Molecular biology of the circadian clock in the rodent heart

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by

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Abstract

Circadian rhythms are physiological and behavioural patterns with a period of approximately 24 hours that allow almost all organisms to anticipate predictable changes in their environment. In mammals, these rhythms are co-ordinated by a hypothalamic pacemaker, the suprachiasmatic nuclei (SCN) that is synchronised to solar time by retinal signals. Rhythms are generated at the cellular level by interlocking auto-regulatory transcriptional/post-translational feedback loops known as the circadian clock. The same mechanism exists in all mammalian cells investigated, including components of the cardiovascular system (CVS). Signals from the clock are translated into physiological rhythms via the regulation of clock-controlled genes (CCGs) and recent observations suggest that over 10% of the transcriptome is rhythmically expressed. Disruption of the clock is associated with chronic illness such as cardiovascular disease, cancer and diabetes and in the human CVS circadian rhythms in cardiac function and the occurrence of pathological events have been observed. The molecular targets of the clock in the heart are however largely unconfirmed.

The aim of this thesis was to identify CCGs in the mouse heart and investigate their regulation by the clock using *in vivo* and *in vitro* approaches. SCN-dependent temporal expression and up-regulation by clock factors suggests for the first time that *Bnp* is a cardiac CCG and, with the putative CCGs *Anp* and *Ms1*, implicates the cardiac clock in regulating hypertrophy, and cardio-protection. Treatment of SCN-ablated mice with dexamethasone re-established circadian expression of *Bnp* and altered expression of most genes investigated, implicating glucocorticoids in synchronisation of the cardiac clock and target processes. Cardiac circadian expression of *Pai-1* was confirmed and direct transcriptional regulation by the clock was demonstrated. A novel E-box-containing distal region in the *Pai-1* promoter was identified which may be sufficient to generate cycling in dexamethasone-synchronised cells. E-box dependent activation of this distal region and the proximal promoter by clock and hypoxic factors suggests the E-box provides the molecular interface between circadian and stress pathways and that *Pai-1* is a key integrator of the circadian clockwork and diverse physiological processes in the CVS.

Together these and previous findings suggest the cardiac clock can control complex co-ordination of gene cascades and integrate diverse processes with adaptation to the temporal environment, providing a molecular explanation for the diurnal variation in cardiovascular events and suggesting new therapeutic targets.

Publications and presentations from the work presented in this thesis

Publications

Singletary, J.H., Chan, D., Samani, N.J. and Chong, N.W.: The canonical E-box motif: A target for glucocorticoid action that drives rhythmic mouse *Pai-1* transcription *in vitro*. Gene (2008) Volume 420, pages 42-7.

Presentations

[1] Singletary, J.H., Chan, D., Samani, N.J. and Chong, N.W.: Regulation of the mouse plasminogen activator inhibitor-1 (PAI-1) promoter by circadian and hypoxic factors – a conserved mechanism with humans. Acta Physiologica (2007) Volume 191, Supplement 658 :OTH12-47

Presented as an oral communication at the Joint Meeting of The Slovak Physiological Society, The Physiological Society and The Federation of European Physiological Societies, September 2007, Bratislava, Slovakia, and at the European Young Physiologists Symposium of the same meeting.

[2] Singletary, J.H. and Chong, N.W.: The Clock is ticking – a wake-up call for heart disease? Selected poster presented at the University of Leicester "Festival of Postgraduate Research", June 2007.

Contents

List of figures and tables

Abbreviations

ABRA	actin-binding Rho activating protein
ANOVA	analysis of variance
ANP	atrial (or A-type) natriuretic peptide
ANS	autonomic nervous system
apo	apolipoprotein
ARC	Apoptosis Repressor with CARD domain
ARNT	aryl hydrocarbon receptor nuclear translocator
ARNTL	ARNT-like
ATF	activating transcription factor
bHLH	basic helix-loop-helix
BHLHB	bHLH domain containing, class B
BMAL	brain and muscle ARNT-like protein
BNP	brain (or B-type) natriuretic peptide
cAMP	cyclic adenosine monophosphate/adenosine 3',5'-monophosphate
CARD	caspase recruitment domain
CCG	clock-controlled gene
c-erbAα	thyroid receptor α gene
CHF	cardiovascular helix-loop-helix factor
ChIP	chromatin immuno-precipitation
CKI	casein kinase
CLIF	cycle-like factor
CLOCK	circadian locomotor output cycles kaput
CNS	central nervous system
CRY	cryptochrome
CT	circadian time
CTGF	connective tissue growth factor

CVD	cardiovascular disease
CVS	cardiovascular system
DBP	D-element binding protein
DD	dark-dark
DEC	differentially expressed in chondrocytes protein
DEX	dexamethasone
DIG	digoxigenin
DMV	dorsal motor nucleus of the vagus
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DR	dim red light
E4BP4	adenovirus E4 promoter ATF site binding protein 4
ECR	evolutionary conserved regions
EMSA	electromobility shift assay
EPAS	Endothelial PAS domain protein
FASPS	familial advanced sleep phase syndrome
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATA4	GATA binding protein 4
GLUT	glucose receptor
GRE	glucocorticoid response element
GSK	glycogen synthase kinase
HAND2	heart and neural crest derivatives expressed 2
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia-Inducible Factor
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA

