Circadian Rhythms in Cardiovascular Disease
From Bench to Bedside

Bastiaan du Pré

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Circadiane Ritmes in Hart- en Vaatziekten:
Van Pipet naar Patiënt.

(met samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 1 maart 2018 des middags te 12.45 uur

door

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geboren op 31 maart 1988 te Utrecht

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Il n’est point nécessaire pour ce phénomène qu’elle soit au soleil
de Marain, 1729, Observation Botanique

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Preface

"Let there be light", is the first thing God said according to Genesis, the first book of the Bible and Torah, thereby creating day and night; and "Sol Iustitiae Illustra Nos" is the motto of this university. It is no coincidence that the most-read book of all time and the motto of our university start with the difference between day and night. Light shines on our planet in a 24-hour cycle and all life, from bacteria to plants and mammals, experience two completely different environmental conditions every day, requiring continuous adaptation.

People have been interested in 24-hour rhythms throughout history. 3.5 millennia ago, the people of Babylon already used sundials to analyse time. In 1729, Jean Jacques d'Ortous de Mairan discovered, by studying the diurnal opening and closure of the leaves of Mimosa Pudica, that life possesses an intrinsic mechanism that anticipates to diurnal changes.[1] In the 1980's, pioneers Jeffrey Hall, Michael Rosbash, and Michael Young elucidated that this intrinsic mechanism consists of a molecular clock.[2, 3] More recently, various researchers throughout the world realised that circadian rhythms, 24-hour rhythms regulated by the molecular circadian clock, are not only an interesting phenomenon; circadian rhythms play a major role of in the development of various diseases, including infections, cancer, and cardiovascular disease.

Cardiovascular disease is the leading cause of morbidity and mortality throughout the world. The last decades, major progress has been made in prevention and treatment, with a reduction in mortality of approximately 50%.[4] This is the result of societal healthcare changes, the introduction of percutaneous coronary interventions and medication such as antiplatelet, antihypertensive, and antihyperlipidemic drugs. The last decade however, further reduction in cardiovascular mortality is slowing.[5] In addition, reduced mortality in combination with an aging, fattening, and more diabetic population has led to an increase in cardiovascular morbidity.[6] This trend occurs despite an increasing amount of money spent and articles published on cardiovascular research. Novel concepts are necessary to better understand cardiovascular (patho) physiology, invent novel therapeutic strategies and improve treatments already available.

This thesis aims to introduce circadian rhythmicity as a novel concept in the (patho) physiology and treatment of cardiovascular disease and addresses the following questions:

1. Are circadian rhythms present in the heart / cardiovascular cell types?
2. What is the physiological role of circadian rhythms in these cells?
3. What is the role of circadian rhythms in the development of cardiovascular disease?
4. Can we use circadian rhythms to treat the cardiovascular patient?

Chapter 1 introduces circadian rhythms, the molecular clock machinery, and provides an overview of current literature about the role of circadian rhythms in mammalian physiology, especially the cardiovascular system. Development of circadian clocks, both the central clock in the brain and peripheral clocks in somatic cells is described in **Chapter 2**. Literature about physiological *in utero* development and *in vitro* differentiation is summarised and compared. **Chapter 3** is an introduction to the first experimental chapter (4) and gives a detailed overview regarding the principles of stem cell based cardiac repair. In this chapter we discuss cell types used and summarize challenges in both pre-clinical and clinical research. In **Chapter 4** we analyse the presence of a molecular clock in SCA1+ cells, a cell type frequently used in pharmacological and cardiac repair studies. Also, we investigate whether 24-hour rhythms are present at a functional level. Pre-clinical tests that analyse circadian effects on the heart are currently performed in animals. In **Chapter 5** we hypothesise that neonatal rat cardiomyocytes can be used as an *in vitro* model for circadian rhythmicity in the heart. In our experiments, we test whether these cells mimic an *in vivo* situation at the molecular and functional level, and if compounds known to influence the circadian clock, alter molecular and functional rhythmicity in the model. 24-hour rhythmicity is present in the incidence and outcome of myocardial infarction that previously showed to be regulated by the molecular clock. This rhythm however, is not present in all studies and the moment of maximal damage differs. In **Chapter 6** we investigate whether these differences can be attributed to the model of myocardial infarction used. **Chapter 7** addresses the final question of this thesis. In two retrospective studies, we compare 24-hour variation in ventricular repolarization to the occurrence of ventricular arrhythmias and potential dysfunction of the hERG channel, an important component for ventricular repolarization. In **Chapter 8**, the overall results of this thesis are discussed and put into retrospect.

Chapter 1

Circadian clocks, rhythms and function

Chapter 1 - Circadian clocks, rhythms 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.

Here, we review the function of circadian rhythms. We describe the molecular circadian clock and how the circadian clock influences myocardial biology.

Introduction

Our planet turns around every 24 hours. Anticipation to changes caused by this rotation, such as dark / light and temperature changes, has advantages on a population, organism, and cellular level. Information about the time-of-day allows preparing for an event before it starts and enables a rapid and adequate response. During the Babylonian, Egyptian, Greek, and Roman ages, people already understood the importance of knowing the time of the day and developed sundials (shadow clocks) to estimate the time. Diurnal patterns in organisms, such as closure of Tamarind tree leaves in the evening and diurnal differences in pulse rate have been appreciated and documented over many centuries.[7, 8] The idea of an endogenous clock was first raised in 1792. Jean–Jacques d'Ortous de Mairan described that the 24-hour pattern in the opening and closure of leaves of the Mimosa pudic plant continued in complete darkness.[9] Other scientists however, could not believe that a biological process can create a non-variable period of 24 hours over a wide variety of temperatures (known as temperature compensation) and attributed the findings of d'Ortous de Mairan and others to changes in temperature, humidity, or an unknown factor X; it was only in the 1970s that there was consensus about the existence of an internal clock that anticipates diurnal environmental cues.[10]

Circadian Rhythms

Circadian rhythms, diurnal rhythms, and circadian clocks are terms used in chronobiology, which are interchanged in literature and this review but have a different meaning. Diurnal rhythms are patterns that recur every 24 hours, whereas circadian rhythms are biorhythms that persist with a period of ~24 hours in the absence of external cues, but remain responsive to environmental conditions such as light and exercise.[11] Circadian rhythms thus differ from diurnal rhythms based on their self-sustainability.

Circadian rhythms are regulated by circadian, endogenous clocks. These endogenous clocks can be subdivided into a 'master clock' that is located in the suprachiasmatic nucleus (SCN) of the brain and 'peripheral clocks' that are present in essentially all mammalian cells. (Figure 1.1) The core clock components in cells of the master clock and peripheral cells are similar, but input and output signals can differ. The master clock was discovered in 1972 by SCN lesion studies and is the most commonly studied endogenous clock.[12, 13] Presence or absence of light is the main input signal (Zeitgeber or timekeeper) of the master clock in adults.[14] Retinal photoreceptors in the eye receive photic information and convey this information via the retino-hypothalamic tract to the SCN. Non-photic SCN Zeitgebers in adults include exercise and social behaviour.[15] The output of the master clock consists of several neurohumoral signals such as TGF-α, cardiotrophin-like cytokine, and prokineticin 2 which orchestrate circadian rhythms throughout the organism, either directly or by regulating peripheral circadian clocks.[16-18]

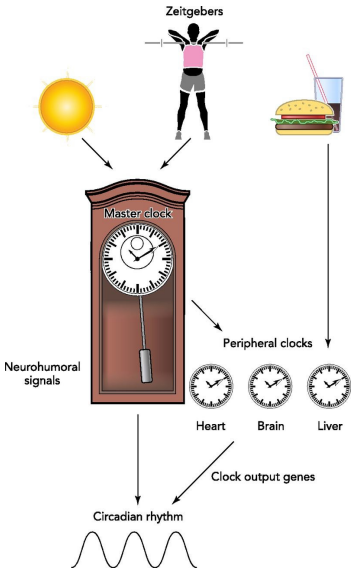

Figure 1.1. Circadian rhythms are regulated by (1) the master clock via neurohumoral signals and by (2) peripheral clocks that are located in almost every cell.

Following their first description approximately 15 years ago, it is now apparent that peripheral clocks are present in almost every mammalian cell. Perception of the relationship between peripheral clocks and the master clock has changed relatively recently.[19, 20] Traditionally, peripheral clocks were considered to be 'executors' of the central clock. In this view, peripheral clocks simply receive time-of-day information from the master clock and respond accordingly ("master-slave model"). However, peripheral clocks are now considered autonomous in nature, being able to function independently of the SCN. The master clock functions as a conductor and harmonizes peripheral clocks ("orchestra model").[21] A good example of the autonomy of peripheral clocks is restricted feeding. When food is offered at 'inappropriate' times (e.g., restricted to the sleep phase), peripheral clocks (e.g., in the liver) adjust their phase to the new feeding regime, while the master clock will remain in phase with the light/dark cycle.[22]

Diurnal rhythms have a marked impact on health. Physiological parameters such as blood pressure, heart rate, body temperature, metabolism, and hormone levels have diurnal patterns, while at the cellular level ~10% of the transcriptome exhibits circadian oscillations in expression.[23-26] Disruption of diurnal rhythms is associated with disease in multiple organ systems, including the cardiovascular system, kidneys, gastrointestinal system, skeletal muscle, endocrine system, immune system, and reproductive system.[20] In the cardiovascular system for example, the occurrence of several diseases (e.g., the onset of myocardial infarction) has a diurnal pattern. Similarly, disruption of normal day-night rhythms (e.g. by shift work or mutation of clock genes) results in an increased cardiovascular risk, and outcome of cardiovascular events such as myocardial infarction is time-of-day dependent.[27-33]

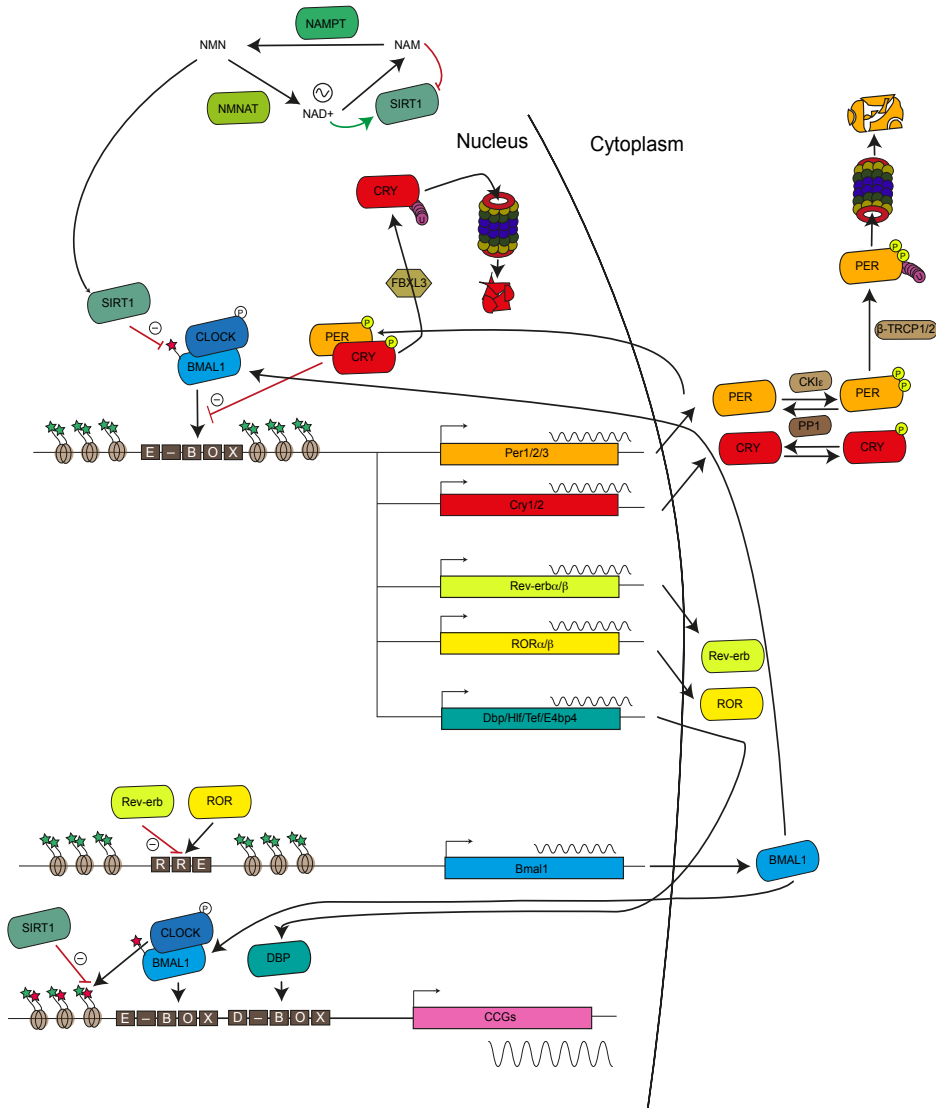

Figure 1.2. Schematic representation of the transcriptional/translational feedback loop of the circadian core clock pathway in mammals. The central proteins BMAL1 and CLOCK form a heterodimer and bind E-box elements in the promoter of period (Per1 / Per2) and cryptochrome (Cry1 / Cry2) genes. PER and 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 dimerise 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 hours, 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

The core clock machinery

A complex transcriptional feedback loop defines the molecular clock

The circadian clock is a molecular clock, that 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. 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-Arntl-Single-minded*) proteins and form the centre of the activating limb of the circadian clock pathway. Upon heterodimerisation via their PAS domains, they drive gene expression through docking on two types of enhancer box elements (E-Boxes): E-box (5'-CACGTG-3') and E'-box (5'-CACGTT-3'). These E-boxes are located near or in the promoter of their targets, termed clock controlled genes.[34-37] Knocking out *Bmal1* results in the complete loss of behavioural rhythmicity.[38, 39] *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 behaviour,[40] which is lost in *Clock/Npas2* double knockouts.[41]

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.[42, 43] PER and CRY proteins accumulate and dimerise in the cytoplasm, where their presence is tightly regulated. They can either be stabilized by phosphatase1[44] or phosphorylated by casein kinases[45-47] resulting in active degradation. For CRY1 this process occurs via ubiquitination by F-box and leucine-rich repeat protein 21.[48-50] Phosphorylated PER2 can be polyubiquitinated by β-TRCP1/2 resulting in proteasomal degradation.[51] In general, dimerization of PER:CRY in the cytoplasm protects both proteins from degradation.[52] Upon stabilization, the PER:CRY dimer translocates into the nucleus[53] forming a nuclear complex.[54] There, the dimer binds to the NuRD (nucleosome remodelling deacetylases) transcriptional repressor complex and directs NuRD to BMAL1:CLOCK.[55] A functional NuRD repressing complex is established, which inhibits BMAL1:CLOCK driven transcription. Through this process, PER and CRY inhibit multiple CCGs as well as their own transcription.[36,56, 57] PER initiates this negative feedback loop by functioning as a molecular scaffold, that brings CRY into contact with BMAL1:CLOCK.[58] 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 in a mutant model that causes enhanced inhibition of BMAL1:CLOCK-based transcription and results in period lengthening.[59] 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 RORαβ and REV-ERBα/βencoded 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.[60-62] 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.[23, 63] Binding of RORs drives transcription of *Bmal1* while REV-ERBα inhibits its transcription.[60, 62, 64] Although *Rorα* mRNA levels are only slightly oscillating, RORα is necessary for rhythmic *Bmal1*.[64]

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).[65] Albumin D-site-binding protein (DBP), thyrotroph embryonic factor (TEF), and hepatic leukaemia 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 1.2.

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.[66] H3K9ac and H3K14ac are both markers for permissive transcription. Therefore, BMAL1:CLOCK also regulates transcription of CCGs via modifying their histones.[67] This function is neutralized by several histone deacetylases (HDACs), as described in more detail by Steven Brown.[68]

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.[69] Additionally, SIRT1 is now known to deacetylate the proteins BMAL1, PER2[70] and histone 3 (H3) on the promoter of clock output genes like *Dbp*.[71] 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.[72]

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 fibre, controlling CCG expression. One of these histone methyl transferases (HMTs) is mixed lineage leukaemia 1 (MLL1) that can recruit the BMAL1:CLOCK dimer to the DNA of target genes and cause rhythmic H3K4 trimethylation.[73] 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.[74] The same holds true for LSD1, another histone demethylase whose activity depends on circadian phosphorylation by PKCα.[75]

Additional regulatory systems fine-tune circadian rhythmicity

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). This RNA binding protein regulates circadian rhythmicity during temperature oscillations. CIRP binds to Clock mRNA and stabilizes it, linking temperature to the circadian clock.[76] Next, cyclic alternative splicing[77] as well as light inducible alternative splicing[78] are common factors that regulate circadian rhythms. Third, rhythmic polyadenylation that stabilizes mRNA molecules, facilitates circadian rhythmicity in protein translation.[79] Fourth, fluctuations in m(6)A-RNA methylation affect the circadian transcriptome [80] and finally, non-coding RNAs such as anti-sense RNAs and miRNAs affect circadian rhythms. This latter is demonstrated by a disrupted circadian transcriptome in cells that lack *Dicer*, a gene involved in miRNA processing.[81, 82] In conclusion, the circadian clock is a molecular complex orchestrated interplay between genetics, epigenetics and translational processes.

Circadian rhythms in tissue/organ physiology

Tissue specific control of clock output

The core clock machinery is conserved in all peripheral organs. Peripheral cellular clocks drive extensive rhythms of gene transcription, with 3–10% of all mRNAs in a given tissue showing diurnal rhythms.[25, 83, 84] Remarkably, there are significant differences between tissues in the relative contributions of the clock components, as well as in output pathways that are under their control; genes under circadian control are largely non-overlapping in different tissues. 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.

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.[85] In the adrenal glands, many genes involved in the biosynthesis of corticosterone are clock-controlled. Adrenal gland-specific disruption of *Bmal1* interrupts the ability of the organ to maintain proper oscillatory secretion of corticosterone.[86] In pancreatic islets, the circadian clock helps regulating glucose-stimulated insulin secretion, the loss of which impairs glucose tolerance because of β-cell dysfunction.[87]

Peripheral clocks also play a profound role in the cardiovascular system.[88, 89] 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.[90] 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.[91] *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.* showed that the time to thrombotic vascular occlusion in response to a photochemical injury displays diurnal variation.[90] 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.

Circadian rhythms in the heart

In the heart, rhythmic physiology has mostly been studied in mice harbouring 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. Expression of various ion channels was found to be clock dependent. Furthermore, Connexin 40, a gap junction protein critical in atrial-ventricular conduction, showed oscillatory expression in WT hearts, that was absent in CCM hearts.[92]

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 fuelling the contraction of the myocardium are fatty acids and glucose.[93] 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.[92] 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 absent in CCM hearts. Furthermore, CCM mice display an altered response to high fat diet, demonstrating the role of the cardiomyocyte circadian clock in the regulation of nonoxidative fatty acid metabolism.[94] Similarly, epinephrine-induced glycogenolysis has a time of the day dependent activity in WT hearts that is suppressed in CCM hearts.[92]

Furthermore, hearts from 22-months-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 signalling pathway (Akt, GSK-3β, and p70S6K) all oscillate in hearts over the course of the day, and are continually elevated in CCM hearts.[32] 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.[92]

In summary, peripheral clocks allow for tissue-specific rhythmic gene expression that enables organs to anticipate on their diurnal tasks. In the heart, metabolism and growth are under control of the cardiomyocyte circadian clock, which allows optimal performance during periods of activity and rest.

Chapter 2

Circadian rhythms in cell maturation

Chapter 2 - Circadian Rhythms in Cell Maturation

Bastiaan C du Pré, Toon AB Van Veen, Martin E Young, Marc A Vos, Pieter A Doevendans, Linda W Van Laake

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Abstract

Circadian rhythms are of major importance in mammalian physiology and disease. Circadian rhythms are regulated by circadian clocks. A central, 'master' clock is located in the brain and orchestrates time-of-day dependent function of nearby and distant tissues via neurohumoral signals. Each mammalian cell also has its own 'peripheral' clock that can regulate circadian variations in cell function autonomously. As such, the central and peripheral clocks together determine the time-dependent state of the mammal from the molecular level to the whole organism. Research concerning circadian rhythms has been fragmented. Three decades ago, the role of developing circadian rhythms was investigated using animal models in which the central clock was disrupted mechanically. This revealed the importance of maternal circadian rhythms in foetal development. Later, molecular techniques showed the master clock is a self-sustainable, molecular mechanism effectuated by 12 core proteins. Core clock proteins are already present at the embryonic stem cell stage, but their expression levels increase and start oscillating during embryonic development. Molecular techniques also demonstrated that circadian clocks are present not only in the brain, but in almost all mammalian cells. This notion shifted chronobiology research from the brain to many other organ systems. Recently, regenerative medicine has renewed attention to developmental biology, including the origination of circadian rhythms. In this review, we give an overview of the current knowledge on origination of circadian rhythms. We discuss the development of both master and peripheral clocks and compare the origination of circadian rhythms *in utero* and *in vitro*.

Introduction

In the previous chapter, we introduced circadian rhythms, clocks, and their function in the body and heart. Recently, biological rhythms have gained a lot of attention in science. In the last 10 years, more than 20.000 articles involving circadian rhythms have been published and numerous review articles have been written on the subject.[20, 95] Knowledge about circadian rhythms in physiology and disease is accumulating. However, information about the origination of circadian rhythms during embryogenesis or *in vitro* maturation is scarce. Regenerative medicine, which aims to develop tissues *in vitro*, has renewed the interest in developmental processes. In this review, we therefore provide an up-to-date overview on the origination of circadian rhythms during embryogenesis and *in vitro* differentiation. Although circadian rhythms are present in almost all organisms, we will focus on origination of circadian rhythms in mammals. Difficulties and challenges in the study of the developing circadian rhythms are discussed and origination of circadian rhythms *in utero* is analysed. In the second part of the review, we introduce stem cells, provide information about origination of circadian rhythms during differentiation of stem cells and compare origination of circadian rhythms in stem cells to those in the developing foetus. Finally, future perspectives are provided.

Development of circadian rhythms during embryology

Considerations in research of developing circadian rhythms

Circadian rhythms are biorhythms mediated by molecular clocks, a series of molecular feedback loops that drive diurnal oscillations in core clock and output gene expression. The presence of a circadian clock is therefore generally evaluated by quantifying the expression of oscillating clock genes such as Clock, Bmal, Per, and Cry. (Example provided in Figure 2.1). However, the focus on oscillating clock genes to determine presence of a fully functional clock has some caveats. O'Neill and colleagues showed that circadian rhythms are present in cells without a nucleus (and thus without transcription) and persist in nucleated cells when transcription of core clock genes is disabled, suggesting that mechanisms other than the classic molecular clock are able to maintain circadian rhythms.[96, 97] Second, Paulose et al. demonstrated that development of diurnal metabolic rhythms precede rhythms in clock gene expression.[98] It is therefore possible that in early cell maturation, mechanisms other than the core clock genes regulate select diurnal patterns. Third, evaluating the presence of oscillating clock genes suggests that there is an abrupt start of oscillation during maturation. In fact, circadian rhythms originate progressively during maturation, in several steps: initially, clock genes oscillate with low amplitudes, followed by higher amplitudes, but sometimes also periods without oscillations and phase shifts.[99, 100] During various disease states, rather than a complete absence of circadian rhythms, there are often changes in the amplitude and phase of the rhythm.[101] Oscillations in circadian clock genes therefore do not automatically mean the molecular clock is working properly. Last, when circadian rhythms are evaluated, often whole body or whole-tissue samples are used. Results of clock gene expression

are the average of many individual cells. Numerous studies suggest that circadian rhythms in one cell or tissue type might be in a markedly distinct phase compared to another region of the body, particularly during development.[102-104] This is supported by the finding that in some experiments, oscillations are found *in vitro*, but not *in vivo*.[105] Rhythms at the cell level might therefore not be detected when analysing results on a tissue scale due to the absence of synchronization. Despite these limitations, it is still believed that rhythmic expression of core clock genes is a good indication of clock function.[96, 98, 102]

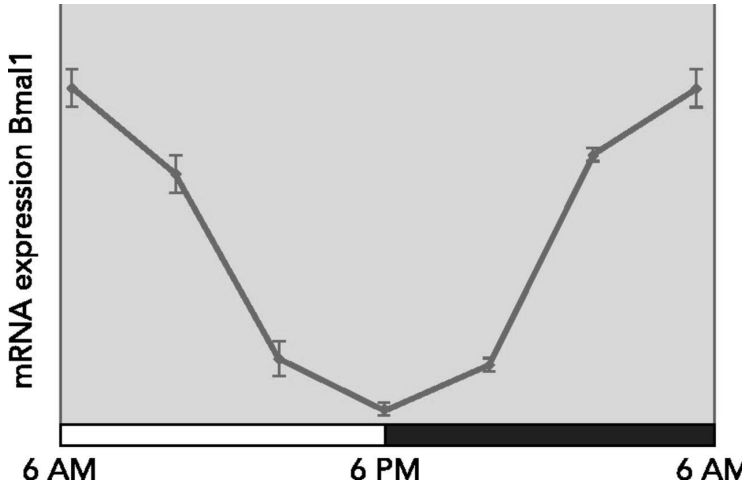


Figure 2.1. Example of oscillating expression of core clock genes: Bmal1 expression in a young, wildtype mouse heart. Data derived from Dorgan et al, 2011.[106]

During embryogenesis, the developing circadian system is exposed to daily fluctuations in maternal signals including temperature, food intake, and hormones (e.g., melatonin).[107] In addition, the fallopian tubes and the uterus of the mother express clock genes.[108] Some suggest that because of their proximity, clock activity in these organs may interfere with developing foetal circadian rhythms, although this has not been demonstrated yet.[108] The developing circadian system should therefore not be considered an immature adult system residing in a closed compartment, but a system capable of anticipating diurnal circumstances *in utero* and preparing for life after delivery.

A last consideration in research of the developing circadian system is that most research is performed in nocturnal animals such as mice, rats, and hamsters. Humans on the other hand are diurnal, which implies that their circadian rhythms are different. In addition, Zeitgebers of rodents differ from Zeitgebers of primates.[109, 110] These considerations should be taken in account when translating animal experiments to the human situation.

Origination of circadian clocks

The molecular circadian clock originates during development *in utero*. The canonical clock genes Per, Cry, Clock and Bmal are already non-rhythmically expressed in the unfertilized mouse oocyte.[108, 111] Expression of these genes diminishes on day 2 after fertilization (2-cell stage), but restores on day 3 (16-cell stage) until day 4 (blastocyst). Next, expression of clock genes increases and around mid- to end-gestation, diurnal oscillations in expression start. The shape, amplitude, and phase of clock gene rhythms change during further development. This development is animal and tissue specific. In general, rodents have a more immature circadian clock at birth compared to primates.[103] An overview of published findings to date regarding the origination of the molecular circadian clock in mouse, rat, hamster, and monkey is given in tables 2.1, 2.2, and 2.3, respectively. In Figure 2.2, a general model for the origination of circadian rhythms is provided.

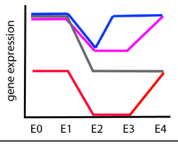
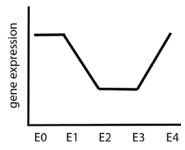
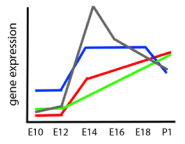
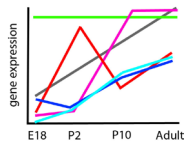
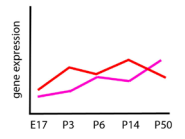
Author	Mode	Tissue	Gene	Start oscillation	Pattern
Johnson et al, 2002	PCR	ESC	Clock Bmal1 Per1 Per2 Per3 Cry1 Cry2	Not measured	
Ko et al, 2000	PCR	ESC	Bmal1	Not measured	
Dolatshad et al, 2010	PCR	Whole body	Clock Bmal1 Per2 Cry1	In vivo no oscillations, in vitro oscillations at E18 (=only time-point measured)	
Ansari et al, 2009	Immuno blot	SCN	Clock Bmal1 Per1 Per2 Cry1 Cry2	No oscillation No oscillation E18 E18 P10 P2	
Shimomura et al, 2001	ISH	SCN	Per1 Per2	E17 P6	

Table 2.1. Expression of core clock components during embryology in the mouse. E: Embryonic day; P: Postnatal day; SCN: suprachiasmatic nucleus; ESC: Embryonic stem cell; ISH: In situ hybridization.

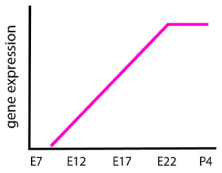
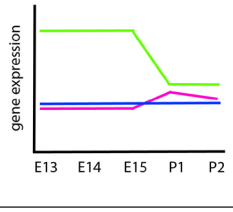
Author	Mode	Tissue	Gene	Start oscillation	Pattern
Rat					
Saxena et al, 2007	Luciferase construct	Whole body	Per1	E12	
Torres-Farfan et al, 2012	PCR	-Whole body -Headless body -Head -Pars Tubularis -Hippocam pus -Pineal -Adrenal -Heart -Liver	Bmal1 Per2	No oscillation E16 (=only time-point measured) E18 (=only time-point measured)	Not measured
Sladek et al, 2004	ISH / immunohis-tochemistry	SCN	Clock Bmal1 Per1 Per2 Cry1	No oscillation P3 P3 P3 P3	Expressed E19-P10 (all time-points measured). No quantitative data in article.
Sladek et al, 2007	PCR	Liver	Clock Bmal1 Per1 Per2 Cry1	P30 P2 P10 E20 P30	Expressed E20-adult (all time-points measured). No quantitative data in article.
Kováciková et al, 2006	ISH	SCN	Clock Bmal1 Per1 Per2 Cry1	After P2 P1 E20 P1 P2	Expressed E20-P2 (all time-points measured). No quantitative data in article.
Sakamoto et al, 2002	Nothern blot	Heart	Bmal1 Per1 Per2	P5 P5 P14	Expressed P2-P30 (all time-points measured).
Torres-Farfan et al, 2011	PCR	Adrenal	Bmal1 Per2	E18 (=only time-point measured)	Not measured
Hamster					
Li et al, 2005	ISH	SCN	Bmal1 Per1 Cry1	No oscillation Post-natal Pre-natal	

Table 2.2. Expression of core clock components during embryology in the rat and hamster. E: Embryonic day; P: Postnatal day; SCN: suprachiasmatic nucleus; ISH: In situ hybridization.

