschillende stresscondities en vervolgens celdood uit te meten, tonen we aan dat de grootte van hartspierschade effectief tijdsafhankelijk is in deze cellen. Dit bewijst dat neonatale hartspiercellen van de rat een eenvoudig en goed model zijn om circadiane cardiovasculaire processen in vitro te bestuderen. Vervolgens hebben we het effect van verschillende stoffen die inwerken op het hart, op de moleculaire klok bekeken. Sommige van deze stoffen waren van invloed op de amplitudes van de klok, hetgeen nefast kan zijn voor het correct functioneren van het hart. We concluderen uit deze studie dat neonatale hartspiercellen van de rat een goed model zijn om de cardiale klok te bestuderen, en daarbij dat dit systeem geschikt is om snel en eenvoudig het effect van verschillende medicijnen op de circadiane klok te testen.

De nucleaire receptoren REV-ERBα en REV-ERBβ zijn twee eiwitten met een belangrijke rol in het aansturen van zowel 24-uurs ritmes als metabolisme, en daarenboven mediëren ze de wisselwerking tussen deze twee fundamentele processen. Hoewel pluripotente embryonale stamcellen van de muis geen functionele klok bezitten, komen beide *Rev-erb* genen wel tot expressie. In **hoofdstuk 5** onderzoeken we de (metabole) rol van REV-ERBα en REV-ERBβ in embryonale stamcellen via het stimuleren van REV-ERB activiteit door toevoeging van de synthetische agonist SR9009. In muizen leidt de activatie van REV-ERB tot een verhoogd energieverbruik, en dit maakt dat SR9009 wellicht gebruikt kan worden als anti-obesitas medicijn. Naast deze metabole effecten is ook aangetoond dat SR9009 somatische celdeling vertraagt, wat doet vermoeden dat REV-ERB een uitgebreidere rol speelt in het integreren van metabole en proliferatieve processen. Via het gebruik van een gespecialiseerde “Seahorse” techniek, laten we in ons onderzoek zien dat SR9009 het metabole profiel van embryonale stamcellen herprogrammeert en de glycolytische flux van deze cellen verhoogt. We observeren ook dat SR9009 de celdelingsnelheid van embryonale stamcellen, die geen functionele klok bezitten, vertraagt. Daarnaast tonen

we via RNA sequencing aan dat SR9009 het transcriptoom van embryonale stamcellen verandert, met grote aanpassingen ter hoogte van aminozuurmetabolisme genen. In het bijzonder genen van de aminozuurnood signaalweg worden geactiveerd, en het gen *Atf4* speelt hierin een centrale rol. Dit alles toont aan dat REV-ERB belangrijke klokonafhankelijke effecten kan veroorzaken in stamcelpopulaties, en dat toediening van SR9009 onverwachte consequenties kan hebben.

Autonome circadiane oscillaties van de cel beïnvloeden vele biologische processen zoals de celcyclus, differentiatie, metabolisme, veroudering, regeneratie, en van uiterst belang voor dit onderzoek, cardiovasculaire functies. In **hoofdstuk 6** beschrijven we dat wanneer men bepaalde aspecten van de circadiane klok wil bestuderen, het van uiterst groot belang is dat het juiste cardiale systeem gebruikt wordt om dit te analyseren. Dat het systeem van tijdsbesef binnen cellen dat lichaamsfysiologie aanpast aan licht/donker cycli van ongeveer 24 uur van grote importantie is, is inmiddels duidelijk. In dit proefschrift is de circadiane klok bestudeerd in stamcellen en daarvan afgeleide hartspiercellen. De studie naar 24-uurs ritmes in deze celtypes levert ons informatie over hartspecificieke oscillerende genexpressiepatronen en functionaliteit in het algemeen. Deze kennis kan (op termijn) gebruikt gaan worden om regeneratieve harttherapie te optimaliseren.

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Curriculum Vitae

Pieterjan Dierickx was born on the 2nd of January, 1988 in Dendermonde in Belgium. In 2006 he earned his diploma in Sciences and Mathematics at the Heilige Maagd College in Dendermonde. In 2009 Pieterjan finished his Bachelors in Biochemistry & Biotechnology. In his last year of his Masters he did an internship and wrote his final thesis on the generation of a conditional and inducible mouse model to study cellular (de)differentiation in the lab of Prof. dr. Jody Haigh. In 2011 Pieterjan graduated with high distinction from his masters in Biomedical Biotechnology. He earned an FWO PhD fellowship and started his PhD in the lab of prof. dr. J. Haigh at the VIB in Gent, Belgium. A few months later, he continued his PhD in the lab of Prof. dr. Niels Geijsen in collaboration and under supervision of dr. Linda van Laake at the Hubrecht Institute, Utrecht, The Netherlands. The results of this research are described in this dissertation.

List of Publications

Pieterjan Dierickx , Pieter A. Doevendans , Niels Geijsen and Linda W. van Laake. (2012) Generation of Pluripotent Stem Cell-Derived Cardiomyocytes for Heart Repair.
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Pieterjan Dierickx and Linda L. W. Van Laake. (2016) Muscle-on-chip: An in vitro model for donor-host cardiomyocyte coupling.
The Journal of Cell Biology 212, 371–373.

Stijn J.A. Aper, **Pieterjan Dierickx** and Maarten Merkx. (2016) Dual readout BRET/FRET-sensors for measuring intracellular zinc.
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Pieterjan Dierickx, Marit W. Vermunt, Mauro J. Muraro, Menno P. Creyghton, Pieter A. Doevendans, Alexander van Oudenaarden, Niels Geijsen and Linda W. van Laake. Functional implications of circadian networks in human embryonic stem cell-derived cardiomyocytes.
(Under Revision)

Pieterjan Dierickx[*], Bastiaan C du Pré[*], Pieter A Doevendans, Marc A Vos, Niels Geijsen, Didi Neutel, Toon AB van Veen and Linda W. van Laake. Neonatal rat cardiomyocytes serve as a good model for in vitro cardiac compound testing on the circadian clock.
[*]*These authors contributed equally to this work*
(In preparation)

Pieterjan Dierickx, Vincent de Boer, Mauro J. Muraro, Peng Shang, Pieter A. Doevendans, Alexander van Oudenaarden, Jaap Keijer, Linda W. van Laake and Niels Geijsen.The REV-ERB agonist SR9009 drives a metabolic shift and activates the amino acid response pathway in mouse embryonic stem cells.
(In Preparation)

Bastiaan C du Pré, Evelyne J Demkes, Dries AM Feyen, **Pieterjan Dierickx**, Joost Sluijter, Pieter A Doevendans, Marc A Vos, Toon AB van Veen and Linda W van Laake. Sca1^{+} Cells from the Heart Possess a Molecular Circadian Clock and Display Circadian Oscillations in Proliferation, Stress Tolerance, and Paracrine Factor Secretion.
(In Preparation)