HRE	hypoxia response element
HRP	horseradish peroxidase
ID	inhibitor of DNA binding
IML	intermedolateral columns
IR	ischemia-reperfusion
KLF	krüpple-like factor
Kv	potassium voltage-gated ion channel
LD	light-dark
Luc	luciferase
MAP	mitogen-activated protein
MEF	myocyte enhancer factor
MI	myocardial infarction
miRNA	microRNA
MOP	member of PAS family
mRNA	messenger RNA
MS1	myocyte stress 1
NAD	nicotinamide adenine dinucleotide
NADP	phosphorylated NAD
NOL3	nucleolar protein 3
NPAS	neuronal PAS domain protein
NPRA	natriuretic peptide receptor A
NR1D1	nuclear receptor subfamily 1, group D, member 1
NTS	nucleus of the solitary tract
PAI	plasminogen activator inhibitor
PAS	period-arnt-singleminded
PBN	parabrachial nucleus
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PER	period
PEST	peptide sequence rich in proline, glutamic acid, serine and threonine
PGC1a	PPARγ co-activator 1α
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PVN	paraventricular nucleus
RAR	retinoic acid receptor
REV-ERBα	reverse expression of c-erbAα gene
RHT	retinohypothalamic tract
RNA	ribonucleic acid
ROR	RAR-related orphan receptor
RORE	REV-ERB/ROR response element
ROS	reactive oxygen species
RT-PCR	reverse transcription PCR
RXR	retinoid X receptor
SCN	suprachiasmatic nuclei
SCNX	SCN-ablation
SD	standard deviation
SEM	standard error of the mean
SHARP	enhancer-of-split and hairy-related protein
SIRT1	silent information regulator 1
SNP	single nucleotide polymorphism
SRF	serum response factor
STRA13	stimulated by retinoic acid 13 homologue
SUMO	small ubiquitin-related modifier protein
TBP	tata box binding protein
TGF	transforming growth factor
TNF	tumour necrosis factor

TPA	12-o-tetradecanoylphorbol 13-acetate
tPA	tissue-type plasminogen activator
TSS	transcription start site
UCP	uncoupling protein
uPA	urokinase-type plasminogen activator
USF	upstream stimulatory factor
VEGF	vascular endothelial growth factor
VSM	vascular smooth muscle
VSMC	vascular smooth muscle cells
WT	wild-type
ZT	zeitgeber time

1. Introduction

1.1. Circadian rhythms

The Earth's rotation around its axis generates environmental cycles, most conspicuously changes between light and darkness. The effects of this 24-hour cycle on diverse organisms have long been noted and studied. The earliest documentation of rhythms in physiology dates back to the 4th century B.C. when a ship's captain, Androsthenes of Thasus, observed and recorded daily movements of the leaves of the tamarind tree (Refinetti, 2005). Around the same time the great physician Hippocrates also noted daily rhythms in the recurrence of fever. It wasn't until 1729 that these rhythms were attributed to an endogenous mechanism when French astronomer Jean-Jacques d'Ortous de Marian placed *Mimosa pudica* plants in constant darkness and noted that the leaves of these plants continued to open during the day and close at night despite the absence of sunlight. His conclusion that these rhythms were an innate property of the plant gave rise to the study of circadian rhythms. The term "circadian", coined by Franz Halberg, comes from the Latin circa, "around", and dies, "day", meaning literally "about a day" and circadian rhythms are defined as those that persist in constant conditions (e.g. constant darkness) and have a period of approximately 24 hours. Maintenance in constant conditions eliminates 'apparent' rhythms that are just a response to external rhythmic cues and demonstrates the endogenous generation of a rhythm. The defining properties of circadian rhythms are shown in Figure 1.1.