Author / Animal	Mode	Tissue	Gene	Start oscillation	Pattern
Capuchin Monkey					
Torres-Farfan et al, 2006	PCR	SCN Adrenal,Pituitary, Thyroid,Brown fat, Pineal	Clock Bmal1 Per2 Cry2	E142, (only time-point measured, =90% gestation.) Exception: Pineal Per2: no oscillating expression	Not measured
Japanese macaque					
Suter et al, 2011	PCR	Liver	Per1 Npas2 (comparable to Clock)	Not measured	Expression at E130 (=only time-point measured)

Table 2.3. Expression of core clock components during embryology in non-human primates. E: Embryonic day; P: Postnatal day; SCN: suprachiasmatic nucleus.

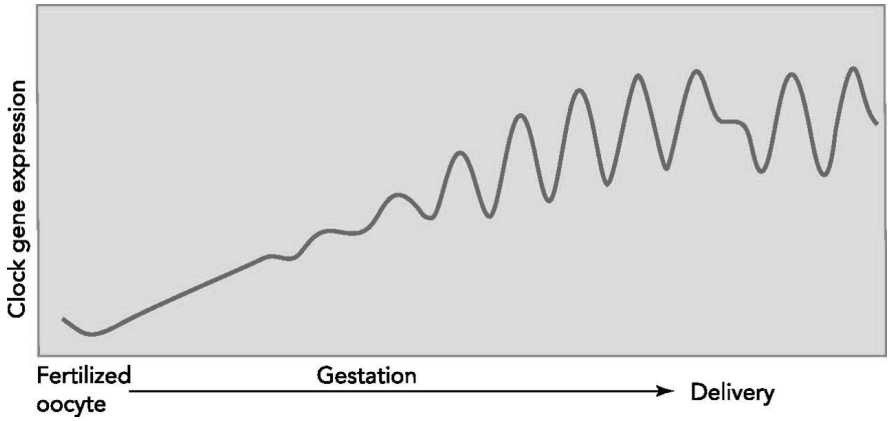


Figure 2.2. Origination of circadian rhythms. Core clock genes are already expressed in the fertilized oocyte. After an initial decrease, expression slowly increases and starts oscillating. At delivery, a phase shift occurs. The exact moment oscillation starts is species and tissue specific. Initial dip based on Johnsen et al. [108] and Ko et al. [111]; gradual increase and start of oscillations based on studies in Table 2.1, 2.2, and 2.3; increasing amplitude and phase shift at delivery based on Saxena et al.[99]

Embryogenesis involves cell divisions. The occurrence of cell division is regulated by circadian rhythms, which restrict mitosis of the cell to specific time frames in the 24-hour cycle *in vivo* and *in vitro* (cell division gating).[112-115] During cell division, circadian rhythms of the mother cell are passed on to the daughter cell. In this process circadian rhythms persist, although amplitude and phase of the rhythm sometimes change.[114,116] Amplitude changes are caused by a wider distribution of periods in proliferating cells compared to non-proliferating cells.[114] Phase changes can be explained by reduced availability of clock proteins. During cell division, transcription halts and cellular components are divided over daughter cells. If cell division occurs in the accumulating phase of Per and Cry, reduction of these proteins will lead to circadian phase prolongation, whereas if this happens in the descending phase of Per and Cry, the circadian phase will be shortened.[114]

Zeitgebers of the developing circadian clock

Zeitgebers of the circadian clock during embryogenesis differ from Zeitgebers for adult cells. This is partly caused by the immature phenotype of clock-regulating components in the embryo. In rats for example, the master clock (SCN) develops from embryonic day 14 (E14) to E17 and is only fully matured at postnatal day 10 (P10).[102] Synchronization of peripheral clocks during gestation must therefore depend on signals that do not come from the foetal master clock. Foetal Zeitgebers also differ because the environment of the foetus is not comparable to the environment of an adult. Light for example is the main Zeitgeber for the adult master clock, but would seem less relevant in the uterus. Surprisingly however, a recent study demonstrated that photic input influences eye development in the mouse foetus. This may also change the traditional view on the role of Zeitgebers and their signalling pathways in circadian development, although it remains unknown whether photic input in the foetus is strong enough to influence its circadian clocks.[117] The established main Zeitgebers during development are maternal Zeitgebers, the most important one being maternal melatonin. Melatonin is mainly produced by the maternal pineal gland and able to pass the placenta unaltered, causing a diurnal rhythm in the foetal circulation. Melatonin receptors are present in the human foetus.[118] Experiments in the hamster show that when the maternal master clock is removed, foetal activity rhythms are desynchronized, but rhythms can be rescued by regular melatonin injections.[119] Maternal steroids, the dopaminergic system, and feeding are also able to synchronize peripheral foetal clocks.[120-122] In spite of the accumulating evidence of a correlation between maternal Zeitgebers and foetal circadian rhythms, phase and time necessary to adapt to changing Zeitgebers differs between maternal and foetal rhythms, indicating that the relation is indirect.[123, 124]

In adults, shift work leads to an increased incidence of several diseases.[28] The exact effect of changes in maternal Zeitgebers such as shift work on pregnancy and the developing embryo however, is unclear. Maternal diurnal rhythms change during pregnancy.[125, 126] Experiments in rat and hamster indicate that although there is asynchrony between offspring, normal circadian rhythms arise in the absence of maternal Zeitgebers.[127-129] Second, when comparing full and preterm babies, presence of circadian rhythms seems to be related to the degree of maturation instead of presence of Zeitgebers.[102] In contrast, studies investigating the effects of maternal shift work during pregnancy show adverse effects on the offspring's health. A meta-analysis from 2011 concluded that infants of pregnant women that do shift work are small for gestational age, but no effects of shift work on the incidence of pre-eclampsia and pre-term delivery were found.[130] Animal studies indicate that shift-work leads to glucose intolerance and insulin resistance in pups.[131] In addition, a dramatic reduction of successful term pregnancy after copulation was observed in female mice subjected to light-dark cycle phase shifts.[132] (Studies in humans that analyse postnatal effects of maternal shift work during pregnancy are currently lacking.

Output of the developing circadian clock

Several physiological parameters such as heart rate and (breathing) movements have diurnal patterns during late gestation.[133, 134] Animal models also show that foetal hormone levels, such as GH, IGF-I, ACTH, and cortisol show time-of-day dependent oscillations, mainly at end-gestation.[135, 136] It is unclear however, whether these oscillations are regulated by autonomous foetal circadian clocks, foetal circadian clocks that function as an executor of maternal signals, or by maternal factors directly.[136, 137] Some studies suggest that this differs between developmental stages.[129] The idea of functional foetal circadian clocks is supported by two findings. First, circadian rhythms are present in preterm infants, even if they are kept under constant external condition and without their mother.[102] Second, intrinsic circadian oscillations in metabolic activity and transcription of clock output genes are present before birth.[138, 139] A summary of the interaction between maternal and foetal circadian rhythms is depicted in Figure 2.3.

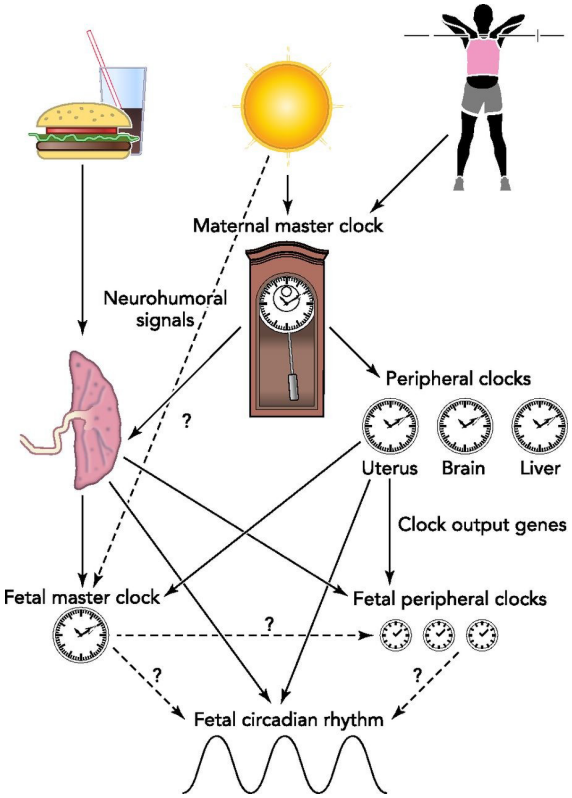


Figure 2.3. Circadian rhythms in the foetus

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

Stem cells

In utero, a single cell can develop into a full organism. The fertilized cell duplicates into 2 cells, followed by numerous other duplications and differentiation steps, which eventually leads to a complex organism with several specialized tissues. In the first stage of development, cells are able to differentiate into cells of all three germ layers (pluripotency). Later, cells can only differentiate into cells of a single germ layer (multipotency), followed by terminal differentiation into adult, somatic cells. Cells that are able to proliferate and differentiate are called stem cells. Pluripotent stem cells are able to proliferate indefinitely, multipotent stem cells generally have a more limited proliferation capacity, and most fully differentiated cell types show very little proliferation or none at all. In adults, under physiological conditions only multipotent stem cells and fully differentiated cells are present. Multipotent stem cells play an important role in tissue renewal and repair. In tissues such as intestines (intestinal stem cell), skin (hair follicle stem cell), and blood (hematopoietic stem cell), multipotent stem cells create a constant renewal of cells [140]. In other tissues such as the heart (cardiac stem cell), multipotent stem cells reside and become active after injury.[141, 142]

In addition to *in vivo* studies, stem cells have been extensively studied *in vitro*. Pluripotent stem cells used for *in vitro* research can be derived from embryos (embryonic stem cells) or can be reprogrammed from somatic cells by forced expression of defined transcriptional genes (induced pluripotent stem cells).[143] Pluripotent stem cells proliferate indefinitely when cultured under the appropriate conditions, thus forming a stable cell line, and can be differentiated into somatic, adult cells. Using pluripotent stem cells, large amounts of somatic cells, such as heart, kidney, or liver cells can be created. Because primary adult human cells are difficult to obtain in sufficient numbers and many cell types cannot be expanded in culture for a prolonged period, stem cell-derived cells provide a better alternative for research and therapy in many situations. Stem cells and their derivatives are now being used to study embryonic development, to mimic adult physiology and pathophysiology, for pharmacological efficacy / toxicity screenings and are being evaluated for their regenerative potential in diseases like heart and liver failure. A short summary of the developmental hierarchy of different stem cells is depicted in Figure 2.4.

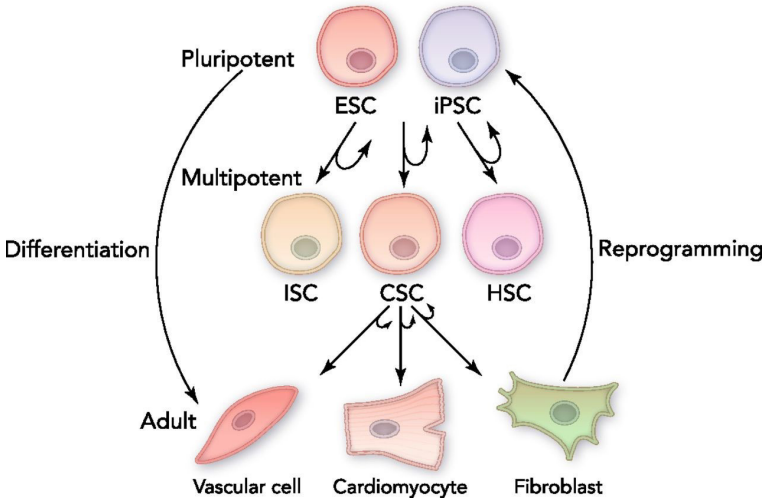


Figure 2.4. Stem cells. iPSC, induced pluripotent stem cell; ISC, intestinal stem cell; CSC, cardiac stem cell; HSC, hematopoietic stem cell.

Circadian rhythms in stem cells

Oscillations in adult stem cell mobilization and trafficking

Circadian rhythms have been described in different kinds of stem cells. A molecular circadian clock was found in human mesenchymal stem cells and epidermal stem cells *in vivo* and *in vitro* after synchronization.[144, 145] In addition, studies in human volunteers show that hematopoietic stem cells and endothelial progenitor cells (EPCs) have diurnal rhythms in mobilization.[146, 147] Oscillating numbers of hematopoietic stem cells *in vivo* are accompanied by diurnal rhythms in hematopoietic growth factors which together lead to diurnal variations in the number of circulating blood cells.[146,148] Mouse studies show that amplitudes of these rhythms are largest at young age and diminish in the aged.[149] Mechanistic studies have shown that stem cell mobilization, via neural signals, is orchestrated by the master clock.[150] Using a circadian mutant mouse model, Mendez-Ferrer et al. showed that diurnal patterns in stem cell mobilization disappear when the master clock is disrupted and that the regulation of mobilization is mediated by circadian rhythms in noradrenalin secretion. Some speculate that diurnal differences in the outcome of cardiovascular disease such as myocardial infarction might be caused in part by diurnal rhythms in homing of stem cells.[147]

Stem cell proliferation

During embryonic development, circadian rhythms initiate at the end of gestation. *In vitro* studies show comparable results. Oscillations of clock genes are not present in pluripotent stem cells, develop during maturation and disappear again when differentiated cells are reprogrammed to their pluripotent state *in vitro*.[151] Circadian rhythms thus seem to be associated with stem cell proliferation and maturation. This hypothesis is supported by several other findings: Core circadian clock proteins and hypoxic transcription proteins, important in stem cell maturation and migration, are both part of the basic helix-loop-helix/Per-Arntl-Sim homology (bHLH/PAS) protein family and have a similar protein structure.[148, 152] Secondly, transcription of nuclear hormone receptors involved in adult stem cell proliferation shows diurnal oscillation.[153] Thirdly, several enzymes that are involved in proliferation and reprogramming of stem cells, such as histone acetyltransferase / deacetylase and glycogen synthase kinase 3 beta (GSK3B), also play an important role in the regulation of circadian clocks.[66,154-157] GSK3B is part of the Wnt signal transduction pathway and is responsible for phosphorylation of clock genes.[154] Inhibition of GSK3B leads to a lengthened circadian period and modified stem cell differentiation and function.[155, 158] Last, the development of tumours is associated with polymorphisms in core clock genes.[148] Recent evidence even suggests that circadian rhythms do not only arise during differentiation, but also play a causal role in this process. Janich at al. found that epidermal stem cells display circadian gene expression rhythmicity with heterogeneous phases. The phase of the stem cell was correlated with their predisposition to differentiate or activate; dormant stem cells were in opposite phase compared to proliferating stem cells.[144] In these stem cells, deletion of core clock genes completely arrested the circadian clock and caused diminished self-renewal, resulting in premature aging and a reduced tumour risk. The exact mechanism that links phase differences in circadian rhythms to differentiation is unknown, but promotor analysis revealed that proteins in the Wnt signalling pathway and TGF-β regulators are likely involved. These results correspond with previous studies, which showed that disruption of the circadian clock leads to reduced proliferation and premature aging, exampled by a reduction of muscle and bone mass.[159-161] In addition, *in vitro* studies found that the ability of cells to differentiate reduces when circadian rhythms are disrupted and *in vivo* and clinical studies link disruption of circadian rhythms to tumour susceptibility.[162-165] Chen and colleagues thoroughly investigated premature aging in circadian disrupted animals. In a first study they found a correlation between Bmal1 protein levels and the proliferative capacity of mesenchymal stem cells, progenitors of osteoblasts. They hypothesized that disrupted circadian rhythms result in a reduced proliferative capacity of mesenchymal stem cells, which in turn leads to reduced osteoblast formation, reduced bone formation and eventually reduced bone mass and premature aging. In a recent publication they demonstrate that the Wnt pathway might mediate the effect of circadian rhythms on stem cell proliferation.[166] In a mouse model, overexpression of Bmal1 led to increased levels of β-catenin, a core component of the canonical Wnt pathway, and an increased proliferation rate. The exact mechanism however, and why disruption of circadian rhythms can lead to both increased and reduced tumour risk, is still unclear.

Future directions

The importance of time and circadian rhythms in biomedical research has been elucidated in the past several decades. In most preclinical and clinical studies however, time is still not considered important. In preclinical research for example, many experiments are performed in nocturnal animals such as mouse and rat during working hours, which is their inactive (sleeping) period. When the same therapy is administered in the active (awake) period of humans, results are often disappointing. Circadian research shows that effects of treatments or stimuli differ between the active and inactive period and poor translation of preclinical studies to the clinic might be partly due to this difference.[106] In clinical studies, timing of a treatment is hardly analysed, whereas studies that do take this in account find that therapies work better if they are administered at specific times.[167] Taking time serious in biomedical research will in future increase translation from laboratory to bedside and maximize the effect of treatments in clinic, such as pharmacological therapies, invasive therapies, and upcoming therapies such as stem cell therapy and other types of regenerative medicine.

Research about the development of circadian rhythms and the role of circadian rhythms in development is slowly accumulating, and much knowledge is still missing. First of all, the origination of circadian rhythms will have to be studied in much more detail. Evidence for example that oscillating clock genes are not present in pluripotent stem cells and the first stages of development is mainly based on conventional PCRs that analyse large amounts of cells. New techniques enable more accurate, real-time measurement of oscillations on a single cell level.[168] Experiments using these techniques will hopefully elucidate whether circadian rhythms are truly absent in the first stages of development, or just not synchronized / prominent yet.

Secondly, the exact role of circadian rhythms in maturation / differentiation needs further investigation. Several papers showed that both in pluripotent and adult stem cells, circadian rhythms are related to differentiation and maturation, but their exact role remains unknown. Is it for example possible to induce differentiation by affecting the circadian clock? Does the absence of Zeitgebers in *in vitro* research prevent or temper maturation of stem cells? Do changes in maternal Zeitgebers, such as shift working mothers, affect physiological development of unborn children? Do interventions such as imposing strict day / night cycles on preterm babies in the neonatal intensive care unit have any beneficial effect on their development and disease risk later in life? *In vitro*, preclinical, and clinical studies will in future hopefully answer all these questions.

Conclusion

Circadian rhythms are present in almost every mammalian cell and play an important role in physiology and pathophysiology. Circadian rhythms are regulated by molecular clocks, which develop during differentiation and maturation *in vivo*. Clock development is a gradual process that enables anticipation to the *in utero* environment and might prepare the foetus for life after delivery. First expression of clock genes increases, then clock genes start oscillating and finally period, amplitude and phase are optimized for the outside world. In stem cells in the adult body, circadian rhythms appear to be closely linked to proliferation. The potential role of circadian rhythms in stem cell maturation was recently discovered. Future studies with respect to stem cell differentiation, *in vivo* studies investigating the effects of disrupted circadian rhythms using circadian-disrupted animal models, and human studies including the effect of shift work, aim to further elucidate the role of circadian rhythms during development.

Chapter 3

Stem cells for cardiac repair

Chapter 3 - Stem cells for cardiac repair

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Abstract

Cardiovascular disease is a major cause of morbidity and mortality throughout the world. Most cardiovascular diseases, such as ischemic heart disease and cardiomyopathy, are associated with loss of functional cardiomyocytes. Unfortunately, the heart has a limited regenerative capacity and is not able to replace these cardiomyocytes once lost. In recent years, stem cells have been put forward as a potential source for cardiac regeneration. Pre-clinical studies that use stem cell-derived cardiac cells show promising results. The mechanisms, though, are not well understood, results have been variable, sometimes transient in the long term, and often without a mechanistic explanation. There are still several major hurdles to be taken. Stem cell-derived cardiac cells should resemble original cardiac cell types and be able to integrate in the damaged heart. Integration requires administration of stem cell-derived cardiac cells at the right time using the right mode of delivery. Once delivered, transplanted cells need vascularization, electrophysiological coupling with the injured heart, and prevention of immunological rejection. Finally, stem cell therapy needs to be safe, reproducible, and affordable. In this review, we will give an introduction to the principles of stem cell based cardiac repair.

Introduction

Repairing the injured body with its own tissue as a substrate has captured human fascination for a long time. In Greek mythology, the Lernaean Hydra was a serpent-like creature with multiple heads that regenerated each time they were cut off and Prometheus, a titan punished by Zeus for stealing fire, had a liver that was able to regenerate each night after it was eaten by an eagle. In 1740, Abraham Tembley discovered that microscopic, freshwater animals had the ability to regenerate their head after amputation, later followed by others who discovered that amphibians have the ability to regenerate their tails, limbs, jaws, and eyes.[169, 170] It took scientists until 1933 before they discovered that some human organs, such as the liver, also have the ability to regenerate.[171]

Regenerative therapies are of major interest in cardiovascular medicine. Most cardiovascular diseases, including ischemic heart disease and cardiomyopathy, are associated with loss of functional cardiomyocytes and in other diseases, such as sick sinus syndrome, specific cardiac cell properties are missing. Unlike the Lernaean Hydra or the human liver, the heart does not have the ability to regenerate itself spontaneously once damaged. Cardiomyocytes are terminally differentiated and have a limited proliferative capacity. Lost cardiomyocytes are replaced by fibroblasts and connective tissue with the remaining cardiomyocytes becoming hypertrophic, which may eventually lead to heart failure. On the contrary, stem cells proliferate indefinitely and can be directed to differentiate into specialized cell types such as cardiomyocytes. The goal of stem cell-based regenerative medicine in cardiovascular disease, therefore, is to create healthy, functional cardiac cells that are able to integrate in the injured heart and restore its function.

In the past decades, several stem cell types have been discovered. These stem cells can be subdivided based on their differentiation capacity. Pluripotent stem cells, such as embryonic stem cells and induced pluripotent stem cells, are able to differentiate into all three embryonic germ layers, whereas multipotent stem cells can differentiate into a number of closely related cell types of a single embryonic germ layer. Cardiomyocytes were derived from several stem cell sources (Figure 3.1). Other types of stem cells do not differentiate into cardiomyocytes themselves, but support cardiac repair by different mechanisms (Table 3.1). In this review, we will refer to all stem cell-derived cardiomyocytes and differentiated cell types enriched for cardiomyocytes as stem cell-derived cardiomyocytes, while we will refer to non-cardiomyocyte derivatives (such as vascular cells) as stem cell-derived cardiac support cells.

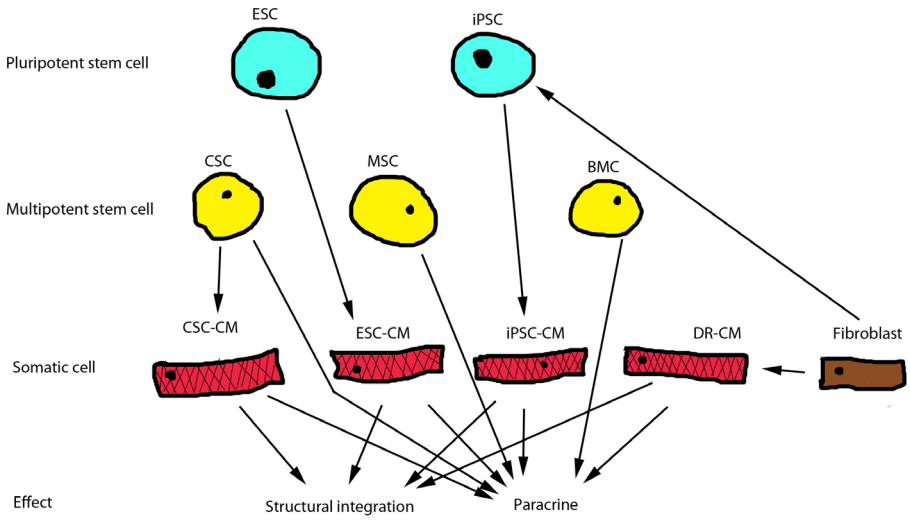

Figure 3.1. Summary of stem cells used for cardiac repair. BMC: bone marrow-derived cell; CSC: cardiac stem cell; CSC-CM: cardiac stem cell-derived cardiomyocyte; DR-CM: cardiomyocyte derived by direct reprogramming; ESC: embryonic stem cell; ESC-CM: embryonic stem cell-derived cardiomyocyte; iPSC: induced pluripotent stem cell; iPSC-CM: induced pluripotent stem cell-derived cardiomyocyte; MSC: mesenchymal stem cell.

Characteristics of different stem cell types						
Stem cell	**Origin**	**Stem cell type**	**Research stage**	**Primary intended effect**	**Immunological status cells**	**Remarks**
Embryonic stem cell	Inner cell mass of blastocyst	Pluripotent	Pre-clinical	Structural integration	Allogenic / matched	Ethical and safety issues
Induced pluripotent stem cell	Somatic cell	Pluripotent	Pre-clinical	Structural integration	Autologous / matched	Safety issues
Cardiac stem cell	(Adult) Heart	Multipotent	Pre-clinical	Structural integration	Auto- and allogenic/ matched	Limited availability
Mesenchymal stem cell	Bone marrow, fat, and cord blood	Multipotent	Clinical	Paracrine	Tolerated/ autologous	No structural effects
Bone marrow cell	Bone marrow	Multipotent	Clinical	Paracrine	Tolerated/ autologous	Heterogeneous cell population, no structural effects
Directly reprogrammed cell	Somatic cell	No stem cell involved	Pre-clinical	Structural integration	Autologous	Safety issues, limited efficacy of differentiation

Table 3.1. Characteristics of stem cells studied for cardiac regeneration potential.

In this review, we will give an introduction to the principles of stem cell-based cardiac repair. Our aim is to give a concise up-to date overview of the therapeutic possibilities of stem cells for cardiac injury. First, we describe general requirements for stem cell therapy. After that, we will discuss in more detail the different stem cell sources and their therapeutic effects, since these vary for each cell type.

Transplanted cells resemble original cardiac cell types
Combination of different cardiac cells and extracellular matrix
Mature electrophysiological phenotype
Contractile function
Structural integration in damaged heart
Delivery at right time
Right mode of delivery
Electrophysiological coupling recipient heart
Vascularization
Prevention of (immunological) rejection
Appropriate paracrine effect
No adverse effects on host myocardium or even beneficial effects
Acceptable complications and ethical considerations
Reproducible methods on large scale
Affordable

Table 3.2. Requirements stem cell based cardiac regeneration

Requirements for stem cell therapy

In order to be suitable for cardiac repair, stem cell-derived cardiac cells should resemble the original cardiac cell types and be able to integrate in the damaged heart. Integration requires administration of stem cell-derived cardiac cells at the right time using the right mode of delivery. Once delivered, transplanted cells need vascularization, electrophysiological coupling with the injured heart, and prevention of immunological rejection. Ideally there would also be beneficial effects on the host myocardium, for example, by stimulating proliferation or differentiation of local progenitors, neovascularization or by inhibiting apoptosis. The minimum requirement for the donor cells is to have no adverse effects. Finally, stem cell therapy needs to be safe, reproducible, and affordable. Each of these requirements will be discussed separately. (Table 3.2)

Cell type

Appropriate cardiac function requires well-timed, successive contraction of different parts of the heart. These contractions are orchestrated by the cardiac electrical system, which consists of the sinoatrial (SA) node, the atria, the atrioventricular node, the His-Purkinje system, and the ventricles. Each part of the system has a different expression of ion channels, and thus a specific electrophysiological phenotype according to its function in the electrical system.[172] Regeneration therapy of the SA-node, therefore, requires stem cell-derived cardiomyocytes with a different cell mixture, contractile function, and ion channel profile compared to regeneration therapy of the ventricles.

Grafted cells should preferably possess characteristics similar to the original cells they replace. In literature, there is no consensus whether this is best achieved by transplantation of stem and progenitor cells, or of fully differentiated cardiac cells. Cardiac tissue is an organized and dynamic contractile tissue that consists of different kinds of myocytes, vascular smooth muscle cells, fibroblasts, and an extracellular matrix.[173] Undifferentiated stem and progenitor cells have the advantage that they can migrate to injured areas and form all cardiac components; physiological signals in the heart, such as those created by the recently discovered telocytes, may regulate differentiation after transplantation.[174] On the other hand, since cardiac repair does not occur spontaneously in the damaged heart, cardiac signals might not be sufficient for regeneration therapy and an *in vitro* differentiated cell mixture might be preferable. *In vitro* differentiation also has the advantage that cells can be organized to optimize contractile function and that there is no risk of teratome formation associated with use of undifferentiated stem and progenitor cells.

Cardiac cells that were derived from stem cells *in vitro,* at present, consist of a heterogeneous population of cardiomyocytes, often accompanied by other stem-cell derived cardiac support cells like fibroblasts, vascular smooth muscle cells, or more problematic, undifferentiated cells.[175] Purification methods to remove unwanted cell types from the stem-cell derived cardiomyocytes population have been developed and the electrophysiological phenotype of stem-cell derived cardiomyocytes can be determined, although none of the clinically suitable methods to purify cells has been shown to be reproducible, efficient and safe.[176, 177] In addition, there is no method to select stem-cell derived cardiomyocytes with a specific electrophysiological phenotype. Development of such methods will increase standardization of stem cell derived cardiomyocytes and enable selection of stem cell derived cardiomyocytes that have properties similar to original cardiac cells.

Mode of delivery

Although paracrine factor release from the transplanted stem-cell derived cardiomyocytes and cardiac support cells is beneficial even if the donor cells do not survive on the long term, the ultimate success of regeneration by stem-cell derived cardiomyocytes transplantation depends on integration of the donor cells in the injured myocardium. Pre-clinical studies show that survival of transplanted stem-cell

derived cardiomyocytes in the damaged myocardium is poor.[178] In order to solve this problem, several modes of transplantation have been developed.[179]

The first and most commonly used method is injection, which can be targeted at different locations. Injection in the center of the injured myocardium has the advantage that damage is most severe so the potential benefit of therapy is maximal. On the other hand, in the border zone of the injured heart, perfusion is still possible and signals of viable cardiac tissue in the proximity may be useful for survival, differentiation, and integration. This assumption is supported by the finding that after myocardial infarction (MI), a limited number of new myocytes is present in the border zone but not in the infarcted tissue itself.[180] Intramyocardial injection can be performed from the epicardial side of the heart by surgical injection, or from the inside (transendocardially) with guidance using a NOGA system).[181] A third option is intravascular injection.[182-184] Intravenous injection is simple and has a low risk of mechanical damage by the injection, but requires adequate homing to the site of injury in the heart. Injection in one of the cardiac vessels (coronary arteries, veins or sinus) has the advantage that therapy can be combined with percutaneous coronary interventions (PCI), a therapy commonly used by intervention cardiologists during acute MI.

The application of biomaterials is a novel method to transplant stem cell derived cardiomyocytes. Biomaterial application is based on the concept that in order to survive, transplanted cells require a biochemical and biophysical environment comparable to extracellular matrix in healthy myocardium. Extracellular matrix in cardiovascular disease plays a role in angiogenesis, differentiation and maturation of stem cells and mechanical and electrical engraftment of transplanted cells.[185] The goal of biomaterials, therefore, is to substitute healthy extracellular matrix to enable integration of stem cell derived cardiomyocytes. In addition, biomaterials themselves also show beneficial effects, although mostly temporarily.[186-188] Several biomaterials have been developed for this aim, including alginate,[186, 187] matrigel,[189] collagen,[190] fibrin,[191] and self-assembling peptides.[192]

In order to be used for regenerative therapy, biomaterials need to have mechanical properties like the cardiac extracellular matrix and be able to continuously and slowly send signals needed for integration, proliferation, and differentiation.[193] They need to be biodegradable in non-toxic metabolites after a period long enough to enable proper integration and the viscosity needs to be low enough to be transplanted and to permit migration, but high enough to prevent mechanical removal of the stem cell derived cardiomyocytes. Especially in the area of signaling, significant progress has been made over the past few years. Biomaterials that slowly deliver proteins,[193] drugs,[194] plasmids,[195] viruses,[195] and microRNAs[196] to surrounding tissue are currently available.

Time of delivery

Regeneration therapy can be applied during several stages of cardiovascular disease. After MI, fast delivery of stem cell derived cardiomyocytes has the advantage that remodeling, characterized by fibrosis and hypertrophy, has not yet developed and that regulatory mechanisms leading to these conditions can be altered. Anti-apoptotic, immune-modulatory, and pro-angiogenic effects of stem-cell derived cardiomyocytes can therefore be used immediately after MI.[197] On the contrary, ischemic and inflammatory conditions directly after a myocardial infarct do not favor cell survival, cell integration, and immunologic tolerance. For actual tissue regeneration, strategies also including delivery at a later time-point might therefore achieve better results.

Vascularization

Oxygenation via vascularization is essential for the survival of cardiac tissues; both acute and chronic ischemia are associated with cardiovascular disease. After a myocardial infarction, ischemia induces pathophysiological mechanisms leading to cell death or hibernation and fibrosis, and termination of ischemia is believed to be the most important therapy after myocardial infarction.[198]

In regenerative therapy, vascularization is both a prerequisite and a goal. Cardiomyocytes have a high oxygen demand and in order for stem cell derived cardiomyocytes to survive and integrate sufficient oxygen and nutrient supply via blood vessels is required. Several strategies have been developed to promote blood vessel formation. This can be achieved by the formation of new blood vessels (vasculogenesis) and by extension of existing blood vessels (angiogenesis). Co-culture and co-transplantation of stem-cell derived cardiomyocytes with cardiac support cells, such as endothelial cells and fibroblasts, improves *in vivo* vascularization and cell survival.[199, 200] In addition, paracrine factors secreted from the stem-cell derived cardiomyocytes themselves can enhance neovascularization.[185, 201] Biomaterials designed with geometries that promote angiogenesis and vasculogenesis, or addition of angiogenic and vasculogenic growth factors and proteins to biomaterials were also developed to stimulate vascularization.[202-204]

Immunological reaction

Transplantation of genetically unrelated tissues usually results in foreign antigen recognition by T-lymphocytes, immune system activation and in most cases, graft rejection. Most *in vivo* experiments with stem-cell derived cardiomyocytes use immunosuppressed animal models.[205] Before the use of stem cell derived cardiomyocytes can become clinically applicable, however, the issue of graft rejection needs to be dealt with.

Generally, there are four strategies to prevent immune rejection. The graft and host can be genetically matched; the immune system can be adapted to tolerate the graft; the graft can be adapted to remain undetected by the immune system; or the

immune system can be suppressed. Stem cell derived cardiomyocytes can be created using the host as a substrate. Adult stem cells and multipotent progenitor cells, but also induced pluripotent stem cells, differentiated somatic cells that have been genetically reprogramed to resemble embryonic stem cells, can be derived from the host. From these stem cells, stem cell derived cardiomyocytes can be developed that are genetically identical to the host. Alternatively, genetically matched stem cells from stem cell banks can be used.[206] Syngeneity of graft and host prevents detection and rejection of transplanted stem cell derived cardiomyocytes by the host's immune system, although it has been suggested that altering and reprogramming might enhance immunogenicity, even of autologous cells.[207] A second strategy to prevent immune rejection is tolerisation. Immune tolerance is regulated by the acquired immune system, in which regulatory T cells play a central role. Some tissues that are not genetically identical to the host, e.g., an unborn child during pregnancy, are tolerated by the immune system of the host. Methods have been developed to induce donor-specific tolerance, for example, by preconditioning the host's immune system with tolerogenic immune cells, such as dendritic cells, before transplantation of stem-cell derived cardiomyocytes.[208] The third method to prevent immune rejection is to create stem cell derived cardiomyocytes that remain undetected by the immune system.[209] Suppression of the immune system can be used in combination with all three previous strategies to further prevent immune rejection, but because of the side effects of immunosuppression, this strategy is unfavorable.

Apart from its role in graft rejection, the immune system also gained attention in regeneration therapy as a potential therapeutic target. In cardiovascular disease, the immune system plays an important role. After MI, inflammation is accountable for a large part of cardiac damage.[210-212] Stem and progenitor cells have the ability to modulate immune responses and animal ischemia-reperfusion models suggest that these modulations have beneficial effects.[183, 213]

Complications

The use of stem cells involves the risk of tumorigenesis. Stem cells have carcinogenic properties: they have the ability to self-renew, proliferate rapidly, lack contact inhibition, and have an extended life-time due to telomerase activity.[214] Studies show that several oncogenes that are highly expressed in teratomas are also found in embryonic stem cells.[215] The actual risk of tumorigenesis in stem cell therapy in humans was highlighted in 2009, when a child received fetal neural stem cells as a therapy for neurodegenerative disease, but developed multifocal glioneural tumors as a complication.[216] Stem-cell derived cardiomyocytes are differentiated *in vitro* and therefore have a much smaller risk of tumorigenesis, but if not carefully selected, contaminating undifferentiated progenitor cells still form a risk. Appropriate cell selection is essential for primary prevention of tumorigenesis.

As secondary prevention, techniques have been developed to track and eliminate teratomas. A reporter can be added to stem cell-derived cardiomyocytes that allows tracking of transplanted stem cell derived cardiomyocytes and selective targeting

in case of teratoma formation.[217] Alternatively, molecular probes that attach to teratoma cell surface receptors were developed for *in vivo* tracking of teratoma formation.[218] Targeting of teratomas using this technique is not yet possible, but would have the advantage that it circumvents genetic modification of stem-cell derived cardiomyocytes, which can induce tumor formation by itself.[214]

A second feared complication of cardiac regeneration therapy is the development of life-threatening arrhythmias. Transplanted stem-cell derived cardiomyocytes have a different action potential compared to host cardiomyocytes, are electrophysiologically poorly integrated in the host myocardium and in most cases display automaticity which leads to cardiac excitability and arrhythmias.[219-222] Experiments in rodent models show an increased risk of arrhythmias after stem cell derived cardiomyocyte transplantation and data from clinical trials reports ventricular arrhythmias as one of the main complications after myoblast transplantation.[223-225] On the contrary, some studies show that implantation of stem cell derived cardiomyocytes prevents the occurrence of arrhythmias.[226] The risk of arrhythmias depends on the cell type used, the mode of delivery, and the host environment.[219] Improvement of electrophysiological integration, determination of the most suitable cell types, and improved modes of delivery are being developed in order to lower the risk of arrhythmia development.

Stem cell sources for cardiac regeneration

Pluripotent stem cells

Embryonic stem cells

Embryonic stem cells are pluripotent stem cells that are isolated from the inner cell mass of mammalian blastocysts. In 1981, murine embryonic stem cells were first isolated, followed by isolation of human embryonic stem cells in 1998.[227, 228] Currently, most human embryonic stem cell lines were derived from pre-implantation stage human blastocysts that were harvested for clinical use, but were no longer intended for that use and were donated after informed consent.[229] Embryonic stem cells are pluripotent; they proliferate indefinitely, and can be differentiated into somatic cells of all three embryonic germ layers under specific culture conditions, including cardiovascular cell types such as fibroblasts, endothelial cells, smooth muscle cells, and cardiomyocytes.[230] Because of these properties, large quantities of cardiac cells, which are necessary for regeneration, can be created *in vitro*. Animal studies show that cardiomyocytes derived from embryonic stem cells (have the ability to integrate in the recipient heart.[201, 231-235] A limited number of transplanted embryonic stem cell derived cardiomyocytes survive, proliferate and mature *in vivo*. Several weeks after transplantation, embryonic stem cell derived cardiomyocytes form desmosomes and gap-junctions and on the midterm cardiac function as measured by ejection fraction improves. These results are promising, but there are some setbacks. Embryonic stem cell derived cardiomyocytes have an immature electrophysiological phenotype, there are still hurdles regarding cell integration and coupling, and functional cardiac improvement in the long term is not the result of cardiomyocytes specifically.[236] As described previously, the use of allogeneic pluripotent stem cells also involves the risk

of immunological rejection and teratoma formation. Finally, embryonic stem cell use involves ethical and legal issues. So far, no clinical studies using embryonic stem cell derived cardiomyocytes have been performed.

Induced pluripotent stem cells

As described above, pluripotent stem cells can differentiate into somatic cells. In 2006, Takahashi et al.[69] first showed that this process can be reversed *in vitro*. Fibroblasts were transduced with retroviral vectors (Oct 3/4, Sox2, Klf4, and c-Myc), which converted them to a cell type that is highly similar to the ESC, called induced pluripotent stem cell.[143] Thus induced pluripotent stem cells have typical embryonic stem cell markers, proliferate indefinitely, and are able to differentiate in somatic cells of all three embryonic cell lineages, including cardiomyocytes.[237, 238] An advantage of induced pluripotent stem cells is that ethical issues of embryonic stem cells are irrelevant and that autologous cells can be created, preventing immunological rejection. A preclinical study performed in 2009 using induced pluripotent stem cell-derived cardiomyocytes reported promising beneficial effects on cardiac function.[239] However, there are issues that require further attention. The use of viral vectors introduces new potential side effects, as they can induce inflammation, cause cell rejection and, in rare cases, lead to a fatal systemic immune response.[240] Secondly, insertion of viral genomes at unwanted locations can disturb cellular function and cause oncogenic changes.[241] However, induced pluripotent stem cell generation methods are rapidly improving and recently it was shown that induced pluripotent stem cells can be created using non-integrating viruses and even without the use of viral vectors.[242, 243] Finally, similar to embryonic stem cell derived cardiomyocytes, there are still challenges regarding cell maturation, integration and coupling, functional cardiac improvement in the long term, and the risk of teratoma formation.

Recently, novel methods were developed to directly reprogram somatic cells into cardiomyocytes.[244-246] Using cardiac transcription factors (Gata4, Mef2c, Tbx5, with or without Hand2,) fibroblasts were reprogrammed in cells that contracted spontaneously, had cardiac-specific markers and showed gene expression profiles comparable to adult cardiomyocytes. Using this technique cardiomyocytes can be created *in vitro*, but *in vivo* direct reprogramming of cardiac fibroblasts into CMs is also possible.[247] In direct reprogramming, the creation of pluripotent cells is bypassed, which reduces the risk of teratoma formation. Side effects related to viral vectors, however, are still possible with the currently available methods.

Multipotent stem cells

Bone marrow cells

Bone marrow-derived cells are stem cells that can be aspirated from the patient's bone marrow and consist of several stem cell types, including hematopoietic stem cells, mesenchymal stem cells, and endothelial stem/progenitor cells.[248] They are different from previously discussed stem cell types, as they are transplanted directly without cardiomyocyte differentiation *in vitro* before transplantation. Some claim that bone marrow-derived cells have the ability to differentiate into non-hematopoietic

cell types, such as the cardiac cells.[249, 250] Others believe that stem cells found in adult organs, such as bone marrow-derived cells, are not truly pluripotent, but are restricted in their differentiative potential and that cardiomyocytes cannot originate from bone marrow-derived cells.[251-253] Therefore, bone marrow-derived cells are not considered candidates for "true" regeneration, but for the paracrine effects they might effectuate. At this moment, bone marrow-derived cells are the most widely used cell source for cardiac repair in clinical trials. Studies in animals and humans proved that transplantation is safe.[254-256] A recent meta-analysis concluded that transplantation of bone marrow-derived cells on average improves LV function, infarct size, and remodelling, and suggests effects on clinical end points, such as mortality and morbidity.[257] Nevertheless, most studies are small, have a relatively short follow-up period, show heterogeneous results, and some of the more recent conducted trials show no effects of bone marrow-derived cells.[258-260] Powered trials with long-term and patient centred outcomes are underway, but it also seems essential to clarify the mechanisms and use these to enhance the beneficial effects of bone marrow-derived cell therapy.[261]

Mesenchymal stem cells

Mesenchymal stem cells are multipotent stem cells that can be derived from several mammalian tissues, such as bone marrow, adipose tissue, and cord blood. In normal physiology, mesenchymal stem cells participate in organ homeostasis, wound healing, and successful aging.[262] They are easily isolated and cultured, do not have side-effects related to pluripotency and the use of viral vectors, are immunologically tolerated as allogeneic transplant, and have the potential to differentiate into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma.[263] Like bone marrow-derived cells, mesenchymal stem cells are generally not differentiated into cardiomyocytes before transplantation, although efforts have been undertaken to turn mesenchymal stem cells into cardiac cells using a combination of growth factors.[264, 265] Animal studies showed the ability of transplanted mesenchymal stem cells to engraft in the myocardium and to secrete paracrine factors.[266, 267] Some even suggested that unmodified mesenchymal stem cells might differentiate into vascular cells and cardiomyocytes.[268-270] Generally, however, it is thought that the effects of mesenchymal stem cells are not primarily based on trans-differentiation into cardiac cells, but on the secretion of paracrine factors by mesenchymal stem cells.[271] As such, true regeneration from unmodified mesenchymal stem cell derived cardiomyocytes is not to be expected. Nevertheless, animal studies reported beneficial effects of mesenchymal stem cell treatment on several outcome parameters, using multiple ischemia models, different kinds of mesenchymal stem cell administration, directly and up to 4 weeks after cardiac injury.[271] Results of clinical trials using mesenchymal stem cells also show beneficial effects on cardiac function, although results are still preliminary and the question remains whether they will hold true for the long term.[197] Further clinical studies will determine whether the beneficial paracrine effects of mesenchymal stem cells found in animal models are reproducible in humans.

Cardiac stem cells

For a long time, it was believed that cardiac tissue is terminally differentiated. Recently, however, resident cardiac stem cells, progenitor cells in the cardiac lineage, were found in cardiac tissue. Cardiac stem cells can be isolated from foetal and adult cardiac biopsies based on expression of stem cell marker proteins, such as Isl-1, c-kit, and Sca-1. They form cell lines that can be expanded in culture whilst keeping their progenitor state. Subsequently, they can differentiate in cardiovascular cell types, such as cardiomyocytes, endothelial cells, and smooth muscle cells.[272-274] After MI, cardiac stem cells and newly formed myocytes are found in the border area of the infarct, suggesting that the heart has some, although insufficient, regeneration capacity.[180] Transplantation of cardiac stem cells aims to use and enlarge the heart's own regeneration capacity. Cardiac stem cells have the advantage that they can differentiate in cardiovascular cell types *in vivo* without the need of pre-implantation *in vitro* differentiation. Recently, results from the first phase 1 clinical trials using cardiac stem cell infusion were published.[275, 276] Intracoronary autologous cardiac stem cell injection appears to be safe and preliminary results showed an increase in viable myocardium, left ventricular ejection fraction, and other clinical parameters, although results differ between trials. Larger randomized, blinded trials with appropriate controls will have to be performed to fully analyse the clinical effect of cardiac stem cell injection.

Clinical applicability

Results of pre-clinical studies using stem cell derived cardiomyocytes for cardiac regeneration seem very promising. Animal studies show survival, maturation, integration, and sometimes functional coupling of stem cell derived cardiomyocytes, and improvement of various cardiac functions, such as ejection fraction, after transplantation. However, there are reasons to be cautious.

Animal models used in preclinical studies do not always translate to human physiology. In a meta-analysis of 76 positive animal studies published in leading scientific journals, only 28 studies were replicated in humans. Of these 28 studies, only 8 therapies were subsequently approved for use in patients.[277]

In 2004, the NHLBI working group on the translation of therapies for protecting the heart from ischemia investigated the lack of translation of promising animal experiments into clinical practice. They concluded that numerous factors, including the use of imperfect animal models, the lack of reproducibility, standardized research protocols, randomized study design, and blinding of investigators resulted in unsuccessful translational research.[278]

In cardiac regeneration therapy specifically, ischemia in animal models is mostly achieved by coronary ligation in a healthy heart. On the contrary, cardiovascular diseases for which cardiac regeneration might be useful, such as heart failure and atherosclerosis that usually underlies a myocardial infarction, are chronic diseases in which the entire heart is affected. Non-ischemic cardiomyopathy represents yet

another challenge for translational medicine as the disease seems to be based on entirely different, though often unknown, pathological mechanisms. Nevertheless, just as with ischemic cardiomyopathy, non-ischemic cardiomyopathy is characterized by a loss of functional cardiomyocytes and therefore, in theory amenable to stem cell-based repair. Pre-clinical studies using this model are scarce, although one clinical trial using bone marrow-derived cells has recently been reported.[279-281] Moreover, immunodeficient animal models allow transplanted stem cell derived cardiomyocytes to survive but skips the important step of immunotolerance, which is necessary for translation to clinical applicability of allogeneic cells.

Most positive results are based on an increase in left ventricular ejection fraction, whereas results of cardiac regeneration therapy on patient-centred outcomes such as mortality and morbidity are absent. This requires longer follow-ups.

The follow-up period in pre-clinical regeneration studies is limited to a few months maximum and the few long-term studies that were published show less positive results.[282, 283] This finding suggests that currently the results of cardiac regeneration are not predominantly attributable to structural integration of functional cardiomyocytes, but to temporary paracrine release of anti-apoptotic, immunemodulatory, and proangiogenic factors by stem-cell derived cardiomyocytes and cardiac support cells. Although these and other paracrine effects of stem-cell derived cardiomyocytes are promising and clinical studies aiming to use these effects are currently being performed, the ultimate goal of regenerative medicine is to use stem cells to create healthy, functional cardiac cells that are able to integrate in the injured heart and restore its function. Paracrine effects might reduce damage after cardiovascular disease such as MI, but are unlikely to repair the already damaged heart.

In order to clinically use stem cells for cardiac repair, more fundamental and translational research is necessary. Fundamental *in vitro* research, preferably using human cells (e.g., induced pluripotent stem cell derived cardiomyocytes) will improve our knowledge of the mechanisms and requirements regarding differentiation of stem cells into functional cardiomyocytes, whereas translational research using appropriate animal models, long-term follow-up and relevant outcome measures, will hopefully result in clinically applicable cardiac repair.

Conclusion

Regenerative therapy using stem cells is a promising, relatively new modality for cardiac repair. Several stem cell types have been identified and investigated in pre-clinical and clinical research, generally with positive results. However, the degree of success has been variable and attributed to paracrine effects. Questions about optimal cell type, mode of delivery, time of delivery, integration in the heart, and safety have to be answered before true regeneration of cardiac tissue in patients will be possible. The field of regenerative medicine is rapidly developing and the first clinical studies are already being performed. In the meantime, many important fundamental questions are being addressed in pre-clinical research.

Chapter 4

Sca1+ cells from the heart possess a molecular circadian clock and display circadian oscillations in proliferation, stress tolerance, and paracrine factor secretion

Chapter 4 - Sca1+ cells from the heart possess a molecular circadian clock and display circadian oscillations in proliferation, stress tolerance, and paracrine factor secretion

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Abstract

Stem cell antigen-1 positive (Sca1+) cells (SPCs) have been investigated in cell-based cardiac repair and pharmacological research although improved cardiac function after injection has been variable and the mode of action remains unclear. Circadian (24-hour) rhythms are biorhythms regulated by molecular clocks that play an important role in (patho)physiology. Here we describe (1) the presence of a molecular circadian clock in SPCs and (2) circadian rhythmicity in SPC function. We isolated SPCs from human fetal heart and found that these cells possess a molecular clock based on typical oscillations in core clock components BMAL1 and CRY1. Functional analyses revealed that circadian rhythmicity also governs SPC proliferation, stress tolerance, and growth factor release, with large differences between peaks and troughs. We conclude that SPCs contain a circadian molecular clock, which controls crucial cellular functions. Taking circadian rhythms into account may improve reproducibility and outcome of research and therapies using SPCs.

Introduction

Stem cell antigen-1 positive cells (Sca1+ cells, SPCs) are self-renewing cells that have been isolated from fetal and adult heart.[284, 285] Together with c-Kit+[286], and Islet-1+[287] cells, SPCs have been referred to as cardiac progenitor / stem cells, even though their *in vivo* cardiogenic differentiation potential is limited.[288-290] *In vitro* demethylation using compounds such as 5-aza and TGF-beta treatment of SPCs however, does induce cardiomyocyte-like phenotypes with up-regulation of cardiac genes and sarcomeric proteins and the development of functional action potentials.[272] SPCs have been investigated for their efficacy in cell-based cardiac repair. In pre-clinical and clinical studies, injection of these cardiac progenitor-like cells proved to be safe and has been described to lead to structural and functional cardiac improvements.[273, 291]

Despite these promising results, challenges still remain. Sca1, c-Kit, and Islet-1 cannot be used to distinguish a specific cell population.[292] As a result, studies show variability in cell surface markers, proliferation and differentiation, limiting the use of these cells in for example pharmacological studies[293] In addition to cell surface marker, proliferation and differentiation variability, pre-clinical transplantation studies face other drawbacks such as poor engraftment, cell survival and *in vivo* differentiation.[290] Finally, there is an ongoing search for underlying mechanisms and ways to maximize beneficial effects, including novel effect modifiers.

Circadian (diurnal, 24-hour) rhythms are biorhythms with a period of approximately 24 hours. These rhythms are regulated by molecular clocks that consist of several oscillating proteins such as CLOCK, BMAL, PER, and CRY.[294, 295] Many cardiovascular parameters, including heart rate, repolarization duration, and cardiac metabolism display circadian rhythmicity.[88] Circadian rhythms also play an important role in cardiovascular disease. The incidence of many cardiovascular diseases such as myocardial infarction and acute heart failure follows a 24-hour pattern. Second, disruption of 24-hour rhythms leads to an increase of cardiovascular disease, and finally, outcome of disease and efficacy of therapy is influenced by this 24-hour rhythm.[31-33, 296]

In short, circadian rhythms are an important factor in cardiovascular physiology and disease but have not yet been studied in SPCs, a potential cell source for cardiac cells that proved to be useful in pharmacological studies and preclinical cell-based cardiac repair. In the current study, we therefore analyzed the potential presence of circadian rhythms in SPC physiology. More specifically, we (1) sought to determine whether SPCs have functional molecular circadian clocks and (2) whether significant circadian oscillations determine SPC functions, such as proliferation, migration, stress tolerance, and paracrine factor secretion.

Methods

SPC isolation

SPCs were isolated and cultured as previously described.[272, 297] In short, fetal hearts, obtained from elective abortion with prior informed consent and approval of the ethical committee of the University Medical Center Utrecht, were enzymatically dissociated. Magnetic cell sorting with an iron-labeled anti-Sca-1 antibody was performed to extract the SPCs from the dissociated cell suspension.

Synchronization of SPCs

Confluent SPCs were exposed to the synchronizers "serum shock" (SS, 50% culture medium / 50% horse serum, Gibco), forskolin (10µM, Sigma), or dexamethasone (100nM, Sigma) at the start of the experiments to align circadian clocks.[298, 299] Non-synchronized SPCs, with medium change more than 2 days before start of the experiment, served as controls.

Quantitative RT-PCR, Western Blotting, and Immunofluorescence

Detailed techniques and protocols used for quantitative reverse transcription polymerase chain reaction (qRT-PCR), western blotting (WB), and immunofluorescence, including primer sequences and antibodies, are described in the supplemental methods and Table 4.3

Bioluminescent reporter

Lentiviral plasmids harboring luciferase reporters of BMAL1 and CRY1 promoters were described previously and kindly provided by professor Liu.[300-302] Viral particles were concentrated via ultracentrifugation after 3 harvests in HEK293T cells. SPCs were then transduced with BMAL1-dLuc or CRY1-dLuc lentivirus and selected with 10ug/ml blasticidin for at least 5 days. Stable polyclonal lines were propagated. Bioluminescence was monitored via the use of a LumiCycle32 device (Actimetrics). In short, cultures of SPCs were grown confluent and synchronized. Then medium was switched to recording medium (Phenol Red-free DMEM, 10%FCS, 10mM HEPES, 0.035% Bicarbonate, 4.5g/L glucose and Pen/Strep +100µM D-Luciferin (Promega). Culture dishes were sealed with high vacuum grease (Dow Corning) and put in a LumiCycle32, kept in a 37°C incubator for recording. Bioluminescence from each dish was continuously recorded (integrated signal of 70 seconds with intervals of 10 minutes). Raw data (counts/seconds) were base line subtracted (polynomial order 3).

Cell detachment assay

SPCs were cultured in 0.1% gelatin coated plastic 75cm^2 flasks and synchronized using serum shock. After subsequent time-periods (9-51 hours with 6 hour intervals), the culture medium was removed temporarily and stored under sterile conditions during

the time needed for trypsinisation. Cells were washed twice with PBS, treated with trypsin (Trypsin-EDTA 0.25%, Lonza) or collagenase (1mg/ml) , and re-plated in their own (temporarily removed) medium to prevent medium-induced re-synchronization. After 12 hours, when cells were reattached, cells were lysed and BMAL1 was quantified using western blotting (supplemental methods).

Matrix assay

SPCs ($2x10^6$ cells per ml) were combined with collagen-type1 (1mg/ml, ThermoFisher) and plated in 50µl drops in a 24-well plate. After 30 minutes, culture medium was added. Wells with SPC loaded matrixes were synchronized using serum shock (or served as controls) and were collected after subsequent time-periods (9-51 hours) for western blot analysis.

Proliferation assay

Incorporation of 5-Bromo-2-deoxyuridine (BrdU) was used to quantify proliferation. For each time-point, SPCs were plated in several dilutions on 1% gelatin coated 12mm Ø glass cover slips. At the start of the experiment (t=0), culture medium was synchronized for 30 minutes. After subsequent time periods (2-50 hours with 4 hour intervals), BrdU (final [10µg/mL], BD Pharmingen) was added to the medium for 6 hours. Identical culture confluency (+/- 30%) was used for all time points. After this period, cells were fixed with 4% paraformaldehyde. BrdU quantification is described in the supplemental methods.

Migration assay

SPC migration was measured using a scratch wound cell migration assay.[303] Cells were cultured in a 0.1% gelatin coated 24-wells plate and synchronized at 80-90% confluency. After subsequent time periods (2-50 hours with 4 hour intervals) a horizontal and vertical scratch were made within the wells using a standard 1ml pipet tip. Pictures of 4 standardized locations were taken directly and 6 hours after the scratch. Migration back into the scratch was measured by calculating the surface area right after the scratch (=100%) minus surface area after 6 hours using ImageJ software (Version 1.48u4). Percentages were averaged per well and reduced by the moving average to correct for any non-circadian variations.

Cell death assay

To measure stress tolerance, SPCs were cultured in a 96-well plate and synchronized. After subsequent time periods (9-51 hours with 6-hour intervals) doxorubicin (10uM, 6 hours Sigma) or tert-butyl hydroperoxide (10 µM, 1 hour, Sigma) was added to the culture medium. Subsequently, apoptosis was measured using a luminescent Caspase-Glo 3/7 assay (Promega) according to the manufacturer's instructions.

Cellular secretion assay

To measure growth factor secretion, SPCs were cultured in a 6-well plate and synchronized. After subsequent time periods (12-62 hours with 5-hour intervals), medium was changed and conditioned medium by SPCs was collected for 2 hours. The growth factor levels of SPCs were determined using a 11-multiplex panel (eBioscience, EPX110-12170-901), measured with a Luminex-200 instrument (Bio-Plex 200). The luminex assay was performed according to manufacturer's protocol.

Statistical analysis

Data are presented as mean ± standard error of mean. Circadian rhythmicity was analyzed using Cosinor analysis in R.[304] To compare differences between 2 groups, (2-tailed) Student's T-test was used. P-values < 0.05 were considered statistically significant.

Results

Figure 4.1 shows the (simplified) experimental setup of the proliferation, migration, stress tolerance, and paracrine secretion experiments.

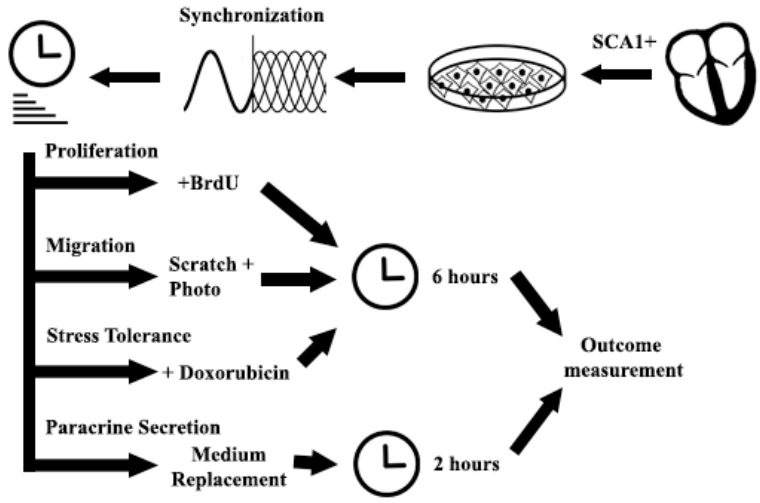

Figure 4.1. Sca1-expression was used to isolate cells. Cells were cultured and synchronized before the experiment. After subsequent time-periods, BrdU was added for the proliferation experiment. For migration, a scratch was made in the culture dish and a picture of the well was taken. For stress tolerance, doxorubicin was added and for paracrine secretion, medium was replaced. After a subsequent period (2 or 6 hours), outcome was measured according to procedures described in the methods

SPC Isolation

SPCs were derived from human fetal and adult hearts based on reaction to a Sca1 antibody, as described previously.[272] Although Sca1 itself is not present in the human heart, this technique has been shown previously to isolate a subset of proliferative and clonogenic cells with cardiogenic potential depending on the addition of exogenous factors as characterized extensively in previous work.[288, 290, 305] Sca1+ cells from the human heart display a large degree of similarity with other progenitor-like cells previously referred to as cardiospheres and C-kit+ cardiac stem cells.[306]

The presence of early cardiac transcription factors Homeobox protein Nkx-2.5 (NKX2.5) and GATA4 in combination with the absence of cardiomyocyte markers β-Myosin Heavy Chain (bMHC) confirmed their successful isolation (Table 4.1).

	PPIA	NKX2.5	GATA4	bMHC
HFH	23.7	29.13	21.34	25.43
SPC	25.43	35.53	32.66	ND
hESC	22.91	ND	ND	ND
H2O	ND	ND	ND	ND

Table 4.1. Cycle threshold values of cardiac transcriptional factors, NKX2.5 and GATA4 and cardiomyocyte marker bMHC. Human undifferentiated embryonic stem cells and H_2O are used as negative controls, human fetal heart tissue as positive control for transcription factors. HFH: Human fetal heart; SPC: human fetal Sca1+ cell; hESC: human embryonic stem cell;

SPCs have a functional molecular clock

To analyze the presence of a molecular circadian clock in SPCs, sequential mRNA and protein expression levels of core clock components BMAL1 and CRY1 were measured. After clock synchronization, BMAL1 and CRY1 mRNA levels oscillated in a 24-hour periodical pattern and were in counter phase with each other (Figure 4.2A and B; A: fold changes 2.25 and 2.28, peaks at 22.4h and 10,6h resp B: fold changes 1.79 and 1.40, peaks at 20.8h and 10.0h resp; $P<0.001$ for all; n=4), in concordance with predicted expression patterns in adult tissues. [307] This translated into corresponding 24-hour oscillations in protein levels of BMAL1 and CRY1 (Figure 4.2C and D; fold changes 1.26 and 1.34, peaks 13.8h and 22.7h, $P<0.001$ and $P=0.014$ for BMAL1 and CRY, respectively; n=4).

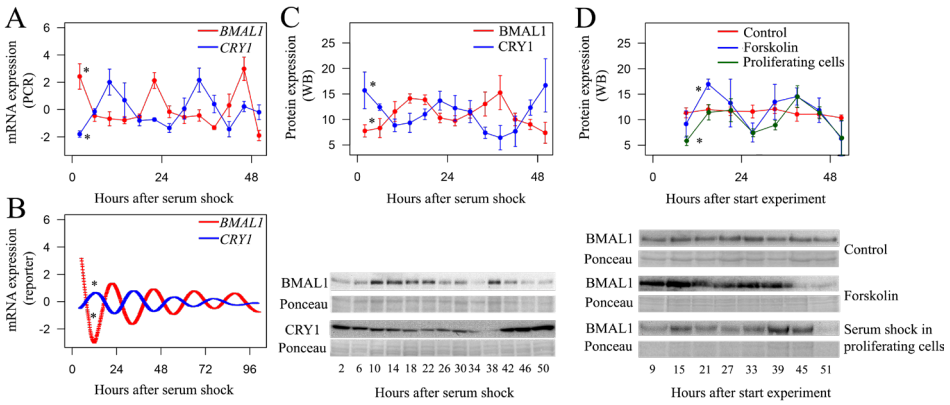

Figure 4.2. Molecular circadian clockwork within SPCs. 24-hour oscillations in core clock components BMAL1 and CRY1 were present at the transcriptional and protein level. A) 48-hour analysis of mRNA using qRT-PCR ($P<0.001$ for BMAL1 and CRY1; n=4). B) 100-hour analysis of mRNA using bioluminescence reporters ($P<0.001$ for BMAL1 and CRY1). C and D) BMAL1 and CRY1 protein expression during 48 hours. Counter phased 24-hour rhythms were present ($P<0.001$ and $P=0.014$ for BMAL1 and CRY1, respectively; n=4). E and F) BMAL1 protein expression during 48 hours. 24-hour rhythmicity was absent when SPCs are unsynchronized ($P=0.734$; n=4). Presence of rhythmicity was independent of synchronizer substance (Forskolin $P=0.01$; n=4) and high / low proliferation rate (proliferating cells $P=0.002$; n=4). * Indicates $P<0.05$ for significant 24-hour oscillation. Data are represented as mean ± SEM.

To exclude the possibility that 24-hour fluctuations were caused by methodological artifacts, we confirmed that oscillations were absent when molecular clocks were not synchronized and were independent of the type of synchronizing substance (Figure 4.2D; fold change 1.38 and peak at 15.5h for synchronized-synchronized SPCs; $P=0.734$ and 0.010 for circadian rhythmicity in unsynchronized and forskolin-synchronized SPCs, respectively; n=4). In addition, proliferating SPCs had similar 24-hour fluctuations with respect to amplitude and phase as non-proliferating confluent SPCs (Figure 4.2E and F; fold change 1.38 and peak 17.3h $P=0.002$ for circadian rhythmicity; n=4).

Next, we analyzed the presence of intracellular shuttling of BMAL1, one of the other characteristics of a functional molecular circadian clock.[308] Indeed, there was a 24-hour rhythm in the nuclear/cytoplasmic BMAL1 fraction in the first 24 hours after serum shock (Figure 4.3; fold change 1.36 and peak at 13.0h; $P=0.003$; n=3).

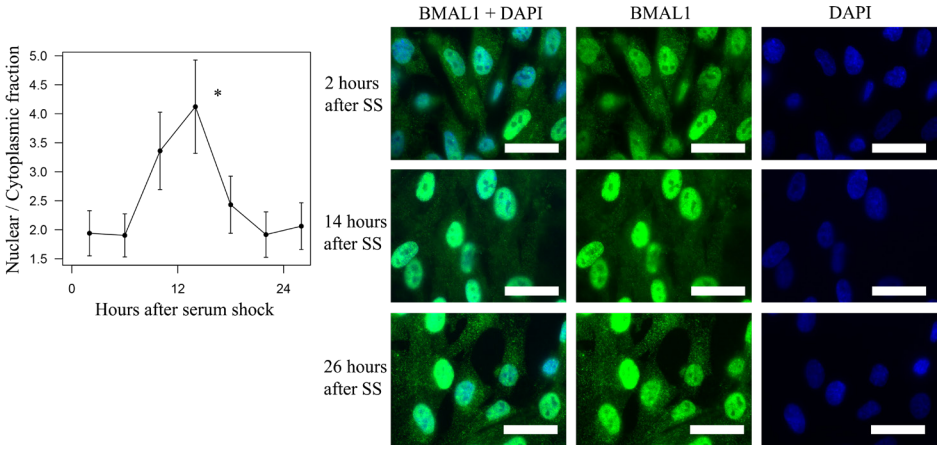

Figure 4.3. Intracellular shuttling of BMAL1. During 24-hours after serum shock (SS), BMAL1 was measured using immunofluorescence. There was 24-hour rhythmicity in the localization of BMAL1, as quantified by the nuclear / cytoplasmic fraction (P=0.003; n=3). * Indicates P<0.05 for significant 24-hour oscillation. Data are represented as mean ± SEM. Scale bars, 40µm

Because circadian rhythms are associated with ageing, we repeated the clock component measurements in adult SPCs and found that similar to fetal cells, BMAL1 levels varied in a 24-hour periodical pattern. (Figure 4.4; fold change 1.59 and 1.38, peak at 2.85h and 15.5h P<0.001 and 0.01 for reporter and WB; n=3)

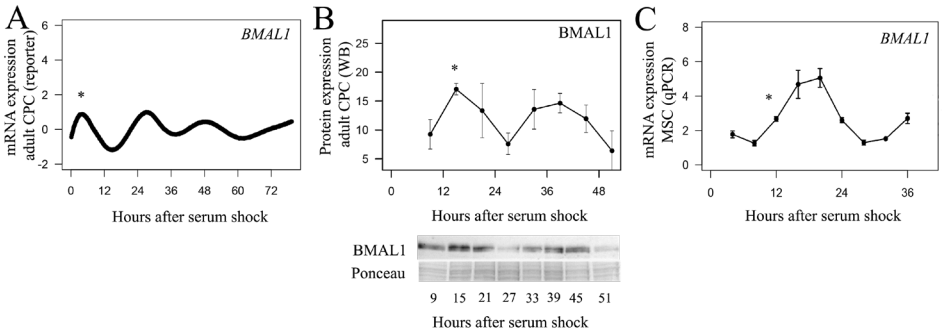

Figure 4.4. Molecular circadian clockwork within adult SPCs and MSCs. 24-hour oscillations in core clock component BMAL1 was present at the transcriptional and protein level. A) 80-hour analysis of mRNA in adult SPCs using bioluminescence reporters (P<0.001; n=3). B) BMAL1 protein expression in adult SPCs during 51 hours. A 24-hour rhythm was present (P<0.01; n=3). C) Bmal1 expression in fetal MSCs. (fold change 1.64, peak at 18.2h, P<0.001; n=3). SPC: human fetal Sca1+ cell; MSC: Mesenchymal stem cell. * Indicates P<0.05 for significant 24-hour oscillation Data are represented as mean ± SEM.

All together, these observations demonstrate that the molecular circadian clockwork is present and active in SPCs.

Cell dissociation disrupts circadian rhythmicity

We analyzed whether cell dissociation using trypsinisation or collagenase treatment of SPCs influences 24-hour rhythms of BMAL1. After SPCs were trypsinised or treated with collagenase, BMAL1 protein expression no longer oscillated, indicating that cell dissociation disrupts the synchrony of circadian rhythmicity in the cell dish (Figure 4.5 and Figure 4.6A; P=0.589 and P=0.344 respectively, n=3).

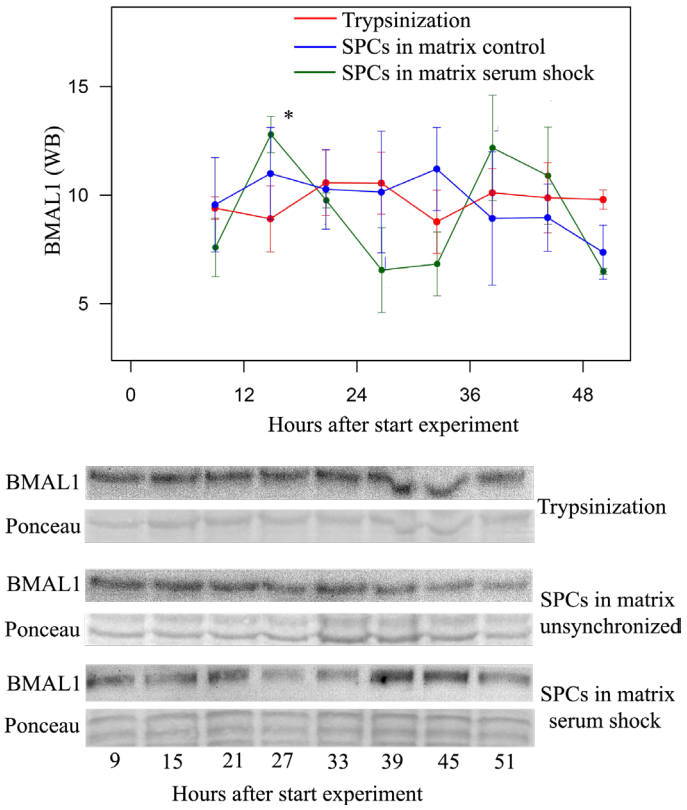

Figure 4.5. Dissociation of SPCs disrupts the 24-hour rhythm in BMAL1 protein expression. (P=0.589; n=3). SPCs were also loaded in collagen matrixes that do not require cell dissociation. After synchronization, SPC in these matrixes showed 24-hour oscillations in BMAL1 similar to SPCs plated in culture dishes (P=0.014 and 0.734 for serum shock and unsynchronized cells in matrix, respectively; n=3). * Indicates P<0.05 for significant 24-hour oscillation. Data are represented as mean ± SEM.

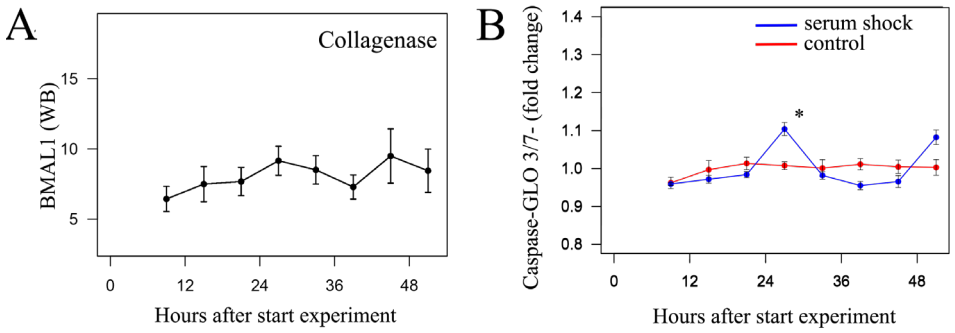

Figure 4.6. A) Collagenase of SPCs disrupts the 24-hour rhythm in BMAL1 protein expression. (P=0.344; n=3). B) Apoptosis was measured after serum shock, subsequent time-periods (as depicted on x-axis) and 1 hour of hydroxyperoxide exposure using a Caspase-Glo 3/7 assay. Apoptosis was 13% higher 27 and 51 hours compared to 15 and 39 hours after serum shock (P<0.001; n=4). In non-synchronized cells, no circadian rhythm was present (P=0.312; n=4). Data are represented as mean ± SEM.

This effect of dissociation could limit the use of circadian rhythmicity in SPCs. Synchronization and use of the most optimal time-window for subsequent applications is problematic if SPCs have to be detached from the culture dish before injection. To circumvent this potential problem, we loaded SPCs in matrixes, since those do not require dissociation before application. After loading of SPCs and synchronization, BMAL1 expression oscillated comparable to cells attached in a culture dish (Figure 4.5; fold change 1.38, peak at 15.5h, P=0.014 for serum shock; P=0.734 for unsynchronized cells in matrix; n=3). This shows that by using extracellular matrix based scaffolds, disruption of circadian rhythms by dissociation can be prevented.

Circadian rhythms are present in proliferation but not migration

In order to explore the functional consequences of the molecular circadian clock, we analyzed circadian rhythmicity in SPC proliferation and migration. We found that BrdU incorporation, an indicator of proliferation, occurs in a clear circadian pattern with two peaks per 24 hours (Figure 4.7A and B; fold change 1.08 and peak at 15.5h for serum shock, P<0.001 and P=0.776 for serum shock and non-synchronized negative controls, respectively; n=4). At its peaks, proliferation was 16.2±6.9% higher compared to troughs (P=0.015). We considered the possibility that clock synchronization also synchronizes cell cycle and that differences in BrdU incorporation were caused by changes in cell cycle phase instead of proliferation. To analyze this possibility, we quantified the amount of cells during 48 hours of cell culture and found that SPC doubling time was 19.5±0.8 hours (n=4), making it an unlikely cause for the 12-hourly peaks in proliferation.

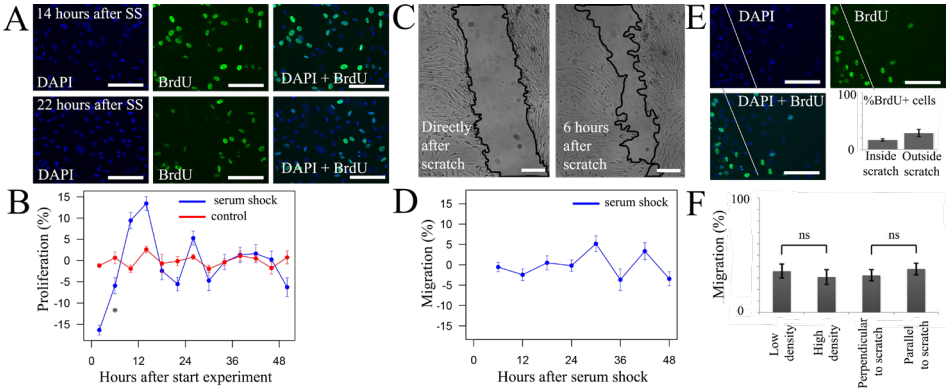

Figure 4.7. Analysis of circadian rhythmicity in SPC proliferation and migration. A) Two examples of BrdU staining showing BrdU incorporation 14-20 hours (47%) and 22-28 hours (31%) after serum shock. B) Circadian biphasic rhythm in BrdU incorporation of SPCs after serum shock (period 12 hours; P<0.001; n=4) but not in unsynchronized cells (P=0.776; n=4). C) Example of scratch wound migration assay picture taken directly and 6 hours after scratch. D) No rhythmicity was present in migration of SPCs into the scratch (P=0.442, n=4). E) Combination of proliferation and migration experiments showing that the majority (83.1±2.0%) of cells present in the scratch after 6 hours did not proliferate. White line indicates border of the scratch. F) Percentage of growth back into scratch in conditions with lowest and highest cell density and in scratches perpendicular and parallel to cell orientation. No significant differences were found between groups (low density 36.1±6.0 vs. high density 31.0±6.5, P=0.59; perpendicular orientation 32.4±4.9 vs. parallel orientation 37.9±5.2; P=0.11; n=12 for all). * Indicates P<0.05 for significant circadian oscillation. Data are represented as mean ± SEM. Scale bars, 100µm

SPC migration did not demonstrate any circadian fluctuations (Figure 4.7C and D; P=0.442; n=4). To rule out the possibility that concurrent proliferation clouded the results of our migration assay, we combined the migration assay with BrdU incorporation and found that 83.1±2.0% of cells in the scratch were BrdU negative (Figure 4.7E; n=12). This indicates that the majority of SPCs within the scratch originate from migration of cells, rather than from proliferation of cells residing originally in the scratch borderzone. In addition, we analyzed whether SPC density or orientation to the scratch (parallel or perpendicular to the orientation of the scratch) interfered, but these factors did not influence migration (Figure 4.7F; low density vs. high density 36.1±6.0 vs. 31.0±6.5; P=0.59; perpendicular orientation vs. parallel orientation 32.4±4.9 vs. 37.9±5.2; P=0.11; n=12 for all).

Circadian rhythmicity in cell death/apoptosis

To further explore functional rhythmicity in SPCs, we analyzed rhythmicity in stress tolerance by exposing SPCs to doxorubicin, an anti-cancer drug well known for its cardiac toxicity.[309] We found a clear circadian rhythm in doxorubicin-induced apoptosis that was not present in unsynchronized control cells. (Figure 4.8A; fold change 1.13 and peak at 27.2h, P<0.001 for doxorubicin-treated SPCs, P=0.396 for controls; n=4). At its peaks, apoptosis as expressed by Caspase-Glo 3/7 was 27.4±2.0% higher compared

to the troughs (P<0.001; n=4). To test whether these findings were limited to the stressor doxorubicin, we repeated the experiment with or tert-butyl hydroperoxide as an alternative and found similar results (Figure 4.6B; fold change 1.06, peak at 26.9 hours, P<0.001, n=4)

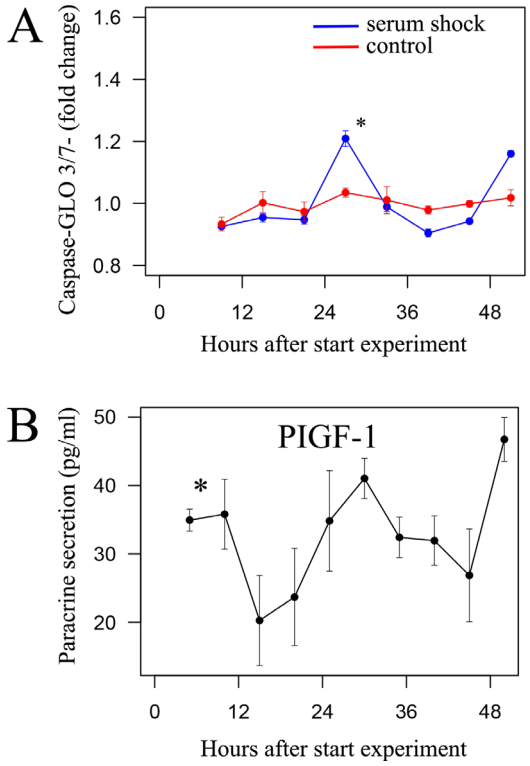

Figure 4.8. Circadian rhythmicity was present in stress tolerance and 4/10 paracrine secretion factors. A) Apoptosis was measured after serum shock, subsequent time-periods (as depicted on x-axis) and 6 hours of doxorubicin exposure using a Caspase-Glo 3/7 assay. Apoptosis was 27.4±2.0% higher 27 and 51 hours compared to 15 and 39 hours after serum shock (P<0.001; n=4). In non-synchronized cells, no circadian rhythm was present (P=0.396; n=4). B) Circadian rhythm in the excretion of paracrine factor PIGF-1 (placental growth factor); * Indicates P<0.05 for significant circadian oscillation. Data are represented as mean ± SEM.

Circadian rhythm in excretion of growth factors

Finally, we analyzed rhythmicity in SPC function by measuring the release of ten growth factors by SPCs during 48 hours. Four factors (beta-nerve growth factor (bNGF), human placental growth factor (hPGF), stem cell factor (SCF), vascular endothelial growth factor A (VEGFA)) showed a clear circadian rhythm, whereas in the remaining six factors (Brain-derived neurotrophic factor (BNF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF) and vascular endothelial growth factor D (VEGFD)) no rhythmicity was found. The 4 oscillating factors have not been reported to influence circadian rhythmicity themselves. An example of a circadian rhythm in paracrine factor release is

depicted in Figure 4B. A complete overview of all ten factors is given in Table 4.2. Peak release of the four oscillating factors occurred at the same time-points (around 25 and 50 hours after synchronization). The amplitude of the four oscillating factors was 24.3±1.2%, indicating that at its circadian peak, paracrine factor release was almost twice as large as compared to the circadian trough.

Paracrine factor	24h Average (pg/ml)	Significance of 24h rhythm (P-value)	24h Amplitude (pg/ml)
BDNF	98.4	0.374	na
bNGF*	244	0.024	56.5
EGF	29.2	0.523	na
FGF-2	67.4	0.149	na
HGF	$10x10^3$	0.054	$3.17x10^3$
LIF	830	0.269	na
PIGF-1*	32.5	0.005	8.28
SCF*	19.6	0.020	4.94
VEGF-A*	$5.28x10^3$	0.017	$1.23x10^3$
VEGF-D	61,1	0.056	ND

Table 4.2. Release of paracrine factors from SPCs. bNGF, PIGF-1, SCF, and VEGF-A release has a 24hour rhythm. SPC: Sca1+ cell. * Indicates $P<0.05$ for significant circadian oscillation. n=3 per paracrine factor.

Discussion

Our data demonstrate that Sca1 reactive cells, as an example of progenitor-like cells from the heart, possess a molecular circadian clock that is functional both at the transcriptional and protein level, resulting in circadian oscillations of core clock genes and proteins. In addition, we show that circadian rhythms are present in the important cell functions proliferation, stress tolerance, and paracrine factor secretion, but not in migration.

Taking circadian rhythmicity into account could improve the potential of progenitor-like cells in research and future therapeutic applications, and can potentially clarify some of the discrepancies that have been found between studies. *In vitro* experiments investigating proliferation, stress tolerance, and paracrine secretion will be more reliable and reproducible when circadian rhythmicity is included. For optimal testing, all different experimental conditions should be performed at the same molecular time, and preferably even at different time-points. In our stress tolerance experiments for example, we showed that the timing of adding a low dose of doxorubicin to SPC cultures is crucial: doxorubicin hardly increased apoptosis when applied 21 hours after the start of an experiment, whereas it did increase apoptosis significantly when added only 6 hours later, 27 hours after the start of the experiment.

In vivo experiments that apply transplantation of cultured stem- or progenitor derived cells could also potentially benefit from knowledge about circadian rhythms: similar to *in vitro* experiments, reproducibility will likely be improved when all experiments are

performed at the same (and preferably more) time frames. This may for example help translate results from nocturnal rodents to diurnal larger animals or patients, which are known to have opposite circadian rhythms. In addition, physiological circadian rhythmicity has the potential to improve SPC effects, with the advantage that it does not require genetic modifications, which often compromise translation from the experimental lab to the patient.

Circadian rhythms have previously been associated with cell proliferation and stress tolerance *in vivo*. Osteoblasts, adult stem cells responsible for bone formation, possess a molecular circadian clock which, when disrupted, inhibits proliferation.[160] In epidermal stem cells, the phase of the molecular circadian clock determines whether stem cells are receptive to proliferative cues, or stay in a dormant state.[144] The link between stress tolerance and circadian rhythms was most prominently shown by studies investigating myocardial infarction. Several independent pre-clinical and clinical studies showed that infarct size and remaining left ventricular function depend on the time of day a myocardial infarction occurs.[32, 33] Reiter et al. quantified the potential impact of a circadian rhythm in infarct size for patients, with a difference of >7% in ejection fraction one year after the infarct between the best and worse time-of-day, even when taking possible confounders into account.[32, 33] Studies of Durgan et al, Xiao et al, and Bonney et al. provided mechanistic insight into this phenomenon by demonstrating the relation to the cardiomyocyte circadian clock and its output pathways, including possibly paracrine excretion.[32, 310-313]

The studies of Janich, Fu, Durgan, and others nicely demonstrated that genetic or environmental disruption of the molecular circadian clock in various stem cells and cardiac cells results in disrupted proliferation or stress tolerance.[32, 144, 160] Although our data is in line with these results, the set-up of our experiments differs: we did not focus on (long-term) results of molecular clock disruption, but were interested in physiological circadian fluctuations in SPC functions which had not been investigated previously.

In conclusion, to our knowledge, this is the first time that circadian rhythmicity has been linked to a cell type with potential for cardiac *in vitro* research and cell-based cardiac repair. Our findings indicate that significant circadian rhythmicity is present in SPCs both at the molecular and functional level and may influence the results of experiments and therapies using SPCs and other progenitor-like cells.

Supplemental methods

Quantitative RT-PCR

qRT-PCR was used for gene expression analysis. RNA was isolated with a phenol-chloroform (Merck) extraction method, DNase-treated, and quantified using a spectrophotometer. After cDNA synthesis, quantitative measurements of Bmal1, Cry1, NKX2.5, GATA4, Troponin, and bMHC were done using SYBR-green (BIORAD) RT-PCR. PPIA was used as housekeeping gene. Primer sequences are given in Table 4.3.

Gene	Forward primer	Reverse primer
PPIA	TTCTGCTGTCTTTGGGACT	CACCGTGTTCTTCGACATTG
Bmal1	GGCTCATAGATGCAAAAACTGG	CTCCAGAACATAATCGAGATGG
Cry1	TTGGAAAGGAACGAGACGCAG	CGGTTGTCCACCATTGAGTT
NKX2.5	TTCTATCCACGTGCCTACAGC	CTGTCTTCTCCAGCTCCACC
GATA4	CGACACCCCAATCTCGATATG	GTTGCACAGATAGTGACCCGT
Troponin	GCGGGTCTTGGAGACTTTCT	TTCGACCTGCAGGAGAAGTT
bMHC	TCTTTCCCTGCTGCTCTC	GACTGCCATCTCCGA ATC

Table 4.3. Primer sequences used for PCR

Western Blotting

RIPA-buffer was used to lyse cells for proteins analysis as described previously.[314] Protein concentrations were measured using BCA assay. Lysates were 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, #93806, Abcam) or anti-CRY1 (1:2000, #13474-1-A, Proteintech) antibodies, followed by a peroxidase-conjugated antibody (1:7000, #170-6515, Biorad), and ECL chemiluminescence (sc-2048, SantaCruz) for detection. Ponceau-corrected protein quantification was performed with Image Lab (Version 5.1, Biorad).

Intracellular shuttling of Bmal

SPCs were plated on 1% gelatin coated 12mm Ø glass cover slips and synchronized using serum shock. At subsequent periods after synchronization (2-26 hours with 4-hour intervals) SPCs were fixed with 4% paraformaldehyde. BMAL1 was quantified using a standard immunofluorescence protocol. In short, SPCs grown on coverslips were permeabilized using 0.5% Triton-X100, treated with 50mM Glycine, and incubated with an anti-BMAL1 antibody (1:50, #93806, Abcam) overnight. After incubations with a secondary antibody and 4',6-diamidino-2-phenylindole (DAPI) for nuclear labeling, cells were mounted in Vectashield. Pictures were taken with a Nikon Eclipse 80i light microscope at 4 standardized, equally distributed locations on each coverslip. BMAL1 fluorescence intensity was measured in ImageJ software (Version 1.48u4) by a blinded researcher. Nuclear/ cytoplasmic ratio was calculated and all locations on a coverslip were averaged. Subsequent time-periods were compared.

BrdU quantification

To quantify BrdU incorporation, coverslips were incubated with 2N HCl for 30 minutes to allow for DNA denaturation. Samples were blocked with 5% goat serum (Dako) in 0.1% Triton X-100 (VWR chemicals) and incubated with a primary anti-BrdU antibody (1:200, #347580, Pharmigen) overnight. After incubation with secondary antibody (1:100, #715-095-150, Jackson), cells were co-stained with DAPI (1:40.000, Invitrogen) and mounted with Vectashield (Vector laboratories). Pictures of all coverslips were taken with a Nikon Eclipse 80i light microscope at 5 standardized locations equally

distributed within the coverslip. A blinded researcher manually counted the amount of DAPI and BrdU positive cells using ImageJ (Version 1.48u4). Percentages were calculated, averaged per coverslip, and reduced by the moving average to correct for any non-circadian variations. Non-synchronized samples served as controls.

Doubling time

SPCs were cultured in 0.1% gelatin coated 35mm plastic culture dishes for at least 2 days. At the start of the experiment (t=0), half of the culture dishes were synchronized. After subsequent time-periods (0-48 hours with 4-hour intervals) cells were loaded in Bürker's chamber and photos taken. Cell numbers were quantified in ImageJ

Scratch assay sub-analyses

In a sub-analysis, a blinded researcher analyzed the effects of cell density and cell orientation on migration. Cell density was derived from the photos taken directly after the scratch. Using ImageJ, % area covered with SPCs was measured semi-automatically. Migration of the 25% lowest and highest density samples was compared. For cell orientation, photos were manually subdivided in 3 groups: parallel, perpendicular, or a mixed orientation as compared to the scratch. Migration of samples with parallel and perpendicular orientation was compared.

Chapter 5

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

Chapter 5 - 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, disease and therapy, but a convenient *in vitro* model that mimics 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 (24-hour variation in the expression of BMAL1 and PER2, 2 core clock genes / proteins, $P<0.05$ for all) and that shows functional dependence on the clock as indicated by an oscillating response in apoptosis induced by doxorubicin, tBHP or hypoxia ($P<0.05$ for all). In addition, perturbation of the cardiac clock by the use of several compounds including Resveratrol and Ex-527 was found to result in loss of functional rhythmicity ($P<0.05$ for both). This indicates that neonatal rat cardiomyocytes are a good model to investigate the cardiac circadian clock as well as a system that allows for fast and easy preclinical testing of the influence of compounds on circadian rhythmicity.

Introduction

Circadian rhythms allow the body to anticipate diurnal environmental changes.[295] In humans, these rhythms are regulated by multiple clocks: one central master clock located in the suprachiasmatic nucleus of the brain, and distinct peripheral clocks that are present in almost every organ and cell. The clock pathway relies on the oscillatory expression of core clock genes such as *CLOCK, ARNTL, PER and CRY,*[315] which results in the rhythmic expression of clock-controlled output genes (CCGs). According to tissue physiology, CCGs vary per organ and have important functional implications. In the cardiovascular system, circadian rhythms influence various physiological features such as metabolism, electrophysiological characteristics, (cardiac) hormone receptor functionality and coagulation.[29, 88, 316, 317] 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,[30, 318, 319] and disruption of rhythmicity by genetic defects, genetic manipulation or sleep disturbance, is involved in cardiac pathophysiology.[31, 294, 296]

Discovery of the importance of the circadian clock in organ function resulted in several studies that investigated circadian rhythmicity in the heart.[294, 320, 321] In addition, the interest in the use of circadian rhythms in (pharmacological) therapy is rising.[167, 322, 323] Preclinically, several animal models have been applied to uncover the contribution of circadian rhythmicity to cardiac physiology, disease and (pharmacological) therapy.[92, 106, 294] 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 rodent cardiomyocytes to mimic circadian rhythmicity in the heart.[324] Indeed, *in vitro* cultured rodent ventricular cardiomyocytes retain their circadian rhythms, even in the absence of any environmental input.[324] While these studies have provided important insights, adult cardiomyocytes are difficult to obtain: it takes time to breed animals to adulthood, isolation and culture are difficult and time-consuming, and since adult cardiomyocytes do not proliferate, the number of cells derived per heart is relatively small. In addition, adult cardiomyocytes cannot be kept in culture long enough to allow the analysis of several circadian cycles. This limits their suitability for interventional studies, for example to test whether the effect of newly developed drugs is time-dependent and whether they interfere with the intrinsic cardiomyocyte circadian clock.

In the current study, we propose neonatal rat cardiomyocytes as an easy *in vitro* system to study molecular and functional circadian rhythmicity in the heart and prove that it can serve as a model to test clock interfering characteristics of multiple compounds.

Materials and Methods

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 1 g/L, $Na_2HPO_4.2H_2O$ 60 mg/L, KH_2PO_4 60 mg/L, phenol red 20 mg/L and HEPES 4,77 mg/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 $1mm^3$ pieces. Tissue pieces were transferred to 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 (10 mg/L in Solution A, #11243217001, Roche) culture dishes (35 mm, #353001, Falcon). After 20 hours, medium was replaced to remove dead cells.

Bioluminescence reporter recordings

Neonatal rat cardiomyocytes were transduced with Bmal1- and Per2- destabilized luciferase (dLuc) lentiviruses. Lentiviral plasmids, harbouring luciferase reporters of the murine Per2- and Bmal1-promoters, were previously described and kindly provided by Prof. Dr. Liu.[300-302] 1.5 days after transduction, cells were synchronized with 100 nM Dexamethasone for 2 hours[298] and switched to recording medium (Phenol Red-free DMEM, 10%FCS, 10 mM HEPES, 0.035% Bicarbonate, 4.5 g/L glucose, Pen/Strep and 100 µM D-Luciferin (Promega). Culture dishes were sealed with high vacuum grease (Dow Corning) and analysed 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.

Synchronization of nrCMs

nrCMs were synchronized by a 2 hour 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.[298, 299, 325] Non-synchronized cardiomyocytes, that had only a medium change more than 1 day before the start of the experiments, served as controls.

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.

Western Blotting

For protein analysis, nrCMs were washed with PBS and lysed using RIPA-buffer as described previously.[314] 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).

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 (tBHP) 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.

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, objective 10x).

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.

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.[326] Student's T-test was used to compare non-circadian differences between groups. P-values < 0.05 were considered statistically significant.

Results

Neonatal rat cardiomyocytes show a functional molecular clock

In contrast to adult cardiomyocytes, neonatal rat cardiomyocytes (nrCMs) are easy to derive in large quantities and can be cultured for prolonged periods. We therefore investigated the use of these cells to study circadian rhythmicity. To analyze the presence of a circadian clock in nrCMs, we synchronized nrCMs via serum shock.[298] Subsequently, mRNA was sampled every 6 hours across 42 hours, starting 9 hours after synchronization. 24 hour period significance was assessed by the nonparametric algorithm RAIN.[326] *BMAL1* expression, as measured by qRT-PCR, significantly oscillated in a diurnal manner (RAIN, P<0.05; Figure 5.1A). To further validate the presence of a functional clock we investigated anti-phasic oscillatory expression of *Bmal1* and *Per2*, a hallmark of a functional circadian clock, using 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. Opposing bioluminescent oscillations for *Bmal1* and *Per2* were detected over the course of 4.5 days (Figure 5.1B). In addition to mRNA oscillations, western blot analysis for BMAL1 revealed rhythmic protein levels in nrCMs (RAIN, P<0.0005; Figure 5.1C) with peaks that followed gene expression with a delay of approximately 6-12 hours (Figure 5.1A). From these data, we conclude that nrCMs contain a functional molecular circadian clock at the transcriptional as well as at the protein level.

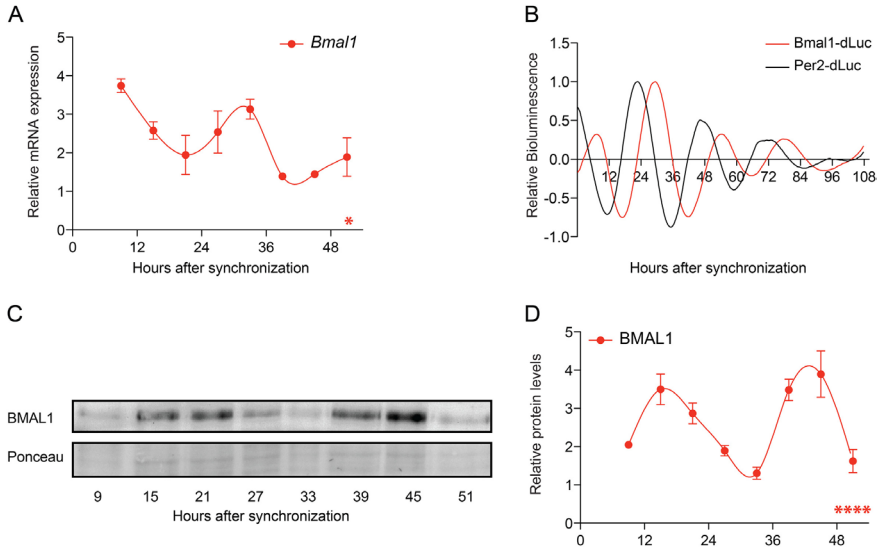

Figure 5.1. Neonatal rat cardiomyocytes possess a functional circadian clock. (A) *Bmal1* mRNA expression levels in synchronized neonatal rat cardiomyocytes (nrCMs) as determined by qRT-PCR. Lowest value was set to 1. Expression levels were normalized to the non-oscillatory housekeeping gene *PPIA*. Significance of rhythmicity across 48 hours was analysed 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 hours was analysed using the RAIN algorithm and is indicated (**** P<0.0005).

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

The circadian clock drives rhythmic output of clock-controlled genes, which allows for functional tissue-specific oscillations. In the heart, central and peripheral circadian clocks together, control heart rate.[327] To test intrinsic functional rhythmicity in nrCMs, we therefore measured beating frequency in a temporal manner. After serum shock-based synchronization, circadian rhythmicity of spontaneous beating frequency was found over the course of two days (RAIN, P<0.05; Figure 5.2A). Unsynchronized cell cultures did not show rhythmicity (RAIN, P=0.99; Figure 5.2A). These results show that nrCMs do not only possess a molecular clock, but display functional rhythmicity as well.

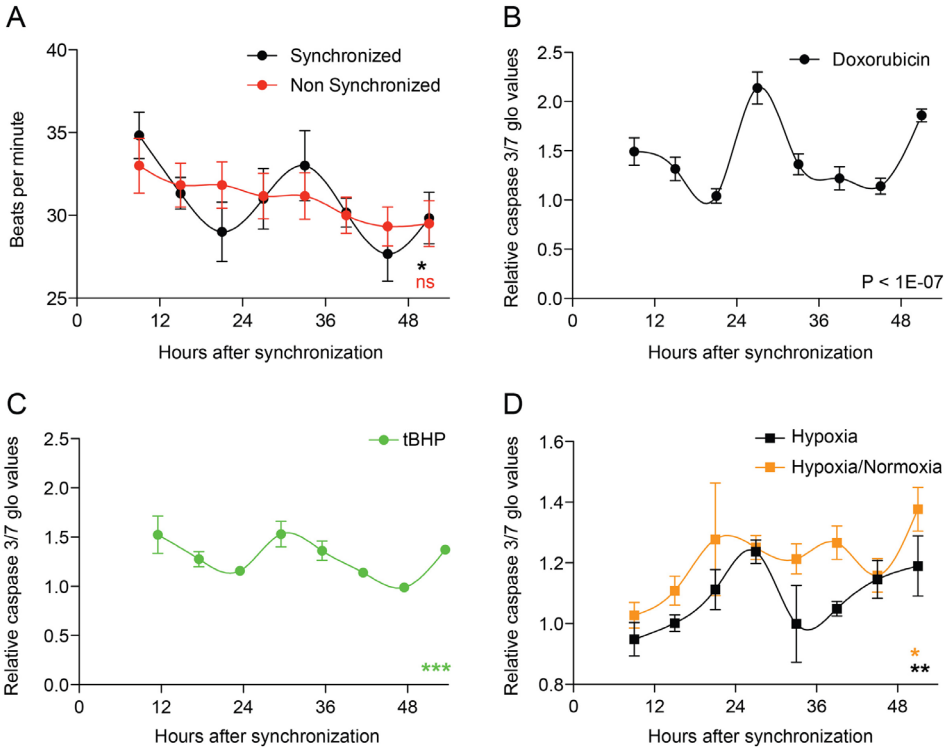

Figure 5.2. Neonatal rat cardiomyocytes show functional oscillations. (A) Counts of spontaneous contraction in nrCMs, measured as beats per minute and monitored across 48 hours (n=6). (B) Relative apoptotic levels in doxorubicin treated synchronized nrCMs measured by Caspase-Glo 3/7 values across 48 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 hours was analysed using the RAIN algorithm and is indicated (ns: not significant, * P<0.05, ** P<0.005, *** P<0.0005).

During myocardial infarction in humans as well as in mice, the amount of damage correlates to the time of the day ischemia (or a different stressor) occurs.[32, 33, 328] We tested whether the stress-response of nrCMs is also rhythmic by exposing synchronized nrCMs to doxorubicin, a known cardiotoxic anti-cancer drug, in a time-dependent manner. Serum shock based synchronization itself did not induce apoptosis as measured by a Caspase-Glo 3/7 assay (two-tailed Student T-Test, P=0.95; Figure 5.3A). When synchronized nrCMs were exposed to doxorubicin however, significant circadian rhythms were found in their damage response (normalized to background values, see Methods, Figure 5.3b) (RAIN, P<1E-07; Figure 5.2B), which were absent in non-synchronized cultures (RAIN, P=0.58; Figure 5.3C). Additional TUNEL staining on doxorubicin treated nrCMs showed a similar oscillating output (RAIN P<0.005 and P<0.0005 for non-treated and doxorubicin-treated samples; Figure 5.3D), confirming the time-dependent vulnerability of nrCMs to doxorubicin.

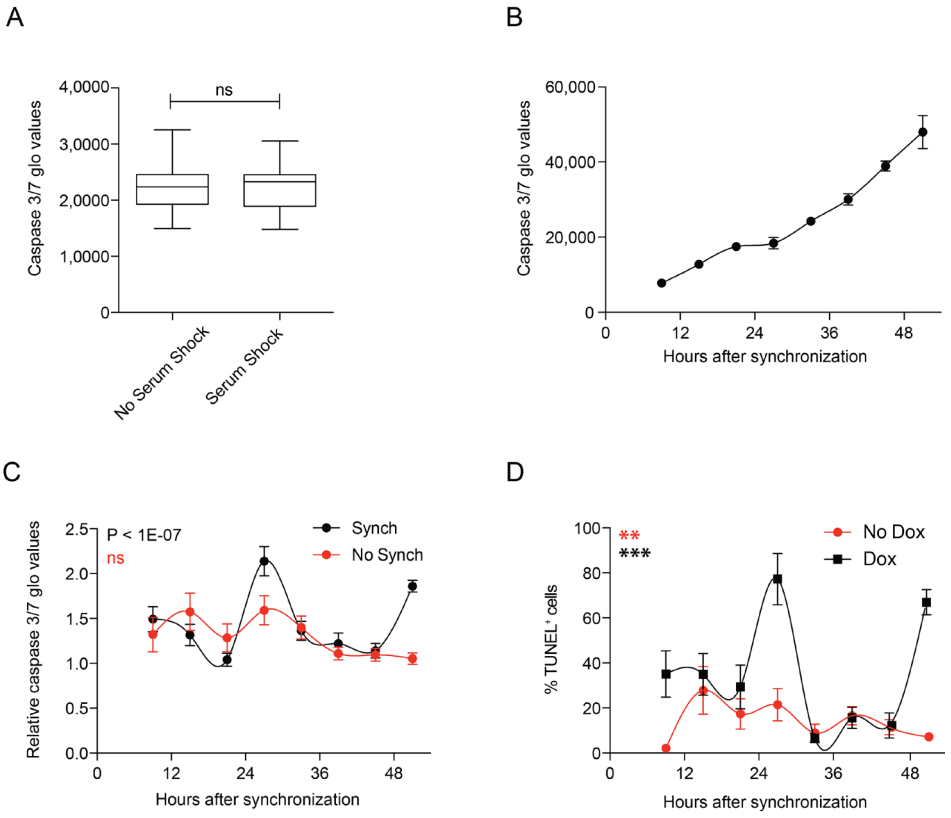

Figure 5.3. Apoptosis in neonatal rat cardiomyocytes. (A) Apoptotic levels in nrCMs > 1 day after medium change (no serum shock) or after a synchronizing serum shock as measured by a Caspase-Glo 3/7 assay. Two conditions were compared via unpaired Student's T-Test (ns = non significant). (B) Basal apoptosis 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 hours before measurement, No Synch) doxorubicin treated nrCMs. The synch (black) curve is the same as the one depicted in Figure 5.2B (D) TUNEL assay on synchronized nrCMs treated with (Dox) or without doxorubicin (No Dox). Significance of rhythmicity was analyzed via RAIN and indicated (** P<0.005, *** P<0.0005).

We hypothesized that this time-dependent vulnerability of nrCMs is not limited to doxorubicin and therefore analyzed whether the use of a different stressor, the pro-oxidant tert-butyl hydroperoxide (tBHP) would lead to equal results. Indeed, a circadian apoptotic response pattern was observed for tBHP (RAIN, P< 0.0005; Figure 5.2C). *In vivo*, the most common cardiac stressors are ischemia and ischemia followed by reperfusion, which are known to follow a diurnal pattern.[32] To mimic ischemia *in vitro*, we cultured nrCMs in 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 as well as hypoxia/normoxia followed a significant 24-hour pattern (RAIN P<0.005 and P<0.05, respectively; Figure 5.2D). These results show that, similar to the *in vivo* situation, nrCMs respond to ischemia in a circadian manner.

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

Many pharmacological drugs and compounds affect the circadian clock. Because of the important role of circadian rhythmicity in cardiovascular physiology and disease, an *in vitro* tool that is able to predict the effect of compounds on the molecular and functional clock could be useful for drug development and testing. To determine whether nrCMs can be used for this purpose, we treated nrCMs with resveratrol, a compound of interest for its potentially beneficial effects on atherosclerosis, hypertension and ischemia/reperfusion,[329] which has also been linked to the circadian clock.[330, 331] Using our Per2-dLuc bioluminescence reporter system, we observed a dose-dependent decrease of Per2 amplitude (Figure 5.4A), which indicates dampening of the molecular clock upon administration of resveratrol. Congruently, we observed a dose-dependent dampening of circadian oscillations upon administration of Ex-527 (Sirtinol) (Figure 5.4B), a compound previously linked to both cardiomyocyte stress,[332] and the core clock system via the sirtuin SIRT1.309 Low concentrations of each compound (resveratrol or 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 5.4C). Additionally, both compounds lengthened the circadian period 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[333] and clock disturbance has been observed in numerous pathologies (hypertension, diabetes, sleep disturbance and cancer).[31, 295] Gaining insight in 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 pharmacological therapeutics affect clock dynamics.

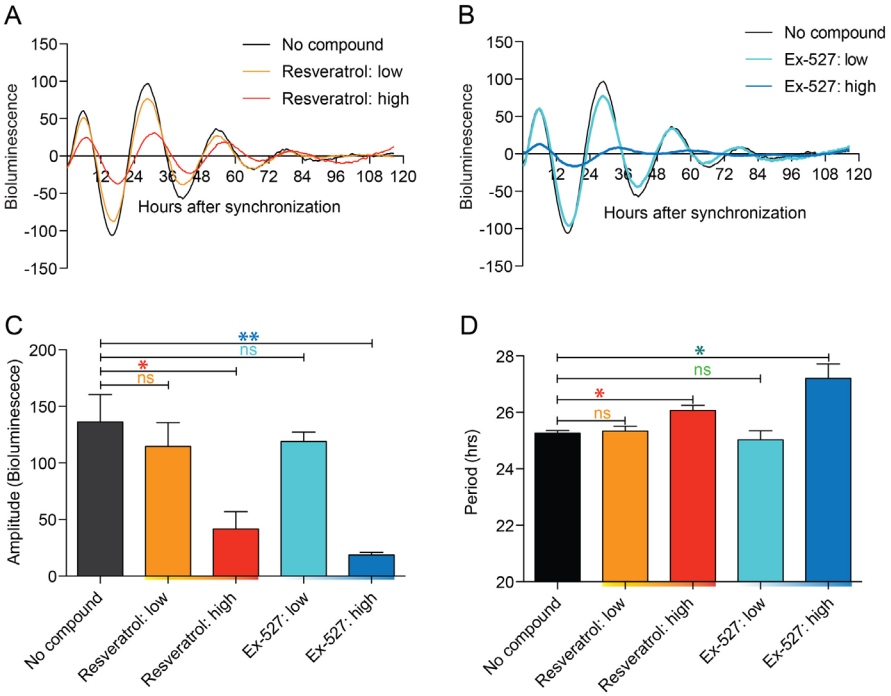

Figure 5.4. Resveratrol and Ex-527 affect the molecular clock of neonatal rat cardiomyocytes. (A) Per2-Luc bioluminescence in synchronized neonatal rat cardiomyocytes (nrCMs) treated with low or high doses of resveratrol. Bioluminescence was measured by a LumiCyle32 across 116 hours. Means of 3 independent 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 analysed using an unpaired Student's T-Test and significance levels are indicated (ns: not significant, * P<0.05, ** P<0.005).

Neonatal rat cardiomyocytes lose functional rhythmicity upon clock disturbance

As both resveratrol and Ex-527 were found to dampen the molecular clock in nrCMs (Figure 5.4A-D), we questioned whether this would also affect functional circadian output. Resveratrol showed a cardioprotective effect on nrCMs under basic culture conditions (Student's T-Test P<0.05; Figure 5.5A). Nevertheless, the strong pro-apoptotic effect of doxorubicin (Student's T-Test P<0.05) could not be reversed by resveratrol (Figure 5.5A). In contrast, when our second compound Ex-527 was added, basal apoptosis levels increased significantly (Student's T-Test P<0.005; Figure 5.5B). Administration of Ex-527 in combination with doxorubicin had an additive apoptotic effect when compared to doxorubicin alone (Student's T-Test, P< 0.0005; Figure 5.5B).

Analysis of the effect of both compounds on the oscillating response to doxorubicin (Figure 5.2C) revealed that both resveratrol and Ex-527 abolished the circadian variation of the apoptotic response (RAIN, resveratrol, P=0.29, Ex-527, P<0.37; Figure 5.5C,D). This shows that disturbing the molecular clock of nrCMs through the use of compounds can lead to impaired circadian functionality.

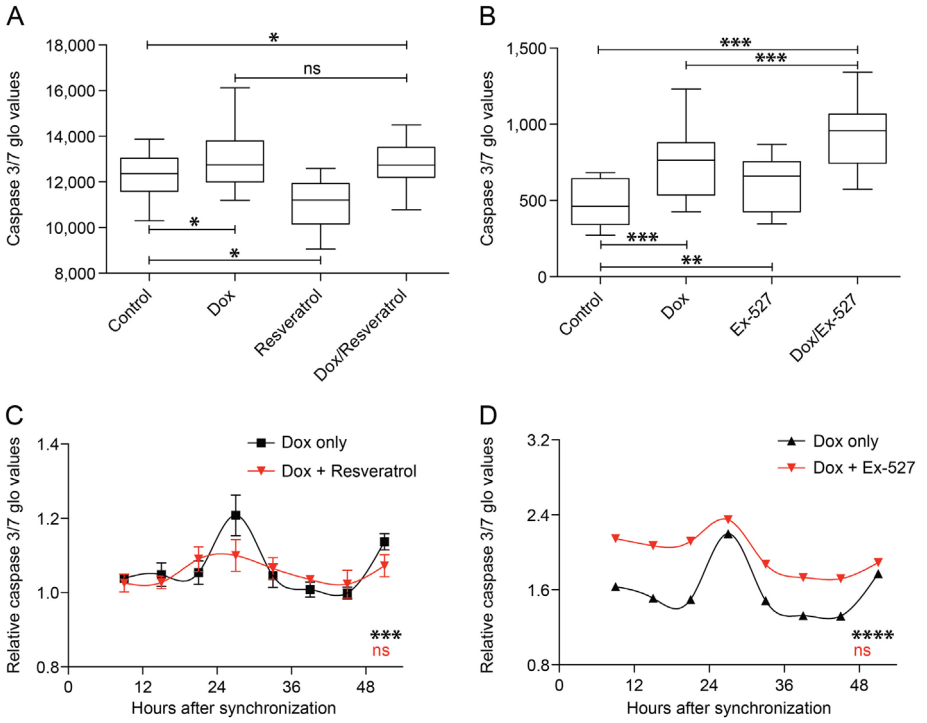

Figure 5.5. Resveratrol and Ex-527 impair time-dependent apoptotic response to doxorubicin in neonatal rat cardiomyocytes. (A) Apoptotic levels in neonatal rat cardiomyocytes (nrCMs) treated with doxorubicin (dox), resveratrol or a combination compared to non-treated cells (control). (B) Similar analysis 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).

Discussion

Circadian rhythmicity plays an important role in physiological, biochemical, and behavioral processes and is increasingly considered in pharmacological studies.[323] Typically pharmacological studies are done in animal models, where time-of-day dependent drug efficacy or toxicity has to be analyzed at various time-points, requiring an undesired, but necessary large number of animals. In addition, there has been a shift in attention from central, neurohumoral control of 24-hour rhythms to peripheral, cellular regulation.[294] In animal experiments, both effects are measured at the same time, possibly blurring circadian effects. Alternative, more specific assays at earlier preclinical test stages are needed. In cardiovascular disease this is challenging because cardiomyocytes are difficult to obtain or to maintain in culture for a sufficient long time without deterioration of intrinsic characteristics.

In the current study, we present neonatal rat cardiomyocytes (nrCMs) as a convenient system to model the circadian clock of the heart. We show that nrCMs have a molecular and functional clock with 24-hour rhythmicity in spontaneous contraction as well as resistance to induced apoptosis by several stressors. Importantly, we demonstrate that different pharmacological compounds influence and disrupt the cardiac clock both at the molecular and functional level.

Our findings correspond to *in* and *ex vivo* studies that investigated circadian rhythmicity the heart. Clock components (BMAL1 and PER2), stress tolerance, and contraction rate are regulated by the cardiomyocyte circadian clock and all follow a 24-hour pattern *in vivo*.[32, 327] More specifically, in a rodent model Durgan et al. demonstrated that cardiomyocyte stress tolerance is lowest when BMAL1 mRNA expression levels start to rise.[32] Our *in vitro* assay shows similar results: maximum apoptosis (doxorubicin exposure 20-26 hours after synchronization) coincided with low, increasing BMAL1 mRNA levels. Second, *in vitro* circadian spontaneous contraction rhythmicity is comparable to physiological rhythmicity: heart rate in mice (both *in* and *ex vivo*) peaks in the middle of the active period, when BMAL1 mRNA expression is high.[92] In correspondence, our NRC experiments show maximal spontaneous beating frequencies when BMAL1 mRNA is high.

Prior to using expensive and long-lasting animal or clinical studies, nrCMs would thus be a good and easy-to-use first stage model to study the cardiomyocyte molecular clock *in vitro*, as well as the effect of compounds on circadian rhythmicity in the heart.

Chapter 6

Variation within variation: comparison of 24-hour rhythm in rodent infarct size between ischemia reperfusion and permanent ligation

Chapter 6 - Variation within variation: Comparison of 24-hour rhythm in rodent infarct size between ischemia reperfusion and permanent ligation

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Abstract

Background: Outcome of myocardial infarction in humans and rodents has a 24-hour rhythm. In some human cohorts however, rhythmicity was absent while time of maximum damage differs between cohorts. We hypothesized that the type of damage influences the 24-hour rhythm in infarct size.

Methods: Ischemia was induced in 12-week-old C57BL/6 mice at 4 different time-points during the day using permanent ligation (PL), 30 minutes of ischemia followed by reperfusion (IR), or no ligation (controls). Infarct size was measured by echocardiography and histology after a 1-month follow-up.

Results: Rhythmicity in infarct size was present in PL at the functional and histological level, with maximal damage when the infarct was induced at noon. In IR, no circadian rhythm was found.

Conclusions: Time of coronary artery ligation determines outcome of myocardial infarction. Our data showed that in rodents, presence of circadian rhythmicity and time of peak infarct size varies between experimental setups.

Introduction

Twenty-four-hour (diurnal, day/night) rhythms are biorhythms that play an important role in cardiac physiology.[88, 295] Cardiovascular parameters, such as blood pressure, heart rate, circulating hormones, and coagulation time, all display 24-hour oscillations.[316] These rhythms are regulated by 2 mechanisms: 1) central regulation via the brain, mainly via neurohumoral signaling, and 2) peripheral regulation via molecular circadian clocks present within individual cells.[334]

Twenty-four-hour rhythms are not only important in physiology, but also play an important role in cardiovascular disease. The incidences of sudden cardiac death and myocardial infarction (MI) for example, have a diurnal rhythm, and disruption of a normal day/night rhythm (e.g. via shift work), leads to an increase in cardiovascular disease.[296, 335] Recently, it was shown that in addition to their role in cardiovascular disease incidence and pathophysiology 24-hour rhythms are crucial in cardiovascular disease outcome as well.[32, 336, 337] In 3 rodent studies, myocardial infarction induced at 4 different time-points showed a 24-hour rhythm in infarct size.[32, 336, 337]

These animal studies were followed by several clinical studies investigating patients suffering from ST-elevated myocardial infarction. Six out of eight studies demonstrated that 24-hour oscillations are present in infarct outcome.[33, 328, 338-341] Two relatively large study however, failed to show rhythmicity in echocardiographic parameters and/or cardiac enzymes.[342] In addition, time of maximum infarct size differed between studies. Symptom onset between midnight and 6AM was associated with the worst outcome in the majority of studies,[33, 328, 338, 341] but two studies had a different peak time (6AM until noon) [339] and two damage peaks, one around 9AM and one around 8PM[340] respectively).

Recently, there has been a debate about what causes these differences between studies. Several factors, such as differences in ethnic background, medication use, statistical methodology, study size, culprit artery, time of ischemia, comorbidities (diabetes), climate and day-night cycle variations, and outcome measure have been proposed to explain variation between those studies.[328, 343-346]

One of the most commonly suggested explanations is the difference in reperfusion damage.[344] In the studies of Ammirati et al. and Mukamal et al. (in which no circadian effects in echocardiographic/cardiac enzyme parameters were observed), most patients did not receive primary percutaneous coronary intervention (PCI).[328, 347] The contribution of reperfusion damage to infarct size in these studies is therefore smaller than in the other cohorts. In addition, data of Durgan et al. and Virag et al. suggest that mitochondrial function mediates the 24-hour rhythm in cardioprotection.[32, 348] Reperfusion after PCI causes an increase in oxidative stress within the heart, which is specifically harmful for cardiac mitochondria.[349]

Based on these findings, we hypothesized that 24-hour differences in reperfusion damage are responsible for 24-hour rhythmicity in infarct size. To test this hypothesis, we induced myocardial infarction in mice at different time points using either 1) ischemia/reperfusion (IR) or 2) permanent ligation (PL).

Materials and Methods

Animals

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

Male, wild-type C57BL/6 mice (Charles River) were housed in a 14-hour light/10-hour dark cycle (Zeitgeber Time 0 (ZT0) = lights on, 14 hours later (ZT14) = lights off). Food and water were provided ad-libitum. At 10 weeks of age, mice were distributed to 3 rooms with different light regimes (light on at 5am, 11am, or 2pm, all in a 14-hour light/10-hour dark cycle) to enable myocardial infarction operations during working hours.

Myocardial infarction

Mice were anesthetized (medetomidine 0.5mg/kg, midazolam 5mg/kg, and fentanyl 0.05mg/kg) and myocardial infarction was induced using an open-chest model as described previously[350]. 3 different types of myocardial infarction were used:
1) PL: permanent left coronary artery ligation; 2) IR: 30 minutes left coronary artery ligation in which a small tube was placed within the stitch, followed by reperfusion (removal of stitch and tube); 3) controls: incision of a needle and a suture thread, but no ligation. Mice that died or were terminated because a humane endpoint was reached during the operation or follow-up were replaced.

Mice were operated at 4 different time-points (ZT1, ZT7, ZT13, and ZT19). To prevent disturbance of the sleep/wake cycle by the (necessary) light during the operations, mice were anesthetized before transport to the operation theatre. For the mice operated at ZT 19 (darkness), cages were completely covered during transport to the operation theatre. Subsequently, mice were intubated and positioned with minimal light and after the head of the mouse was completely covered, operation lights were turned on.

Echocardiography

Echocardiography was performed under 2% Isoflurane anesthesia 5 days before, 5 days after, and 28 days after the operations using a 13-24MHz transducer (Vevo 2100 and MS250, Visual Sonics). All measurements were done at ZT 2. During the procedure, heart rate, temperature, and respiration were continuously monitored. Two independent and blinded researchers performed the echo analyses using the

manufacturer's software. Ejection fraction (EF), end diastolic volume (EDV), and end systolic volume (ESV) values were derived from 3 averaged mid-myocardial long axis recordings in B-mode. Fractional shortening (FS), end diastolic diameter (EDD), and end systolic diameter (ESD) were measured in M-mode. 3 short axis recordings from cardiac base to apex were averaged. An overview of the procedures is shown in Figure 6.1.

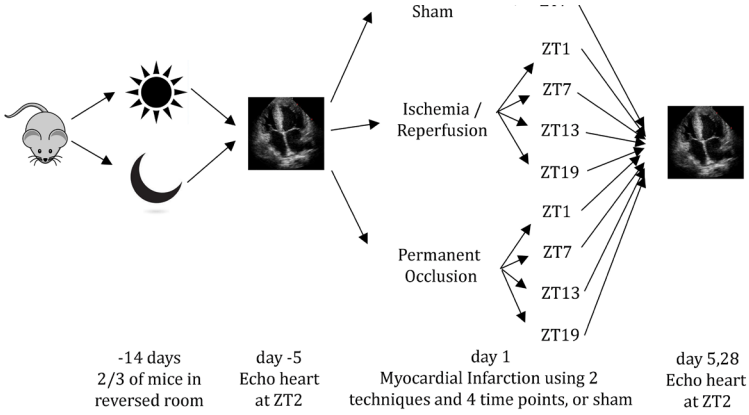

Figure 6.1. Overview of the procedure. Two thirds of all mice were placed in rooms with altered light/dark regimes. Five days before the procedure, baseline echocardiography was performed. On day 1, myocardial infarction (or sham) was induced at 4 different time-points using 2 different techniques. At 5 and 28 days, outcome was measured using echocardiography. ZT: Zeitgeber Time (ZT0 = lights on).

Histology

After the last echocardiography, mice were sacrificed by cervical dislocation. Hearts were collected, washed in cold PBS, weighed (both ventricles), and stored in 4% paraformaldehyde until embedding in paraffin and slicing in 5µm long axis sections with 400µm intervals (total 8-14 sections). To assess the amount of fibrosis, sections were stained with Picrosirius-red, washed with 0.2N HCL, mounted in entellan and digitized by scanning. For each heart, a researcher blinded to the origin of the sections, subsequently quantified the amount of fibrosis by dividing the area of collagen by the total left ventricular surface area.

Statistical analysis

Data are presented as averages ± standard error of mean. Cosinor analysis was used to analyze presence (or absence) of 24-hour rhythmicity in the IR and PL group. Levene's test was used to check equality of variances. When homoscedastic, one- or two-way analysis of variance (ANOVA) was conducted to compare groups. If not, the Kruskal-Wallis test was performed. In analyses with multiple groups, Bonferroni post-hoc analysis was used to compare subjects with controls. Pearson's chi-squared test was used to compare categorical data. P-values <0.05 were considered statistically significant.

Results

Operations

Sixty-five mice were operated. During follow-up, 13 mice (20%; all PL/IR) died or were euthanized because a humane endpoint was reached. 12/13 dead animals, had undergone PL and the majority (11/13) died within the first week after the infarct. Mortality was highest in the animals operated at ZT 7 (middle of inactive/resting period), although differences were not statistically significant (Figure 6.2; P=0,77).

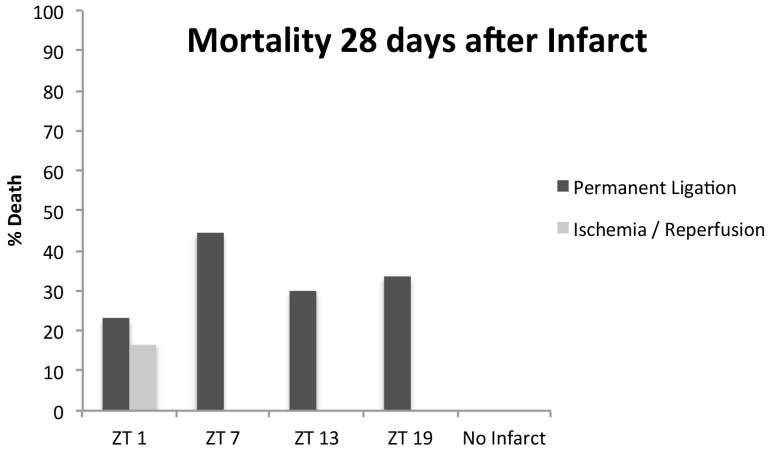

Figure 6.2. Mortality in the 28 days after the infarct. Thirteen mice died, of which 0 underwent sham, 1 IR, and 12 PL. In the PL group, mortality was highest in the ZT 7 group, although differences were not statistically significant. ZT: Zeitgeber Time (ZT0 = lights on).

Body and cardiac weight

Of the 52 mice that completed follow-up, 20 were in the PL group, 18 in the I/R group, and 14 were controls. Body weight decreased slightly in the 5 days after the infarct (0.6±0.1g), but increased in the overall 1-month follow-up (27.5±0.1 to 28.3±0.1g). No significant differences in body weight were found between types of ischemia and time-points.

Biventricular weight of mice that had undergone PL was significantly increased compared to sham, whereas no significant differences were found between IR and controls (biventricular weight/body weight ratio 6.00±0.27, 5.34±0.53, and 4.96±0.09mg/g for PL, IR, and sham respectively; P=0.003 and 0.086 for PL and IR vs. sham respectively). In the PL mice, there was a significant difference in biventricular weight (P=0.048, n=20) depending on the time of coronary artery ligation, which was absent in the IR group (Figure 6.3; P=0.543, n=18).

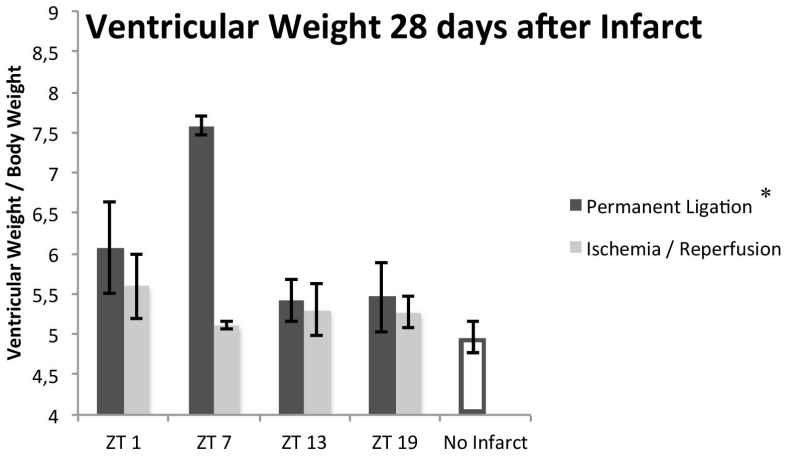

Figure 6.3. Ventricular weight 28 days after the infarct. Compared to sham controls, PL mice had an increase in biventricular mass that showed a significant 24-hour rhythm peaking at ZT 7 (middle of inactive/resting period). In IR mice, no significant increase or 24-hour rhythm was present. n=20, 18, and 14 for PL, IR, and sham respectively, n=3-5 per time point. Striped line indicates control value; * Indicates P<0.05 for presence of 24-hour rhythm. ZT: Zeitgeber Time (ZT0 = lights on).

Echocardiography

Cardiac function decreased in the PL and IR groups and was significantly worse compared to the sham group 28 days after the infarct (Table 6.1 and Figure 6.4). Cosinor analysis revealed that functional impairment varied significantly with a 24-hour period based on the timing of coronary artery ligation in the mice receiving PL, with a peak damage occurring after coronary artery ligation at ZT7 (middle of inactive/resting period; P=0.041, P=0.049, and 0.021 for EF, FS, and EDV respectively, n=20). In IR mice however, no circadian dependence was observed (P=0.598, 0.127, and 0.368 for EF, FS, and EDV respectively, n=18).

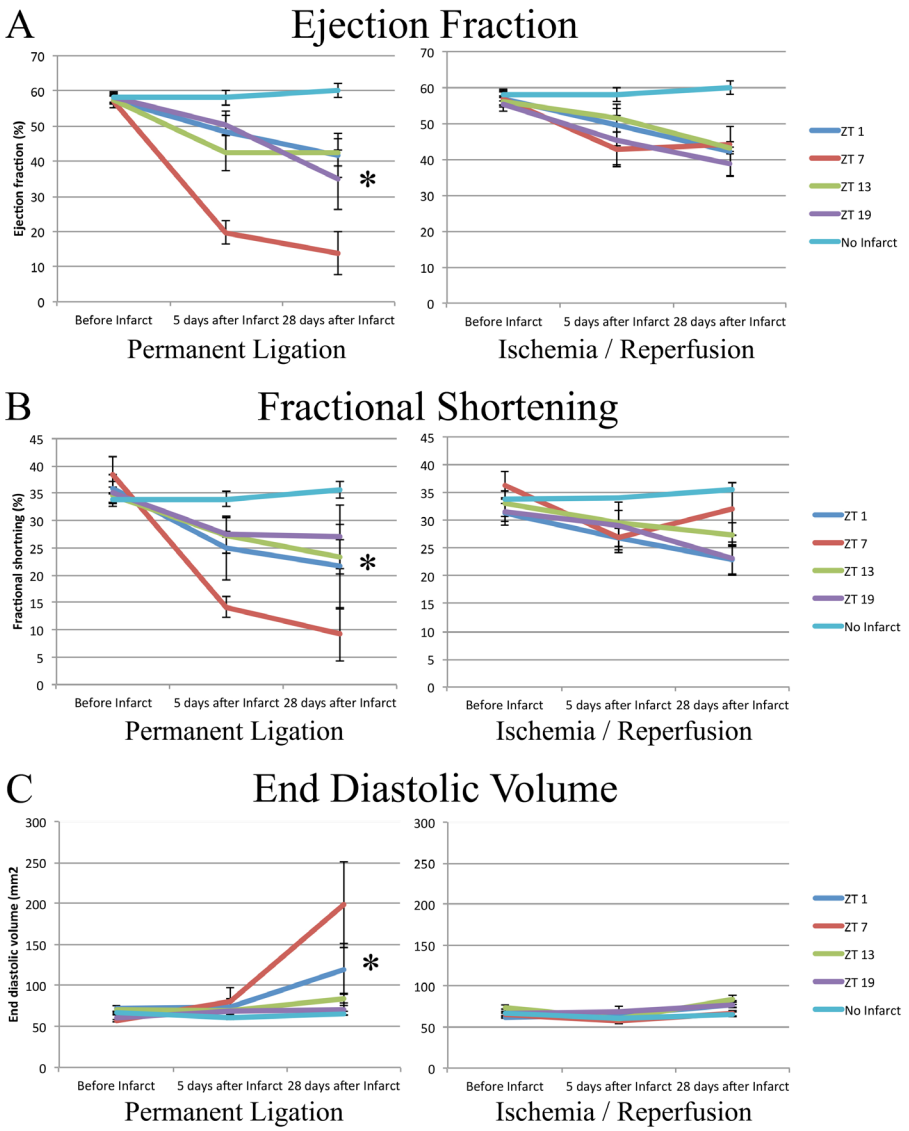

Figure 6.4. Echocardiographic parameters 5 days before, 5 days after, and 28 days after myocardial infarct. n=20, 18, and 14 for PL, IR, and sham respectively, n=3-5 per time point. * Indicates P<0.05 for presence of 24-hour rhythm. ZT: Zeitgeber Time (ZT0 = lights on);

Function	PL	IR	Sham	PL vs Sham	IR vs sham
EF (%)	31.8±4.0	42.3±1.6	60.4±1.0	P<0.001	P=0.012
FS (%)	19.9±2.6	26.5±1.6	35.7±1.4	P<0.001	P=0.014
EDV (ml)	118.1±18.1	77.3±2.9	64.1±2.8	0.011	1.000

Table 6.1. Cardiac function 28 days after operations. PL: Permanent ligation; IR: Ischemia/reperfusion; EF: Left ventricular ejection fraction; FS: fractional shortening; EDV: end diastolic volume

Histology

Fibrosis quantification confirmed the echocardiographic findings and a showed a significant 24-hour rhythm in the amount of fibrosis based on the time of coronary artery ligation, with peak fibrosis at ZT 7 in the PL group (middle of inactive/resting period; P=0.024, Figure 6.5A,B, n=20). In IR mice, no rhythm was present (P=0.110, n=18; Figure 6.5A,C).

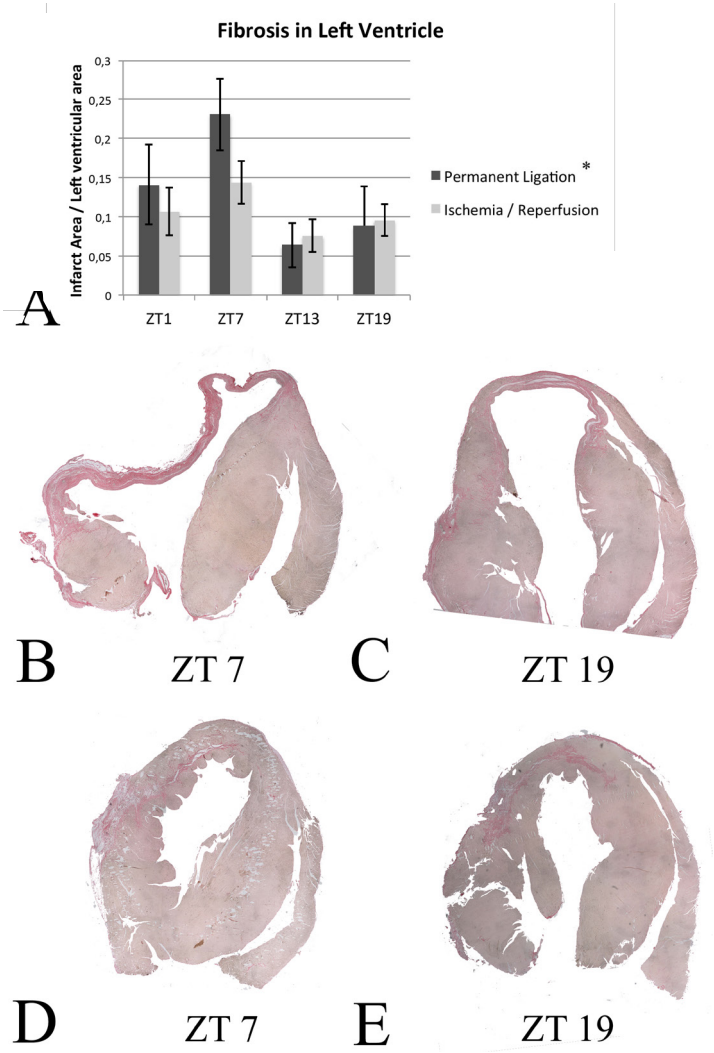

Figure 6.5. Picosirius-red staining of myocardial infarcts. The pink tissue represents fibrosis. A) Fibrosis quantification; a 24-hour rhythm in amount of fibrosis, based on the time of coronary artery ligation was present in PL but not IR. n=20, 18, and 14 for PL, IR, and sham respectively, n=3-5 per time point B,C) Representative examples of permanent ligation. Infarcts induced at ZT 7 (B, middle of inactive/resting period) were larger compared to infarcts induced at ZT19 (C, middle of the active period). D,E) Representative examples of Ischemia/reperfusion. No differences were found between time points. * Indicate P<0.05 for 24-hour rhythm. ZT: Zeitgeber Time (ZT0 =lights on).

Discussion

In the current study, we analyzed 24-hour rhythms in outcome of myocardial infarction. For the first time, we directly compared circadian influences in two infarction models that differed with respect to the absence or presence of reperfusion.

Thereby, we eliminated all other variables, such as mouse strain, gender, age, anesthesia, or housing conditions, that may have influenced previously published controversial results.

Our histological and functional data show that a diurnal rhythm in infarct size, as measured by the amount of fibrosis, is present in our mouse model of permanent ligation, but not in ischemia/reperfusion. These findings contradict our original hypothesis that rhythmicity is caused by reperfusion damage and suggest that differences in rhythmicity (presence of rhythmicity and time of peak damage) found in clinical studies are not solely based on this type of damage.

Our results confirm the findings of Durgan et al., Eckle et al. and Schloss et al. that a 24-hour rhythm is present in outcome of myocardial infarction.[32, 336, 337] The timing of coronary artery ligation that was associated with peak damage, however, was different between studies (Durgan et al. ZT 6-12, Schloss et al ZT 13, Eckle et al. ZT 0-6, our data ZT 7). Secondly, Durgan et al. and Eckle et al. found rhythmicity in infarct outcome based on the time of occlusion in IR, whereas we only found a rhythm in PL. There are multiple differences between the studies that might potentially explain these observations. Durgan et al. used a closed chest infarct model (as opposed to the open chest model of Eckle et al.[351,] and our study[350]). In addition, compared to our IR-model, duration of ischemia prior to reperfusion was 50% and 100% longer in the studies of Durgan et al. and Eckle et al. respectively, thereby inducing more permanent damage comparable to our permanent ligation model. Furthermore, there were significant differences in mouse strain, echocardiography device (with different baseline echo data), outcome measures, and light/dark schedule. Direct comparison between studies is therefore not possible. Indeed, similar to the clinical studies, these differences illustrate that variability is present in 24-hour rhythmicity of infarct outcome.

Our data may have various clinical implications. First, it demonstrates that rhythmicity in infarct outcome is not limited to patients receiving PCI, but may be present in all patients with myocardial infarction. Secondly, it confirms that physiological explanations may underlie variation in preclinical and clinical rhythmicity studies: Absence of rhythmicity or peak differences do not have to be attributed to flaws in study-setup or statistics. Finally, it shows that when studying 24-hour rhythms in infarct outcome, physiological parameters such as ethnicity, outcome measures, infarct size, and day-night cycle variations may influence rhythmicity and should therefore be taken into account. Understanding which parameters influence rhythmicity in infarct outcome may lead to novel ways to minimize infarct damage.

In conclusion, our data confirmed that outcome of myocardial infarction in rodents depends on the time-of-day of the ischemic event and showed that this diurnal rhythm is present in the absence of reperfusion. Furthermore, our study demonstrated that similar to clinical studies, presence and peak of 24-hour rhythmicity is variable.

Chapter

Analysis of 24-h rhythm in ventricular repolarization identifies QT diurnality as a novel clinical parameter associated with previous ventricular arrhythmias in heart failure patients

Chapter 7 - Analysis of 24-h rhythm in ventricular repolarization identifies QT diurnality as a novel clinical parameter associated with previous ventricular arrhythmias in heart failure patients

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Abstract

Introduction: Cardiac repolarization abnormalities are among the major causes of ventricular arrhythmias and sudden cardiac death. In humans, cardiac repolarization duration has a 24-hour rhythm. Animal studies show that this rhythm is regulated by 24-hour rhythms in ion channel function and that disruption of this rhythm leads to ventricular arrhythmias. We hypothesized that 24-hour rhythms in QT duration can be used as a predictor for sudden cardiac death and are associated with ventricular arrhythmias. Secondly, we assessed a possible mechanistic explanation by studying the putative role of hERG channel dysfunction.
Materials and Methods: In 2 retrospective studies, measures of the 24-hour variation in the QT and QTc intervals (QT and QTc diurnality, QTd and QTcd respectively) have been derived from Holter analyses and compared between groups: 1) 39 post-infarct patients with systolic heart failure (CHF: EF<35 %), of which 14 with, and 25 without a history of ventricular arrhythmias and 2) 5 patients with proven (LQTS2) and 16 with potential (Sotalol-induced) hERG channel dysfunction vs 22 controls.
Results: QTd was 2-fold higher in CHF patients with a history of ventricular arrhythmias (38±15ms) compared to CHF patients without VT (16±9ms, P=0.001). QTd was significantly increased in LQT2 patients (43±24ms) or those treated with Sotalol (30±10ms) compared to controls (21±8ms, P<0.05 for both).
Discussion: QT diurnality presents a novel clinical parameter of repolarization that can be derived from Holter registrations and may be useful for identification of patients at risk for ventricular arrhythmias.

Introduction

Sudden cardiac death, often caused by ventricular arrhythmias, is a major health problem, which affects 1 in every 500 persons per year [352]. Several parameters to identify patients at risk for sudden cardiac death have been proposed in the last decades. In patients with primary prophylactic implantable cardioverter/defibrillator (ICD) therapy however, the number needed to treat is still high (11 in MADIT2[353] and 14 in SCD-HeFT[354]) and possibly underestimated. Criteria that can decrease the number of patients needed to treat will reduce the ICD burden, which consists of regular ICD device checks, battery changes, occupational / lifestyle restrictions and the risk of surgical complications or unnecessary shocks.[355] Furthermore, better risk prediction will make ICD therapy more cost effective. Clearly, there is a high demand for better sudden cardiac death prediction in patients currently treated with a primary prophylactic ICD.

24-hour rhythms are biorhythms present within many cardiac electrophysiological parameters, including QRS duration, QT interval, and heart rate variability.[356] These rhythms are regulated by diurnal changes in ion channel function, such as the hERG channel.[357] In addition, 24-hour rhythmicity has been linked to the incidence and pathophysiology of ventricular arrhythmias and sudden cardiac death, which display an increased incidence in the morning. In animal studies, disruption of the normal 24-hour rhythm leads to a severely depressed functioning of hERG channels conducting the repolarizing current IKr and a concomitant increased susceptibility to ventricular arrhythmias.[29, 357] Ventricular repolarization is humans however, significantly differs between humans and animals (ICH S7B guidelines, 2015). [358] So far, it remains unknown whether the presence and nature of 24-hour rhythms in electrophysiological parameters in humans possess predictive value for the manifestation of ventricular arrhythmias and sudden cardiac death.

We hypothesized that disruption of physiological 24-hour rhythms in ventricular repolarization duration is associated with ventricular arrhythmias. In the current retrospective study, we therefore compared 24-hour rhythms in QT interval duration between heart failure patients with and without history of ventricular arrhythmias. Our data suggest that the amplitude of the 24-hour rhythms in QT and QTc interval (QT and QTc diurnality, QTd and QTcd respectively) is not only associated with ventricular arrhythmias, but also linked to disturbed functioning of I_{Kr}.

Material and Methods

The study consists of 2 analyses. In the first analysis, we compared 24-hour rhythms in QT interval duration between heart failure patients with and without history of ventricular arrhythmias. In this first part, we introduce 2 novel parameters QT and QTc diurnality, which are measures for the amount of 24-hour variation (amplitude) in the QT and QTc interval (Figure 7.2). In the second analysis, we studied whether these parameters are related to hERG channel functionality. We compared QTd and QTcd levels between subjects with genetic or drug-induced hERG channel dysfunction and controls.

Patient selection

1. 24-hour rhythms in repolarization and ventricular arrhythmias (n=39).

Patients with systolic heart failure (HFrEF; heart failure NYHA class II and above with an LVEF <35 %) and a history of a myocardial infarction were included from 3 different sources: 1) Patients of the UMC Utrecht participating in the EUTrigTreat study.[359] Holter analysis was part of the EUTrigTreat study protocol and already performed in all patients before the start of the current study. 2) Patients of the UMC Utrecht who received a guideline-indicated ICD implantation as primary prevention (EF<35 %) between 2009-2011,[360] and that received a Holter registration after ICD implantation. 3) Patients who attended the outpatient clinic of the UMC Utrecht between September and November 2014 and had a history of ventricular arrhythmias. In patients from the third source (outpatient clinic UMC Utrecht, all with ICD upon inclusion) Holter analysis was performed after informed consent was obtained.

Exclusion criteria for all patients in this study were: 1) Use of antiarrhythmic drugs (all classes, slow–release beta-blockers excluded) during or in the 6 weeks before the Holter registration. 2) Supraventricular arrhythmias during Holter registration. 3) Myocardial infarction in the 3 years prior to Holter registration.
All patients included in this study were subdivided in 2 groups, based on history of ventricular arrhythmias. VT+ means that the arrhythmia occurred after the moment that EF was reduced below 35%. All VT+ were confirmed by either an electrocardiogram or manual check of the ICD-recording.

2. 24-hour rhythms in repolarization and hERG channel functionality

<u>Genetic hERG channel dysfunction: LQTS2 (n=5)</u>

Patients clinically diagnosed with LQTS type II carrying a loss-of function mutation of the KCNH2 gene encoding the protein that constitutes the hERG channel, which attended the outpatient clinic of the UMC Utrecht and received a 24-hour electrocardiogram recording (Holter) between 2009-2015, were included.

<u>Drug-induced hERG channel dysfunction: Sotalol users (n=16) (and controls, n=22)</u>

Patients who successfully underwent pulmonary vein isolation for treatment of atrial fibrillation, and received a Holter between May and September 2015, were screened. Subjects that used Sotalol for at least 2 weeks or were devoid of anti-arrhythmic medication for at least 2 weeks (controls) during their Holter were included. Exclusion criteria were the use of other anti-arrhythmic drugs (all classes, beta blockers excluded), a left ventricular ejection fraction (LVEF) of less than 45%, and presence of supraventricular arrhythmia during Holter registration.

A summary of patient source and stratification is provided in Table 7.1.

Source	Analysis 1		Analysis 2		
	VT/VF-	VT/VF+	Control	Sotalol	LQTS2
EUTrigTreat	11	7			
ICD implantation database 2009-2011	14	2			
Outpatient Clinic Cardiology: Sep – Nov 2014		5			
Outpatient Clinic Cardiology: All					5
Holter database: PVI patients May - Sep 2015			22	16	

Table 7.1. Patient source and stratification. ICD: Implantable cardioverter/ defibrillator, PVI: Pulmonary Vein Isolation, VT/VF: Ventricular Tachycardia/ Ventricular Fibrillation

The investigation conforms to the principles outlined in the Declaration of Helsinki. The Medical Ethical Committee of the UMC Utrecht approved both retrospective studies (reference #WAG/mb/15/036026)

Patient characteristics

Age, gender, medication use, echocardiography parameters, NYHA classification, comorbidities (diabetes mellitus, renal failure), medication history, and history of ventricular arrhythmias at the time of the Holter were collected from the electronic patient file. Ventricular arrhythmias (Ventricular fibrillation or sustained ventricular tachycardia) were only qualified as such when recorded on an electrocardiogram (ECG) lasting at least 30s. Ventricular arrhythmias detected by the ICD were manually checked for accuracy.

An verview of both analyses is depicted in Figure 7.1.

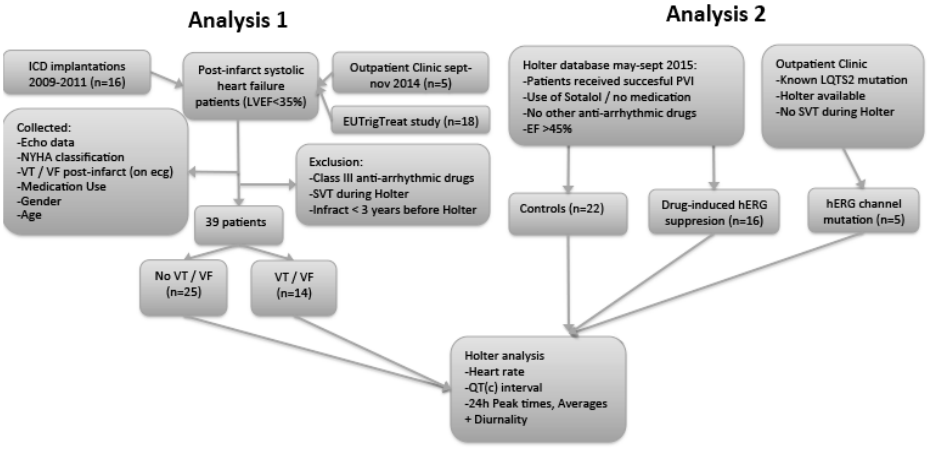

Figure 7.1. Flowchart of retrospective analyses. LVEF: Left Ventricular Ejection Fraction, ICD: Implantable Cardioverter/Defibrillator, LQTS2: Long QT Syndrome Type 2, PVI: Pulmonary Vein Isolation, SVT: Supraventricular Arrhythmia, VT / VF: Ventricular Tachycardia/ Ventricular Fibrillation.

Holter

Holters recordings were obtained with a standard 125Hz 3 lead Holter registration system (SEER Light Holter Recorder, GE healthcare, UK). A trained nurse attached all devices and placed leads at locations V1, V3, and V5. All registrations were done outside the hospital and patients were instructed to follow their normal routine. RR, QT (measured both until the peak (QTp) and end (QTe) of T-wave) and QTc (corrected with the Bazett's Formula) interval were measured every 15 seconds. Hourly averages were calculated using the manufacturers software (MARS version 7.2, GE Healthcare, UK). Based on the hourly averages, the best-fitting cosine curves with a period of 24 hours were calculated for all parameters using a non-linear regression model (R statistics Version 3.0, R Development Core Team, New Zealand).

From this cosine curve, 24-hour averages, peak time, and diurnality were derived. We defined diurnality as the amplitude of the cosine curve, or half of the difference between peak and trough of the 24-hour cosine curve. QT analysis and a visualization of diurnality of one of the patients are depicted in Figure 7.2.

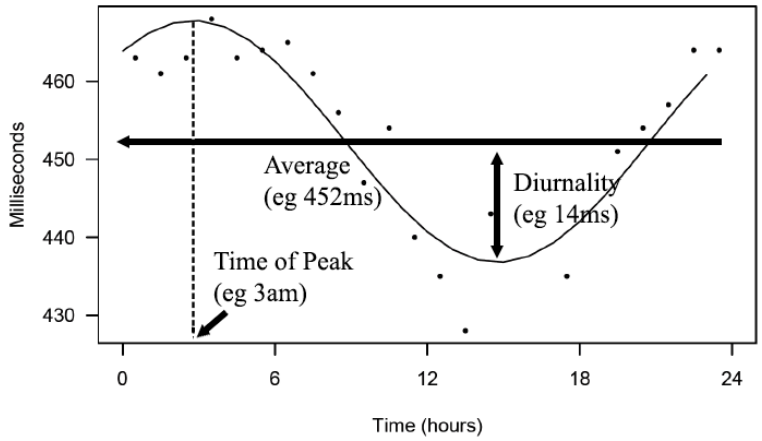

Figure 7.2. Depicted is a 24-hour rhythm in QT of one of the patients. From hourly averages, an optimal fitting cosine curve with a period of 24-hours was calculated. 24-hour average, peak time and diurnality were derived from this curve.

Statistical analysis

Data is presented as averages ± standard deviation. Pearson's correlation coefficient was used to compare variables. Levene's test was used to check equality of variances. When homoscedastic, one- or two-way analysis of variance (ANOVA) was conducted to compare groups. If not, the Kruskal-Wallis test was performed. In analyses with multiple groups, Bonferroni post-hoc analysis was used to compare subjects with controls. Pearson's chi-squared test was used to compare categorical data. P-values <0.05 were considered statistically significant. Predictive power of parameters that were significantly different between groups was quantified using area under curves (AUC) of the receiver-operator characteristics.

Results

1. 24-hour rhythms in repolarization and ventricular arrhythmias.

Patient collection

Thirty-nine patients with systolic heart failure and a history of myocardial infarction were included (see Table 7.1). Baseline characteristics of all patients are depicted in Table 7.2. The only significant difference between patients with and without arrhythmias was the prevalence of Diabetes Mellitus (DM). Our main parameters, QTd and QTcd however, did not differ between patients with and without DM (QTd 20±19 vs 23±11ms, P=0.90; QTcd 13±20 vs 13±7ms P=0.99, respectively).

Baseline Characteristics	VT/VF- n=25	VT/VF + n=14	Significance
Age (years)	51±16	55±10	ns
Gender (% male)	80	79	ns
Ejection Fraction (%)	29±5	26±5	ns
LVEDD (cm)	6.3±0.9	6.2±1.2	ns
LVESD (cm)	5.2±1.0	5.3±1.0	ns
EDV (ml)	181±74	198±91	ns
ESV (ml)	120±42	150±73	ns
SV (ml)	60±29	51±24	ns
β-blocker (used in all patients, converted to mg metoprolol)	113±102	98±75	ns
GRF (ml/min)	51±16	55±10	ns
DM (%)	**44**	**7**	**P=0.017**
NYHA Classification	2.1±0.8	1.9±0.5	ns

Table 7.2. Patient characteristics at time of Holter. DM: Diabetes Mellitus, EDV: End Diastolic Volume, ESV: End Systolic Volume, GFR: Glomerular Filtration Rate, LVEDD: Left Ventricular End Diastolic Diameter, LVESD: Left Ventricular End Systolic Diameter, ns: not significant, SV: Stroke Volume, VT/VF: Ventricular Tachycardia / Ventricular Fibrillation

Accurate automatic 24-hour measurements

Previous studies showed that automatic measurements of the QT interval can be challenging.[361] To assure the accuracy of the automated measurements in this current study, we performed several validations that are specified in the supplementary data. (Figure 7.7, 7.8, and 7.9)

Ventricular arrhythmias are associated with high QTd/QTcd

24-hour rhythms in RR and QT(c) interval duration were compared between patients with and without history of ventricular arrhythmias. History of ventricular arrhythmias was associated with a 2-fold increase of QTd/QTcd as compared to CHF VT- patients

(QTd 38±15 vs. 16±9 ms P=0.001 and QTcd 19±17 vs. 9±6ms, respectively, P=0.003). In addition to these large differences, overlap of QTd values between the 2 groups was low: the lowest QTd of patients with ventricular arrhythmias was 18ms, whereas approximately two thirds (17/25) of the VT- patients had a QTd below this value (Figure 7.3 and Table 7.3). This resulted in a high discriminative power (Area under curve (AUC) of receiver operating characteristic (ROC) curve QTd and QTcd, 0.932±0.038 and 0.818±0.068, respectively).

Baseline Characteristics	VT/VF- n=25	VT/VF+ n=14	Signifi-cance
RR Peak time (hours)	4.5±3.9	3.8±2.2	ns
QT Peak Time (hours)	5.4±6.3	4.4±2.7	ns
QTc peak time (hours)	8.8±7.2	9.6±6.1	ns
RR Average (ms)	861±107	895±133	ns
QT Average (ms)	425±29	428±33	ns
QTc Average (ms)	460±25	455±20	ns
RR Diurnality (ms)	70±41	111±43	ns
QT Diurnality (ms)	**16±9**	**38±15**	**P=0.001**
QTc Diurnality (ms)	**9±6**	**19±17**	**P=0.003**
QRS duration (ms)	119±24	112±31	ns
HRV (SDNN)	114±38	107±35	ns

Table 7.3. Holter parameters of systolic heart failure patients with and without history of VT/VF. HRV: Heart Rate Variability, ns: not significant, VT/VF: Ventricular Tachycardia / Ventricular Fibrillation.

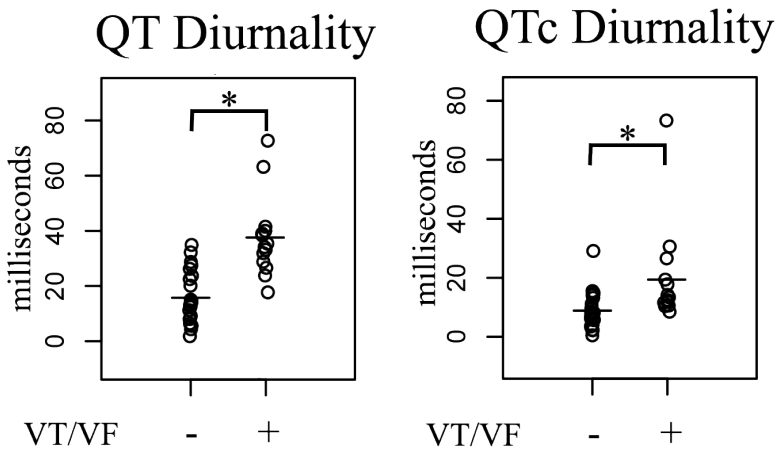

Figure 7.3. Comparison of 24-hour Holter parameters in post-infarct systolic heart failure patients subdivided in 2 groups based on history of ventricular arrhythmias. In arrhythmia patients, QT(c) diurnality (QTd and QTcd, respectively) were 2 fold higher compared to non-arrhythmia controls. VT/VF: Ventricular Tachycardia / Ventricular Fibrillation. * P<0.05 vs no arrhythmia.

2. 24-hour rhythms in repolarization and hERG channel functionality

Cardiovascular disease is associated with, in general, tempered or disrupted 24-hour rhythms.[29, 88, 167] Schroder et al. published a study which showed that normal expression of KCNH2, a gene encoding the hERG (human Ether-à-go-go-Related Gene, $K_V11.1$) protein, has a 24-hour rhythm in mice.[357] hERG is the α-subunit of the delayed rectifier current (I_{Kr}) channel, a potassium channel that mediates repolarization duration of the action potential.[362] Schroder et al showed that the 24-hour rhythm in hERG channel expression suppresses a rhythm in repolarization duration under physiological conditions. Consequently, when rhythmicity in hERG channel expression was disrupted, the 24-hour rhythm in repolarization increased.

Based on this observation, we hypothesized that in humans, a rhythm in ion channel functioning, such as the hERG channel, could also be of importance to control a 24-hour rhythm in repolarization. If so, alike in mice, this could explain why an increase of QTd (and not a decrease) is associated with ventricular arrhythmias. To test this hypothesis, in a second retrospective study, we investigated whether depression of hERG channel functionality in patients resulted in an increase of QTd by comparing QTd of patients with a proven hERG channel dysfunction (LQTS2) or those in which depressed hERG channel functionality is anticipated (Sotalol treated), to controls.

Patient collection

Thirty-four patients were included. Twenty-two Holters were recorded during control conditions (no LQTS2 or Sotalol use), sixteen during a regimen of Sotalol treatment and five in patients with LQTS2. In nine patients, two separate Holters were performed, one Holter before administration of Sotalol and one Holter during Sotalol usage. Time between those two Holters was 12±11 months. As such 43 Holters were available in total. Age and gender were similar between control patients and Sotalol users (59±10 vs. 62±12 years and 59% vs. 63% male, respectively). Four out of five patients diagnosed with LQTS2 were female, these patients were 49±6 years old at the time of their Holter and all used beta-blockers. LQTS2 mutations were c.2959_2960delCT (2x), c.260T>C, c.2887C>A, and c.2354G>T. LVEF was similar in all groups (57±5 % vs. 57±6 % vs. 58±4 %).

Sotalol usage is associated with high QTd/QTcd

In the nine patients in whom two Holter recordings were available (with and without Sotalol), 24-hour averages, QT diurnality, and peak time of RR, QT, and QTc were analyzed. Peak times, 24h averages, and RR diurnality did not differ significantly between the two Holters (Table 7.4). Remarkably, QTd and QTcd increased with Sotalol use (10±13 and 4±6 ms increase, P=0.02 and 0.04 respectively). Data are depicted in Figure 7.4.

Baseline Characteristics	Sotalol- n=9	Sotalol+ n=9	Significance
RR Peak time (hours)	2.8±2.4	2.9±2.0	ns
QT Peak Time (hours)	2.6±2.4	2.6±1.9	ns
QTc peak time (hours)	3.8±5.7	3.5±7.0	ns
RR Average (ms)	948±133	932±101	ns
QT Average (ms)	434±44	440±39	ns
QTc Average (ms)	447±28	457±30	ns
RR Diurnality (ms)	70±46	89±39	ns
QT Diurnality (ms)	18±8	29±10	P=0.02
QTc Diurnality (ms)	7±2	10±6	P=0.04

Table 7.4. Holter parameters of patients with Holter with and without Sotalol. ns: not significant.

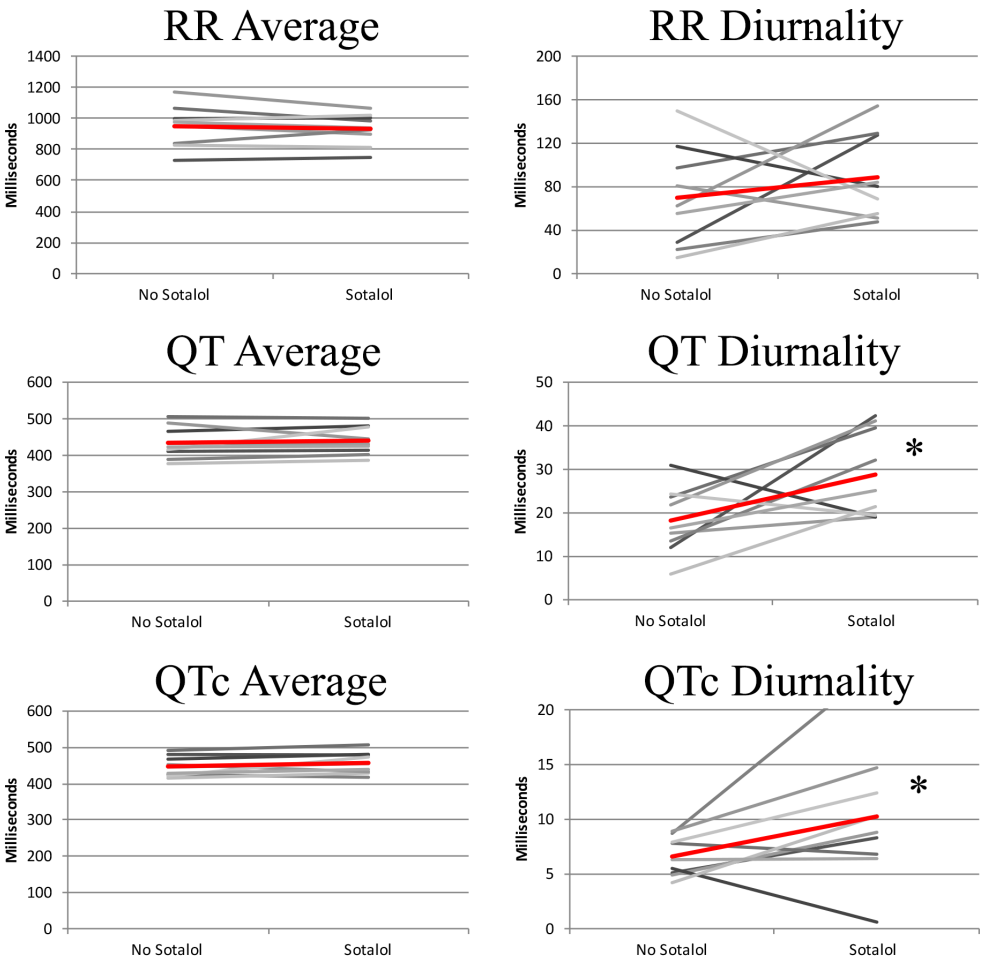

Figure 7.4. 24-hour QT and QTc diurnality (QTd and QTcd, respectively) is higher in patients using Sotalol compared to the same patients without anti-arrhythmic drugs. Red line indicates average of all subjects. * P<0.05 for difference between no Sotalol and Sotalol

Sotalol, especially at lower doses, does not lead to full hERG channel dysfunction[363]. To further explore the relation between repolarization and Sotalol, we therefore compared daily Sotalol dosage to QTd and QTcd. For QTd, no relation was found (R^2 =0.02, P=0.59), but as expected, longer QTcd was associated with higher doses of Sotalol (R^2 =0.35 P=0.02). These findings correspond to the known dose-dependent pro-arrhythmic effects of Sotalol.[364] More importantly, patients with the lowest daily Sotalol dosage (80 mg) all had low (<10 ms) QTcd values (Figure 7.5). Previous studies revealed that low doses of Sotalol have class I (beta-adrenergic) anti-arrhythmic effects, but no effects on repolarization (class III), including pro-arrhythmic side effects.[364, 365] We therefore excluded patients with a daily dose of 80 mg Sotalol from further analysis. Mean dose of the remaining subjects was 196 mg (range 120-320 mg).

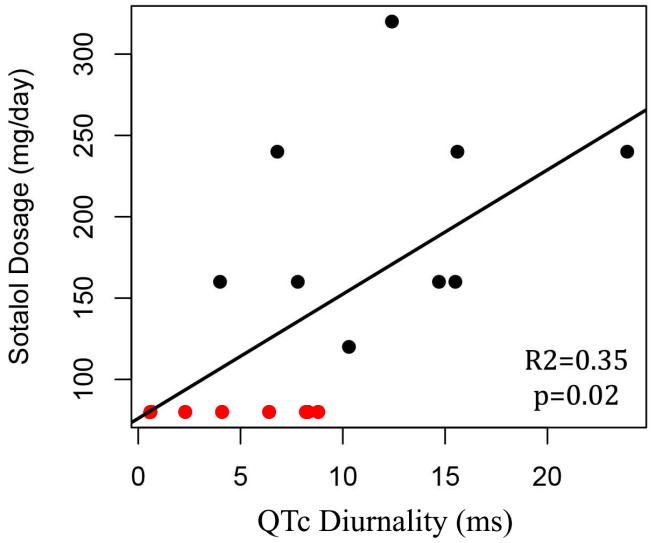

Figure 7.5. QTc diurnality correlates with Sotalol dosage. More specifically, patients receiving low Sotalol amounts (80mg/day, indicated in red) had a relatively low QTc diurnality.

hERG channel dysfunction is associated with QTd and QTcd

As compared to controls, QTd and QTcd were increased in both Sotalol users and LQTS2 patients (QTd 21±8 vs 29±9 and 43±24 ms, P=0.031 and P=0.001 QTcd 8±4 vs 12±6 and 12±7 ms, P=0.027 and P=0.049 for controls vs. Sotalol, and LQTS2 resp.). Compared to those controls, 24-hour QT and QTc averages were significantly higher in LQTS2 patients (QT 421±37 ms vs 474±18, P=0.010 and QTc 448±20 vs 494±33 ms, P=0.002 resp.), but no other significant 24-hour differences were found between Sotalol / LQTS2 vs controls (Data in Table 7.5 and Figure 7.6). These data confirm our hypothesis that hERG channel dysfunction is associated with QTd and QTcd and illustrates that in humans, an increase in rhythmicity (instead of the usually seen decrease) can be associated with disease.

Baseline Characteristics	Controls (n=22)	Sotalol (n=9)	LQTS2 (n=5)	Significance (controls vs Sotalol and LQTS2 resp.)
RR Average (ms)	897±144	954±73	936±104	Both ns
QT Average (ms)	**421±37**	442±37	**474±18**	ns, **P=0.010**
QTc Average (ms)	**448±20**	454±29	**494±33**	ns, **P=0.002**
RR Diurnality (ms)	83±36	89±47	119±66	Both ns
QT Diurnality (ms)	**21±8**	**29±9**	**43±24**	**P=0.031 and P=0.001**
QTc Diurnality (ms)	**8±4**	**12±6**	**12±7**	**P=0.027 and P=0.049**

Table 7.5. Holter parameters of Sotalol users, LQTS2 patients and control patients.LQTS2: Patients with proven mutation in hERG channel; ns: not significant.

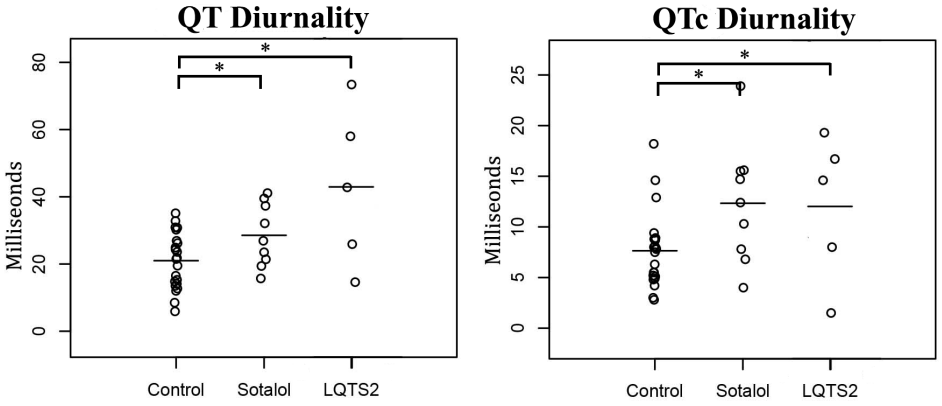

Figure 7.6. Comparison of 24-hour Holter parameters between controls, Sotalol users, and LQTS2 patients. QT and QTc diurnality are increased in Sotalol users and LQTS2 patients. * indicates P<0.05 compared to controls

Discussion

In this study, we investigated 24-hour rhythms in repolarization and introduced the new clinical parameters QT and QTc diurnality (QTd and QTcd, respectively). In a retrospective study, we showed that QTd and QTcd are associated with ventricular arrhythmias in post-infarct patients with a LVEF <35 %. We found that ventricular arrhythmias are associated with a high QTd and QTcd. About 2/3 of patients without arrhythmias showed values of QTd lower than the value of the arrhythmia-positive patient with the lowest QTd value in that group. In an attempt to deduce a potential causative explanation for these observations, we performed a second retrospective study and revealed that (high) QTd and QTcd seem associated with hERG channel dysfunction.

Susceptibility for repolarization related ventricular tachyarrhythmias is often related to an inherited and/or acquired reduced repolarization reserve, which regularly includes a downregulation of the hERG protein and function. To quantify repolarization reserve, QT length is not optimal and other techniques have been applied in the scientific community: 1) exaggerated increase in QT after a hERG blocking drug,[366] 2) parameters of temporal repolarization dispersion quantified as temporal QTVI/N[367] and STV[368,] 3) alterations in T wave morphology after the administration of a hERG blocking drug.[369] So far however, these parameters are not widely used in daily practice.

Some previous publications confirmed the relation between 24-hour rhythm and repolarization parameters. QTVI and QTVN for example, two short-term repolarization parameters, showed a diurnal rhythm.[370] In addition, Van der Berg et al. showed that at night, maximum ventricular repolarization duration corresponds to an increased incidence of sudden cardiac death in LQT3-Brugada patients.[371] To our knowledge however, the current study is the first to study diurnality (amplitude) of repolarization and identifies this parameter as potentially discriminating/predictive for ventricular arrhythmias in humans.

In the current study, we propose that QTd and QTcd are associated with hERG channel dysfunction. Expression profiles of several cardiac ion channels are disrupted in heart failure patients.[372] We hesitate however, that dysfunction of the hERG channels fully explains the increase in QTc/QTcd and occurrence of ventricular arrhythmias. hERG channel disruption for example, is often associated with other electrophysiological changes such as an increase in (average) QTc or heart rate variability (HRV). The patients with reduced LVEF in the current study that faced arrhythmic events, however, do not show these changes. In addition, differences between arrhythmia and non-arrhythmia patients are larger for QTd than for QTcd, suggesting that other factors such as changes in autonomic drive may have contributed to the differences in QTd and QTcd as well. Also in the patients that used Sotalol for treatment of AF, QT and QTc are not prolonged though in these patients we anticipate only a mild degree of hERG block to avoid triggering of ventricular arrhythmias. In line with that, QTd shows an increased value though to a much lower extent when compared to the patients with LQTS2 or those with a reduced EF. On the other hand, in the small group of LQTS2 patients that do have a proven hERG dysfunction and a history of VT but without a low EF, QTd value was even higher as compared to the QTd of the group with low EF and, in this case also QT and QTc were significantly prolonged.

Correct 24-hours measurement of the QT interval is challenging. Not only is the QT interval under influence of heart rate and has automatic measurement proven difficult, positional changes of heart within the thorax and fluctuations in drug plasma levels during the day complicate accurate measurement for longer periods even further. To minimize the risk of inaccurate measurements, we used several strategies. First, automated measurements were done with validated, commercially used software (MARS version 7.2, GE Healthcare, UK) and we checked whether measurements were accurate (Figures 7.7, 7.8, and 7.9).

Secondly, to prevent any interaction of fluctuating drug plasma levels we excluded all patients with anti-arrhythmic drugs despite those that used slow-releasing beta-blockers, which beta-adrenergic effects have been reported to be relatively constant.[373] In the subgroup of Sotalol patients, we analyzed the effects of fluctuating plasma levels on QTd because of the relatively short half-life (+-12 hours). Peak times of the 24-hour QT rhythm in Sotalol users were similar to controls. In addition, ECG changes reach a maximum at 1.5 hours after Sotalol administration.[365] The single QT peak in the middle of the night that is observed in Sotalol users, is therefore unlikely to be caused by a drug regimen of two intakes in the morning and evening.

Third, because repolarization duration depends on heart rate, we considered an effect of 24-hour heart rate variation on QTd. To measure heart-rate independent repolarization values, several formulas were developed, such as Bazett's Formula, Fridericia's Formula, and individually optimized curvature corrections. Previous studies showed that 24-hour rhythms in QTc depend on the method used.[374] In our study, we used Bazett's formula. We purposely did not correct for 24-hour QT/RR hysteresis since this filters 24-hour rhythms, but other methods such as Frederica's Formula or QT/RR hysteresis taken at a specific time point might have yielded different results. In

addition, differences in heart rate, for example caused by beta-adrenergic effects of Sotalol, have previously shown to affect Sotalol activity.[375] The QTd data of Sotalol users vs controls however, shows similar RR averages and RR diurnality levels in combination with significant differences in QTd, confirming that the differences in QTd found are independent of heart rate.

Finally, there is a risk that the position of the heart in the thorax changes throughout the day. However, patient characteristics of those positive for ventricular arrhythmia patients and controls were similar (age, gender, cardiac function, severity of symptoms). Even though 24-hour variation in cardiac position may have influenced QTd, we could not define any reason why this would be different in arrhythmia positive patients compared to those who were devoid of arrhythmias.

Clinical implications

In the post-infarct patient with a LVEF <35%, differences in QTd between patients with and without arrhythmias were not only statistically significant, but also large: 2/3 of the patients without arrhythmias have a lower QTd than the arrhythmia-positive patient with the lowest QTd value. According to current guidelines, all patients with an LVEF <35 % receive an ICD, but only a minority will get a ventricular arrhythmia and benefit from this ICD. 80% of those patients receive an ICD without any benefit but with the risk of complications such as lead revisions and inappropriate shocks. In combination with the high costs of an ICD implantation, there is an urgent clinical need for a better risk stratification in this patient group. The large difference in QTd values and the small overlap between patients with and without history of ventricular arrhythmias, make QTd a promising candidate to follow up as a new and additive arrhythmia predictor in patients at risk.

Study limitations

Because our study was observational, we are not able to confirm a direct causal relationship between hERG channel dysfunction, QTd, and development of ventricular arrhythmias. Secondly, the retrospective study setup and relatively small patient groups do not allow predictive conclusions. To achieve in that, further prospective studies in large patient cohorts will be required in order to reproduce our findings, evaluate the definite predictive value of QTd, and to the define cut-off values for QTd that discriminate between being at risk or not.

Supplemental data

Accuracy of automatic 24-hour measurements

To check the accuracy of the automated measurements in this study, we compared manual and automated calculations of the 24-hours rhythm in QT interval. Although absolute values slightly differed, the correlation between manual and automated measurements was high ($R^2 = 0.97$, $P<0.001$, Figure 7.7).

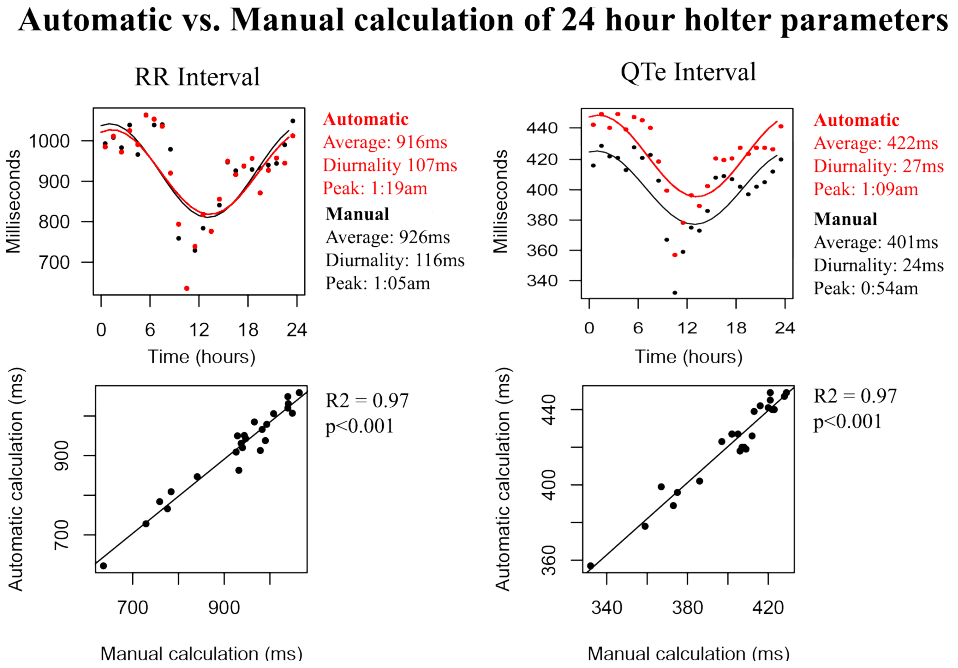

Figure 7.7. Comparison of manual and automated 24-hour Holter parameters. RR interval: Time between 2 QRS complexes, QTe interval: QT time measured until the end of the T-wave.

To further validate the automated measurements, we compared different QT measurement options. QTend values (QT interval from onset of the QRS-complex to the end of the T-wave; used in the current study) were compared to QTpeak values (until the top of the T-wave). QTend values were ±80ms larger than QTpeak values and were strongly correlated (R^2 = 0.99 P<0.001, Supplemental Figure 7.8). Next, QT measurements of different leads were compared. Lead V5 (used in the analyses) and V3, and V5 and V1 have slightly different (±15 ms) absolute values but correlate very well (R^2 = 0.99 P<0.001 and R^2 = 0.97 P<0.001 respectively, V5 vs V3 in Figure 7.8).

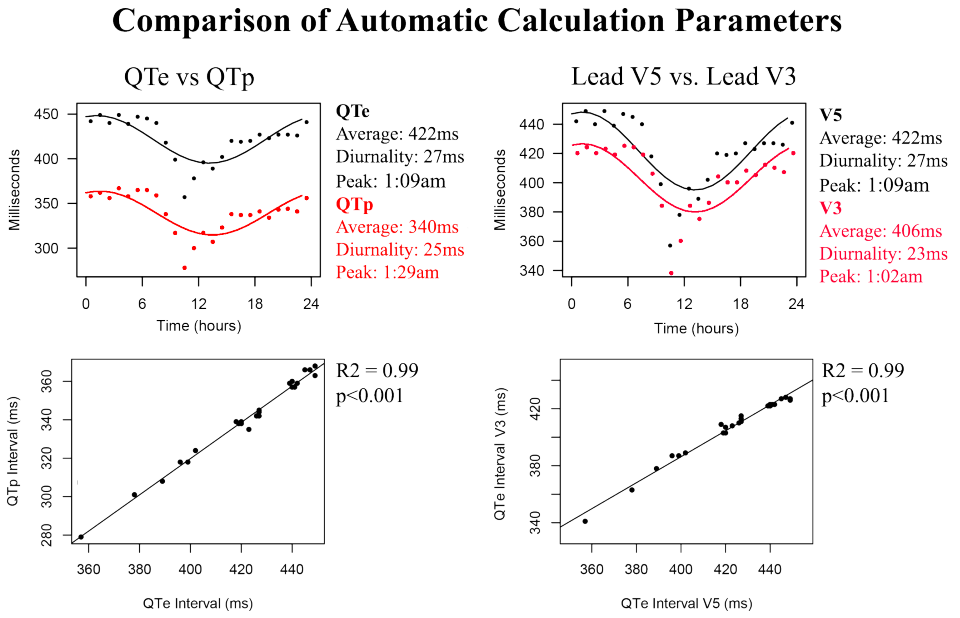

Figure 7.8. Comparison of automated Holter calculations. Left: QT interval from Q-wave to top of T-wave is compared to QT interval from Q-wave to the end of the T-wave. Right: 24-hour parameters from lead V5 are compared to lead V3. QTe: QT interval measured until the end of T-wave, QTp: QT interval measured until the peak of the T-wave.

Finally, we compared cosine curve analysis to other methods of measuring 24-hour parameters. 24-hour averages derived from the cosine curve matched the average of the 24-hourly averages (R^2 = 0.99 P<0.001). Peak times derived from the cosine curve corresponded to the time point of the maximum hourly average (R^2 = 0.87, P<0.001) and diurnality correlated to the difference between the 2 maximal and minimal hourly averages (range, R^2 = 0.83 P<0.001). Importantly, differences between QTd or QTcd, and range were often caused by 1 or 2 outliers (Figure 7.9) that affected range, but not diurnality.

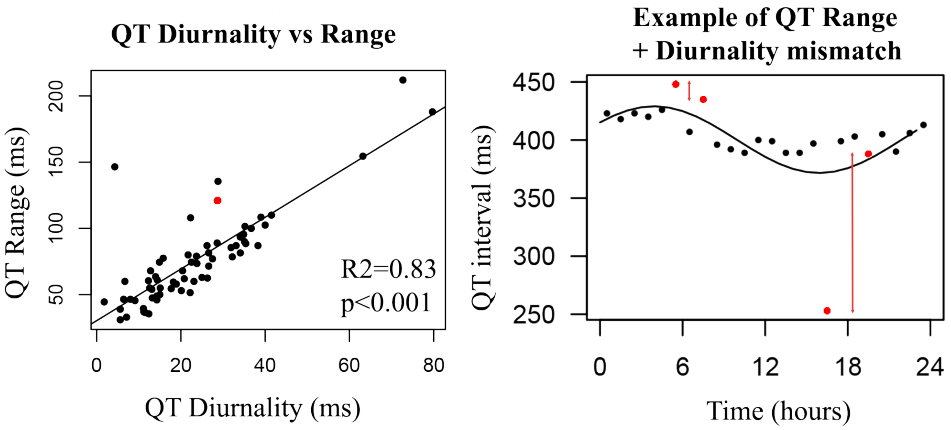

Figure 7.9. Left: 24-hour QT diurnality correlates with 24-hour range. Red dot represents one of the outliers and is further analyzed in right side of Figure. Right: 24-hour rhythm in QT interval of example outlier. QT diurnality was calculated as difference between average and maximum of optimal cosine curve. QT range was calculated as difference between 2 maximal and minimal dots (depicted in red). The relatively high range in the example is caused by one very low value (red dot at bottom).

Chapter 8

General discussion

Chapter 8 - General Discussion

Cardiovascular disease is the number one cause of death around the world, accounting for approximately 30% of all deaths.[376] Although cardiovascular death rate decreased in the recent years, prevalence and as a consequence socioeconomic costs of disease are expected to rise substantially in the upcoming decade.[377] Globally, availability of cost-effective medication such as aspirin, statins, and blood-pressure-lowering drugs would have the biggest effect on further reduction of cardiovascular mortality and morbidity.[378] In the Western world, prevention of disease by reducing the amount of smokers, obese and inactive individuals would have major effects. In patients that already suffer from cardiovascular disease, novel therapies and ways to optimize the current ones are necessary to further reduce the disease burden.

In the last decades, many novel strategies have emerged to treat cardiovascular disease. Two of these therapies, cell-based cardiac repair and the internal cardiac defibrillator made it into clinic, the latter earlier and to a much wider extent. Both therapies however, struggle to fit efficacy in daily practise. Many questions remain about how, and in which patients, these therapies should be applied.

In this thesis, 24-hour biological rhythms are introduced as a novel parameter in the cardiovascular system. Circadian rhythms are regulated by circadian clocks, molecular mechanisms present in the cell that allow anticipation to 24-hour variations.[2, 3] Previous studies showed that these clocks play a major role in the cardiovascular system: they regulate approximately 10% of the cardiac transcriptome. 24-hour variation is present in various cardiovascular functions such as electrophysiology, metabolism, and coagulation.[294] Most importantly, circadian rhythms are associated with cardiovascular disease such as myocardial infarction and arrhythmias: circadian rhythmicity is involved in the incidence, pathophysiology and outcome of these acute events.

We hypothesized that circadian rhythms are not only important in the adult heart, but play a role in cardiovascular immature (stem) cells as well. In Chapter 4, we therefore analysed the presence and effect of circadian clocks in cells used for cell-based cardiac repair. In Chapter 5, we developed a new *in vitro* model for circadian rhythmicity in the heart that aims to optimise translation of new circadian and non-circadian therapies to the clinic. In chapter 6, we further explored the relation between circadian rhythms and myocardial infarction. Finally, we determined whether circadian rhythmicity is an interesting novel tool to predict which patients benefit from an internal cardiac defibrillator and consequently which do not. (Chapter 7)

Cell-based cardiac repair

Cardiovascular disease is associated with the loss of healthy functioning cardiomyocytes. Until recently, the paradigm was that the heart is a terminally differentiated organ and that once cardiomyocytes are lost, they cannot be restored. In the previous decade, there have been major discoveries that challenged this

assumption. Yamanaka discovered that terminally differentiated cells can be reprogrammed to an embryonic-stem-cell-like state.[143] In the heart, multipotent-stem-cell-like cells were found.[379] In combination with studies that proved that many adult cardiomyocytes were created after birth, this led to the widespread idea that injection of endogenous (or other) stem cells could boosts cardiac repair, for example after myocardial infarction.[380] In a relatively short period of time, shiploads of pre-clinical and clinical studies were published, the former with promising results but major methodological shortcomings, the latter often reporting preliminary, dubious results and often considering a limited time of follow-up.[285, 381] In 2014, at the start of this thesis, the tulip mania of cardiovascular research collapsed. Van Berlo et al. convincingly showed that multipotent-stem-cell-like cardiac cells hardly form cardiomyocytes in a normal *in vivo* situation.[382] Second, high-impact papers that formed the basis of the new stem cell paradigm were retracted, potentially because of suspected fraud.[383, 384] After the dust was settled, the scientific community concluded that in their stem cell delusion, they had wanted too much too soon and that both in communication to the public and translation from bench to bedside, many aspects had gone wrong.[385] This thesis therefore starts with an update about the current challenges in the translation of cell-based repair strategies from the basic science laboratory to the patient (chapter 3) and focuses on various cell types, mode and timing of delivery, vascularization, electrophysiological coupling and the host's immunological response, which all require further investigation.

In chapter 3, we stipulate that in addition to these issues, one of the main shortcomings in translation from bench to bedside is a good pre-clinical model to test potential therapies. Often, immune-suppressed, male, young mice without chronic cardiac disease are used with clinically non-relevant outcomes. In addition, therapy is applied during the day (when these animals are in their inactive phase). Although these models can be useful in mechanistic studies, for translational purpose they are the opposite: most studies that use these animals, and showed impressive results at first sight, could not reproduce those observations in humans.[277] In addition, standardisation, randomisation, and blinding of experiments is often lacking in translational, animal studies.

An option to solve the problematic translation from bench to bedside is to better separate the explorative and translational pre-clinical research phase. In the explorative phase, researchers may use various animal models and experiments to prove their (mechanistic) hypothesis, potentially even with multiple procedure variations and outcome measures and not-so-strict statistics. In the translational phase, a core animal facility (with a different researcher) would need to test the therapy with the most promising potential in a clinically relevant animal model. This approach has several advantages. First, it forces translational researchers to stick to clinically relevant animal models without interfering in the explorative phase. Second, it introduces checks and balances: randomisation, blinding, etc., would become standard procedures that apply to all. Finally, it introduces an extra replicate (original researcher plus core lab facility) that improves reproducibility to a much further extent than technical and biological replicates that are used in current daily practise.

In chapters 1 tot 3, we introduce circadian rhythms as a novel parameter in cardiovascular research and cell-based cardiac repair. Circadian rhythms are biorhythms, regulated by circadian clocks that are present in the heart.[386] Previous studies showed that these circadian rhythms play an important role in cardiomyocyte functioning and cell proliferation.[144] We studied the presence of circadian rhythms in Sca1+ cells, a multipotent-stem-cell like cell that can be derived from the adult heart and one of the various stem cell types that has been tested for its regenerative potential. We show that a molecular circadian clock is present in these cells and that Sca1+ cells have a functional 24-hour rhythm in many cellular functions, such as proliferation, stress tolerance, and paracrine factor secretion. These findings are important because they illustrate that circadian rhythms play a major role in many of the functions necessary for cell-based-cardiac repair. In addition, Sca1+ cells or their derivatives are being used in pharmacological studies, where their circadian clock may also influence outcome.

In this study, we did not perform any genetic knockout experiments to reveal the exact pathways responsible for 24-hour rhythms found. Others previously did these experiments in epidermal stem cells and proved that disruption of this clock halts proliferation and alters stress tolerance.[32, 144] In most pre-clinical and clinical experiments, the molecular circadian clock is not (genetically) disrupted, but does vary throughout the day, a condition that is influenced by things as simple as a medium change, temperature differences, or (time of) cell passage. We showed in chapter 4 for example, that a few hours difference in time-after-medium change has major effect on proliferation, stress tolerance and paracrine factor excretion. Most researchers in cell-based cardiac repair or pharmacological studies with stem cells do not realise that this effect exists. When circadian rhythms are not taken into account, for example when control and test samples or animals are not analysed/harvested at exactly the same time, results may not vary because of the hypothesized differences in test and control conditions, but simply because of physiological variations in circadian rhythm. The aim of our study was therefore to show that in physiological, every-day experiments with Sca1+ cells, circadian rhythms influence results. Studies by us/our group (chapter 5) and others show that this is not only true for Sca1+ cells, but applies to almost every cell type.[294] Keeping this in mind may reduce variation, explain previously inconclusive results, improve desired effects and could potentially lead to reconsidering conclusions of previous studies where test and control experiments were not done at the same time.

An interesting question remains whether the circadian clock influences outcome of stem cell injection in a clinical setting. This question was not addressed in this thesis. Rushing our *in vitro* data to a bedside setting hoping for huge effects would mean repeating stem cell research mistakes. We showed presence of functional circadian rhythms in Sca1+ cells, but many important issues remain: how do circadian rhythms in donor cells and the host interfere? Are circadian rhythms in the injured heart comparable to rhythms in a healthy setting, and do other parameters such as the immune system, electrophysiological coupling, etc. interfere with rhythmicity?

Moreover, the importance of circadian rhythms in Sca1+ cells is not limited to their potential in cell-based cardiac repair. On the contrary, the conclusion of this chapter is that circadian rhythms play a role in any experiment using Sca1+ and probably many other cells: *in vitro*, pre-clinical, or clinical.

In vitro model of circadian rhythms in the heart

The study of circadian rhythms requires time. Because time-of-day is taken into account, experimental groups are much larger compared to non-circadian research. For example, to test 3 different drug conditions in non-circadian research, 3 test groups requires limited amounts of animals (e.g. 3 groups x 6 replicates = 18 animals). A similar circadian experiment of these 3 drugs however, necessitates multiple time-points that results in a lot more animals (e.g. 3 groups x 6 replicates x 4 time-points = 76 animals). Not only is this time-consuming, society and research ethics require minimal use of animals. To achieve this, research often turns to *in vitro*, cellular models. For circadian research in the heart however, these are not often used. Human cardiac tissue is scarce and adult cardiomyocytes are difficult to isolate.[387] Some studies use hepatocytes as an alternative and there are novel initiatives by our own group with stem-cell-derived cardiomyocytes, which although promising, are not yet easy to obtain. In chapter 5, we investigated whether cultured neonatal rat cardiomyocytes, an accepted and commonly used *in vitro* model for decades, could also be used as an *in vitro* model for circadian rhythms in the heart. Indeed we show that a molecular circadian clock and 24-hour functional rhythms are present in these cells. Furthermore, we show that these rhythms, for example in stress tolerance and heart rate mimic an *in vivo* situation and illustrate that adding known clock-disrupting compounds to the *in vitro* system alters rhythmicity on a molecular and functional level. To our best knowledge, currently no other *in vitro* system is available that allows analysis of functional rhythms in the adult heart. Of note however, is an article published more than 50 years ago, where Tharp and Folk isolated adult cardiomyocytes and discovered that there was a 24-hour rhythm in the contraction rate of these cells. Although it contains a few methodological flaws, logical for a time-period before the discovery of the molecular circadian clock, their results largely correspond to ours.[388]

Our applied *in vitro* systems proved to be a straightforward method to test circadian effects of chemical compounds or interventions, faster and with less animals that regular cardiac circadian models. In addition, it shows that cardiac circadian effects can be measured at an *in vitro* level. In that regard, when performed with stem cell-derived cardiomyocytes, large amounts of drugs (drug-library) could be tested for their effect on circadian rhythms, and maybe more important, 24-hour variation in efficacy and side effects in the healthy and diseased heart.[389, 390]

Adequate and predictive *in vitro* models that are able to mimic the *in vivo* situation are crucial to get laboratory findings from bench to bedside. Very often (as with cell-based-cardiac repair) high-impact laboratory findings struggle to find their way towards the clinic. Development of one new drug costs approximately 2.6 billion dollar, much of which can be attributed to the fact that almost all drugs 'discovered'

in a researcher's laboratory never make it to the clinic.[391] As a result, the goal of many researchers is no longer to develop a product that can be used to cure a patient, but to get a publication in a high impact journal, which will lead to either more research funds or a pharmaceutical company buying the concept. There are several initiatives to change this phenomenon, for example the 'science-in-transition' movement, which for example aims to grade researchers on the impact they made on society or their research field rather than the impact factor of their publications. Investment in and focus on *in vitro* and *in vivo* models that better predict *in vivo*, clinical situations also have the potential to better and earlier select therapies to cure the patient. Researchers trying to get their discovery from bench to bedside should not prove that their hypothesis works in an imperfect model to get their results published, but rather use a good model that allows true translation to bedside.

Circadian rhythms in myocardial infarction

In the beginning of this decade, various animal and clinical experiments showed that a 24-hour rhythm does not only exist in the incidence, but also the outcome of myocardial infarction.[32, 33, 336] In chapter 6, we summarise pre-clinical and clinical studies that investigated this 24-hour rhythm, and found that rhythmicity is not present in all performed studies, specifically the ones where almost no percutaneous coronary interventions (PCIs) were performed.[342, 344, 347] In addition, the time-of-day with maximum infarct size varies. We hypothesised that the type of infarct is responsible for the variation; more specifically that reperfusion damage causes the 24-hour rhythm in infarct size. We compared two types of myocardial infarction (ischemia/reperfusion and permanent ligation) in a pre-clinical experiment but contrary to our expectation, found rhythmicity in the permanent ligation and not the ischemia / reperfusion model. Reperfusion apparently is not solely responsible for the 24-hour variation in infarct outcome.

Our findings correspond to previous studies, which also found rhythmicity in the outcome of myocardial infarction. There were major differences however in methodology and exact outcome: infarct model, outcome measures, infarct size, time of maximum damage, etc. all differed.[32, 336, 337] In chapter 6, we aimed to answer a simple question, but because of our unexpected outcome and the various difference in methodology, raised multiple new ones instead: are our models the good ones? Is 30 minutes of ischemia in the I/R model long enough? Do we have to use a closed chest model to exclude confounding effects of surgical damage? Was the follow up time long enough? Does our day/night schedule reflect a real-life situation? And finally, did we use correct outcome measures? All these questions are legit and, for a full understanding of what is going on, should be addressed. To answer these questions however, many more follow-up experiments have to be performed. For small experiments with unexpected results, the gain of extra experiments often does not outweigh the effort. When no follow-up experiments are done, results remain inconclusive and risk ending up in a laboratory drawer (not to be used again). In my opinion, results of all experiments, including unexplained and preliminary ones, should be published. Not only does this prevent others from exploring the same dead-end

roads, it is an obligation to the public that paid for, and the animals that were used in the experiments. To get funding for research or to start an animal experiment requires a considerable amount of time, paperwork, reviewing sessions and committees. Once all these hurdles have been taken, there are few more checks that most of the time, do not take publication into account. I propose to shift part of the administrative burden and accountability from the beginning to the end of research, where we have to keep in mind that experiments with unexpected results are rather common than exception, often not fully elaborated and inconclusive.

Circadian rhythms in ventricular repolarization

Next to the consequences of myocardial infarct induced cardiac damage and myocardial remodelling leading to heart failure, ventricular repolarization abnormalities are a major cause of sudden cardiac death.[352] Many electrophysiological (repolarisation) parameters have been investigated for their ability to predict sudden cardiac death. Some of these, such as heart rate, QT-interval and heart rate variability show a 24-hour rhythmicity.[356] In chapter 7, we investigated whether 24-hour variations in the QT-interval are associated with ventricular arrhythmias. In a retrospective study, we discovered that amplitude of the 24-hour variation in QT-interval (QT diurnality) is associated with ventricular arrhythmias. In a second retrospective study, we showed that this QT diurnality is also associated with hERG-channel dysfunction, one of the main channels responsible for repolarisation and, when compromised, for sudden cardiac death. Our study corresponds to pre-clinical, animal studies which show that the hERG channel is responsible for 24-hour variation in ventricular repolarisation.[357] Also, we are not the first to study 24-hour variation in ventricular repolarisation. Dobson et al., and more recently Sprenkeler et al. demonstrated diurnal rhythmicity in short-term variation of ventricular repolarisation.[392, 393] Peak timing of short-term variation of ventricular repolarisation does not coincide with peak QT diurnality, but occurs at the maximum QT diurnality slope. This might suggest that these are 2 different, but potentially related parameters. An interesting hypothesis is that they both represent the so-called 'repolarisation reserve', a combination of repolarisation contributors, that individually are not necessary for repolarisation, but when combined being dysfunctional may cause ventricular repolarisation abnormalities and sudden cardiac death.[394] On the other hand, the fact that peak STV is maximal at the maximal slope of QT diurnality might implicate that the liability in STV represents the phase from max diurnality to minimal diurnality, a phase where repolarisation stability is fragile. Further studies will have to clarify how QT diurnality relates to other circadian and non-circadian repolarisation parameters.

The methodological setup of this chapter results in several limitations. Obviously, our study is retrospective: predictive conclusions cannot be drawn. Also, we included a rather limited amount of patients from various sources in a non-standardised way, most of which used beta-blockers. All these issues potentially influence our results but are not necessarily shortcomings. Our initial goal was not to prove that QT diurnality predicts sudden cardiac death. Rather, we explored various 24-hour parameters of

ventricular repolarization, selected the one with most clinical potential (QT diurnality, little overlap between patient with and without a ventricular arrhythmias) and hypothesised why this parameter is increased in patients with ventricular arrhythmias. There are many fundamental and clinical questions that remain. For example, previous research showed that other ion channels (which in this setting we did not study) could also attribute to a 24-hour variation in repolarisation.[29] Second, at this moment we do not know what the relation between QT diurnality and other 24-hour repolarisation parameters is,[393] These questions, together with the question if QT diurnality predicts sudden cardiac death, will have to be addressed in a large, prospective, study that includes multiple repolarisation parameters.

Conclusion

The sun shines on our planet in a 24-hour cycle to which all life has adapted. Only recently it was discovered that our body has a mechanism, a clock that anticipates to variations caused by this 24-hour rhythm. This thesis showed the circadian clock has a huge influence on cardiac function: cardiovascular cells, including immature types, possess a molecular clock that regulates rhythmicity of various cellular functions. Also, we investigated the role of circadian rhythms in the development of cardiovascular disease and showed that circadian rhythms are important in onset, and potentially, treatment and prognosis of cardiovascular disease. Circadian rhythms are involved in almost every aspect of human physiology, pathophysiology and thereby account for a key to treat the diseased.

Recommendations for the future

At the end of a PhD track, one does not only get to reflect on his/her own work, but also the direction of science as a whole. In the general discussion, I highlighted the problems with translation from bench to bedside and presented several suggestions, such as separating explorative and translational pre-clinical research phases, more attention for randomisation and blinding, more realistic pre-clinical disease models, and publication of negative results. Most of these recommendations have been made before and science is changing; from government subsidies that were awarded to individual researchers a decade ago to a situation nowadays where consortia of academia, corporations, patients and other shareholders from society together dictate how and what research is performed. And although I believe that patients, and science in general, may benefit from this change, and although I also believe that all the recommendations made by others and myself are easier incorporated in these translational agglomerates that when done by individuals, I would like to end with a word of caution.

Almost all scientific and medical discoveries that changed the course of history, where not done out of a societal need or urge, but because of simple interest. When Alexandre Flemming discovered penicillin in 1927, he was not on a translational quest, but interested in the properties of staphylococci. Most Nobel price of Medicine winners that followed also did curiosity-based research without a concrete idea how their research would change society. Recently, similar to Flemming, curiosity-driven bacteria research led to the discovery of CRISPR/Cas9, a gene-editing technique that turned out to have major medical and societal consequences. Henry Ford, inventor of the T-ford car and thereby one of the first to translate science to public use, allegedly said: “If I had asked people what they wanted, they would have said faster horses.” Medical breakthroughs are not the result of societal need, but of smart or lucky people investigating what they feel is interesting. After a laboratory discovery, translation, with all its challenges and shortcomings as described previously, is necessary, but it almost never starts there. At the end of my thesis, I would therefore like to call for a re-appreciation of curiosity-based science, which I believe will lead to major breakthroughs. Do not just invest in research consortia or better translational science, but fund the talented (but often wicked) student or professor interested in something hardly anyone feels is important, let alone may be commercially interesting. Their results will probably end up in a drawer (or PubMed-database), but when enough of those students and professors are funded, one of them discovers something that will change history.

Appendix

References
Nederlandse samenvatting
Dankwoord
List of publications
Curriculum vitae

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Nederlandstalige samenvatting

Circadiane Ritmes in Hart- en Vaatziekten: van Pipet naar Patiënt

Wereldwijd zijn hart- en vaatziekten doodsoorzaak nummer 1. De afgelopen decennia is de mortaliteit sterk gedaald, maar nog steeds sterft 1 op de 3 Nederlanders aan hart- en vaatziekten. Door een rokende, steeds ouder, dikker en minder actief wordende bevolking komen er bovendien steeds meer mensen die niet sterven aan, maar leven met deze ziekte. Wereldwijd is er voor betere bestrijding van hart- en vaatziektes een grotere beschikbaarheid van medicijnen noodzakelijk. In Nederland zou het verminderen van risicofactoren voor het ontwikkelen van ziekte, zoals minder roken, afvallen en meer bewegen een groot en gunstig effect hebben. Daarnaast zijn er nieuwe therapieën, en nieuwe manieren om de huidige therapieën beter te maken, noodzakelijk. In dit proefschrift wordt onderzocht of circadiane (dag/nacht, 24-uurs, diurnale) ritmes hierbij een rol kunnen spelen

Circadiane ritmes zijn bioritmen die worden aangestuurd door een klok. Deze klok zorgt er voor dat mensen, maar ook dieren of planten, kunnen anticiperen op dagelijkse veranderingen in de wereld om hen heen. Mensen hebben twee klokken: een grote (centrale) klok in de hersenen en vele (perifere) klokken die in elke lichaamscel zitten. In het hart speelt deze klok een grote rol. Er is een dag-nachtritme in de aanmaak (transcriptie / expressie) van 10% van alle genen en eiwitten in een hartcel. Deze genen en eiwitten zorgen voor een 24-uurs ritme in functies van het hart en het vaatstelsel, bijvoorbeeld hartslag, bloeddruk, en bloedstolling. Ook spelen dag-nachtritmes een belangrijke rol bij hart- en vaatziektes. Hartritmestoornissen en hartinfarcten komen op bepaalde momenten van de dag vaker voor. Het verstoren van dag-nachtritmes, bijvoorbeeld door het doen van nachtdienst, draagt daarnaast bij aan het ontstaan van suikerziekte, hoge bloeddruk en hartinfarcten. Ook spelen dag-nachtritmes een rol bij de ernst van ziekte. Een infarct in de ochtend zorgt bijvoorbeeld voor veel meer hartschade dan eenzelfde infarct in de avond. Hoofdstuk 1 van dit proefschrift introduceert circadiane ritmes. Het beschrijft de moleculaire opbouw van de circadiane klok en de rol van deze klok in het hart.

Het meeste onderzoek naar circadiane ritmes is gedaan in volwassen mensen of dieren. Circadiane ritmes van onvolwassen mensen of cellen zien er anders uit en werken op een andere manier dan volwassen circadiane ritmes. Hoofdstuk 2 is een literatuurstudie waarin verschillende onderzoeken naar onvolwassen circadiane ritmes worden bestudeerd. Er is daarbij gekeken naar ritmes in de normale ontwikkeling van een bevruchte eicel, via de zwangerschap, tot een volwassen individu en de ontwikkeling van een onvolwassen (stam)cel, die in een laboratorium tot een volwassen cel wordt gekweekt (gedifferentieerd).

Hoofdstuk 3 gaat verder in op deze onvolwassen (stam) cel en de rol van dit type cel in onderzoek. De afgelopen jaren is geprobeerd om met behulp van deze cellen het beschadigde hart te repareren. Onderzoeken uit het laboratorium laten spectaculaire resultaten zien, maar in de mens is deze ‘(stam)celtherapie’ voor het hart tot nog

toe teleurstellend. In hoofdstuk 3 wordt gekeken wat de mogelijke obstakels zijn om celtherapie van het laboratorium naar de patiënt te krijgen en worden aanbevelingen / mogelijkheden voor vervolgonderzoek gedaan.

Een van die mogelijkheden voor vervolgonderzoek is gebruik te maken van circadiane ritmes. In hoofdstuk 4 worden deze ritmes tot in detail bestudeerd in de Sca1+ cel, een van de celtypes waarmee geprobeerd wordt het hart te repareren. We laten zien dat de circadiane klok aanwezig is in deze cel en een belangrijke rol speelt in zijn functie: er is een ritme in de mate van vermenigvuldiging (proliferatie), hoe goed de cel tegen schade kan en hoeveel (voor het hart gunstige) factoren hij uitscheidt. Zowel in onderzoek naar het repareren van het hart als bij geneesmiddelenonderzoek wordt dit celtype vaak gebruikt, maar de gevonden resultaten zijn van belang voor alle onderzoek naar (stam)cellen. 24-uurs variaties spelen een belangrijke rol in cel functie. Door hier rekening mee te houden zal er minder (ongewilde) variatie in onderzoeksresultaten optreden en kunnen gunstige effecten van (cel)therapie optimaal worden benut.

Om onderzoeksresultaten van het laboratorium naar de patiënt te krijgen zijn onderzoeksmodellen nodig die de werkelijkheid zo goed mogelijk nabootsen. Bij circadiane ritmes wordt hierbij vaak gebruik gemaakt van een proefdiermodel, meestal de muis. Omdat er rekening gehouden moet worden met veel verschillende tijdstippen, zijn er voor goed onderzoek heel veel muizen nodig. Zowel vanuit praktisch als ethisch perspectief is dit onwenselijk. In hoofdstuk 5 introduceren we daarom een circadiaans model dat gebruik maakt van gekweekte hartspiercellen. We laten zien dat deze cellen een circadiane klok hebben die net zo werkt als een klok in het hart van een proefdier of mens. 24-uur ritmes in gevoeligheid voor hartschade of hartslag in dit model lijken erg op vergelijkbare ritmes in mensen. Bovendien is het mogelijk om stoffen aan het model toe te voegen die circadiane ritmes beïnvloeden. Door gebruik te maken van dit model kan er relatief gemakkelijk onderzocht worden wat de effecten van een nieuw medicijn, of behandeling, zijn op circadiane ritmes in het hart.

In hoofdstuk 6 hebben we onderzocht wat de precieze rol is van circadiane ritmes tijdens hartinfarcten. Eerdere studies in zowel het laboratorium als bij patiënten hebben laten zien dat er een dag/nachtritme aanwezig is in de schade die ontstaat na een hartinfarct. Vreemd genoeg laten niet alle studies dit effect zien en verschilt het tijdstip van het hartinfarct dat zorgt voor de grootste schade tussen studies. In een dierexperimentele studie hebben we onderzocht of het type hartinfarct deze verschillen kan verklaren. Bij sommige infarcten wordt er een bloedvat afgesloten dat daarna (door een dotterprocedure) weer wordt geopend (ischemie / reperfusie); bij andere infarcten blijft het bloedvat volledig afgesloten en sterft een deel van het hart af (permanente occlusie). Wij hebben deze 2 typen hartinfarcten vergeleken en, tegenovergesteld aan onze verwachtingen, gevonden dat permanente occlusie wel en ischemie/reperfusie geen circadiaan ritme laat zien in infarctgrootte.

Ventriculaire ritmestoornissen zijn gevaarlijke hartritmestoornissen die kunnen leiden tot plotse dood. Vroegere studies hebben laten zien dat stoornissen in de

herstelfase (repolarisatie) van het hart deze ritmestoornissen kunnen veroorzaken. Er zijn verschillende repolarisatie parameters (waardes op het hartfilmpje die iets zeggen over de herstelfase), zoals de QT-tijd, die het optreden van ritmestoornissen kunnen voorspellen. In hoofdstuk 7 vergelijken we 24-uurs ritmes in de QT-tijd van hartpatiënten die in het verleden wel of geen gevaarlijke ritmestoornis hebben gehad. Patiënten met een ritmestoornis hebben een veel grotere dag/nacht-variatie in hun QT-tijd (QT diurnaliteit) dan patiënten zonder ritmestoornis. In een tweede studie hebben we daarnaast ontdekt dat dit te maken zou kunnen hebben met het hERG-kanaal, een van de onderdelen van de hartcel die verantwoordelijk is voor de repolarisatie.

Conclusie

De aarde draait in een periode van 24 uur om zijn eigen as. Het leven op deze planeet heeft als gevolg daarvan te maken met sterk wisselende omstandigheden. Om te anticiperen op deze dagelijkse veranderingen beschikken plant, dier en mens over een circadiane klok, die een groot gedeelte van de processen in ons lichaam aanstuurt. Deze rol beperkt zich niet tot het normale functioneren van ons lichaam; circadiane klokken en ritmes spelen een cruciale rol in het ontstaan van ziekte. In dit proefschrift hebben we onderzoek gedaan naar de rol van circadiane ritmes in hart- en vaatziekten. We hebben gevonden dat circadiane klokken en 24-uurs ritmes aanwezig zijn in (stam) cellen van het hart en een rol spelen in de schade die ontstaat na een hartinfarct. Daarnaast hebben we laten zien dat het mogelijk is deze ritmes in een celmodel te bestuderen en dat ze van belang zijn bij het ontstaan van gevaarlijke ritmestoornissen, waardoor het in de toekomst hopelijk mogelijk is met behulp van circadiane ritmes te voorspellen wie deze ritmestoornis krijgt. Het verschil tussen dag en nacht is groot. Kennis over hoe het lichaam anticipeert op dit verschil is niet alleen interessant, maar biedt de mogelijkheid ziekte te begrijpen, voorspellen en mogelijk te genezen.

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List of Publications

Analysis of 24-h Rhythm in Ventricular Repolarization Identifies QT Diurnality As a Novel Clinical Parameter Associated with Previous Ventricular Arrhythmias in Heart Failure Patients. Du Pré BC, van Laake LW, Meine M, van der Heijden JF, Doevendans PA, Vos MA, van Veen TAB. Front. Physiol. 2017;8:590

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A man with atypical appendicitis. Du Pre BC, Akkersdijk WL. Ned Tijdschr Geneeskd. 2012;156(18):A3432

Curriculum Vitae

Bastiaan Cornelis du Pré was born March 31st 1988 in Utrecht, but lived most of his youth in Susteren, a small village in Limburg. After completing the gymnasium program of Trevianum Sittard cum laude, he moved back to Utrecht to become a Medical Doctor. During his studies, he graduated as a bachelor of Law, finished 2 honours programs and did several research projects at the departments of rheumatology, pathology, and cardiology. In 2012, influenced by dr. Linda van Laake, he got interested in basic and translational science with a focus on the circadian clock. This led him to Birmingham, Alabama where prof. Martin Young taught him the basics of circadian cardiovascular research. After obtaining his medical degree, the Alexandre Suerman Stipendium allowed him to start his own translational research project that led to this thesis. In and around his work as a researcher and clinician, he likes to teach and think about the relation between patient, science and society. Currently, Bastiaan lives and works in Rotterdam. After a year at the Maasstad Ziekenhuis, he now has a position as an internal medicine resident at the Erasmus MC. When clinic and science allow it, he enjoys (marathon) running, scuba diving and travel around the world.