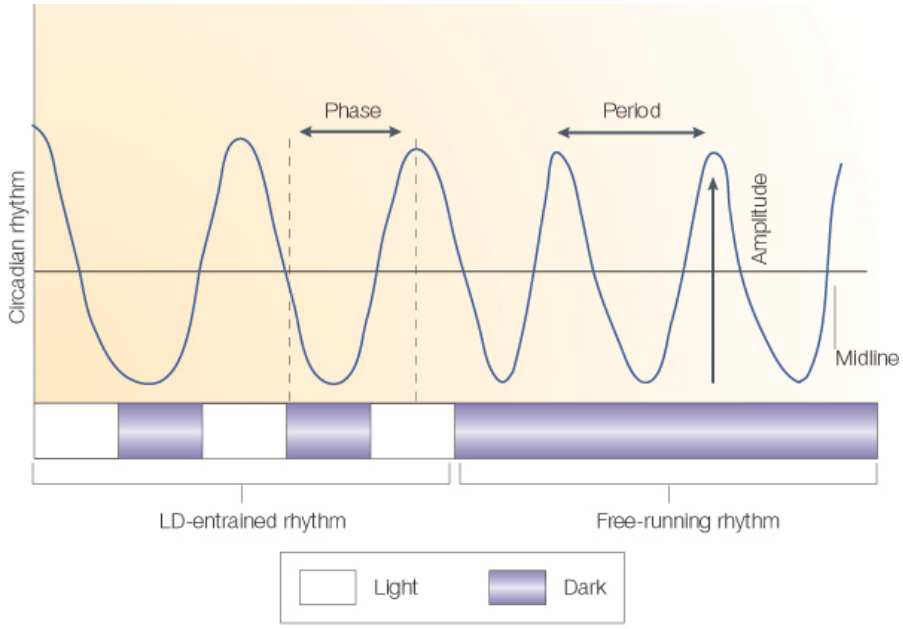


Figure 1.1. Defining properties of circadian rhythms in diverse organisms. Circadian rhythms within an organism, tissue or cell are those which show peak-to-peak intervals, or period, of approximately 24 hours. Rhythms can be entrained to the 24-hour light-dark (LD) cycle but truly circadian rhythms are maintained in the absence of environmental signals (free-running conditions) such as constant darkness. The timings of peaks and troughs in these rhythms can vary from one to another, known as the phase, as can the magnitude between peak and trough, or amplitude (from Bell-Pedersen (2005).

During the 20th century many observations of endogenous circadian rhythms in a variety of species followed, such as temperature and activity in mammals, leaf movement and stomatal opening in plants, asexual spore production in the fungus *Neurospora crassa* and photosynthesis and nitrogen fixation in the cyanobacterium *Synechococcus* (Merrow *et al.*, 2005; Refinetti, 2005). It thus became apparent that nearly all organisms (with the exception of certain prokaryotes) have evolved specific mechanisms to adapt to their rhythmic environments, with environmental cues (called zeitgebers), such as light and temperature, acting as synchronisers. The array of organisms in which circadian rhythms have been studied are shown in Figure 1.2. Much interest was placed on elucidating the underlying mechanisms and a genetic basis to circadian timing, which was first demonstrated in 1971 when Ronald Konopka and Seymour Benzer mapped mutations in three lines of *Drosophila melanogaster* with aberrant circadian behaviour to the same gene, named *period* (Konopka and Benzer, 1971). The *Drosophila period* gene later became the first clock gene to be cloned (Bargiello *et al.*, 1984; Reddy *et al.*, 1984; Zehring *et al.*, 1984), followed by *frequency* in *Neurospora* (Feldman and Hoyle, 1973; McClung *et al.*, 1989). In 1990 Michael Rosbash and his team demonstrated that levels of *period* mRNA, and the resulting PERIOD protein, displayed a 24-hour cycle in wild-type *Drosophila* (Hardin *et al.*, 1990). Around the same time further mutagenesis screening led to the

identification of a second clock gene in *Drosophila* with similar properties to *period*, named *timeless* (Sehgal *et al.*, 1994), and the two protein products of these genes were later found to bind one another and be at the centre of a feedback loop that took 24 hours to complete (Zeng *et al.*, 1996) (see section 1.2.2). This forward genetics approach of isolating circadian mutants and identifying the causative gene led to the identification of additional clock genes in *Drosophila* including the genes *cycle* (Rutila *et al.*, 1998) and *doubletime* (Price *et al.*, 1998).

The first mammalian clock gene, named *Clock*, was identified in the mouse by Joseph Takahashi's group, also using a forward genetics approach (Vitaterna *et al.*, 1994). The same group characterised the *Clock* locus further using two genetic approaches available in the mouse and published the work in two back-to-back papers in Cell in 1997. In one study these authors used positional cloning and subsequent sequencing to show that the mouse *Clock* gene encodes a protein containing a PAS (Period-Arnt-Singleminded) dimerisation domain, similar to that found in *Drosophila* PERIOD, and a basic-helix-loop-helix (bHLH) DNA-binding domain (King *et al.*, 1997). The second study demonstrated *in vivo* complementation, i.e. rescue of an abnormal circadian phenotype, with bacterial artificial chromosomes (BAC) containing the *Clock* gene (Antoch *et al.*, 1997). These techniques together with the investigation of *Clock* transcript expression and evolutionary conservation of the gene demonstrated that *Clock* is an integral part of the mammalian circadian pacemaker system. The first human clock gene, *period2* was discovered in 2001 in people with familial advanced sleep phase syndrome (FASPS) (Toh *et al.*, 2001). In the last 10 years a host of additional clock genes have been identified through mutagenesis screening and molecular genetic screens in model organisms such as humans, rodents, fish, frogs, insects, plants and cyanobacteria (e.g. Young and Kay (2001); Bell-Pedersen *et al*., (2005). Many of these genes are conserved between species but even where sequence or function differs, these genes form the basis of transcriptional-translational feedback negative loops that are able to generate molecular circadian rhythms in all examined organisms (see section 1.2.2) (Dunlap, 1999). As the availability of genetic and biochemical tools increases the mechanisms of clock function, and how molecular rhythms are converted into physiological rhythms can be further investigated, alongside the biological significance of these important conserved mechanisms that confer the advantage of environmental anticipation and adaptation to most organisms.

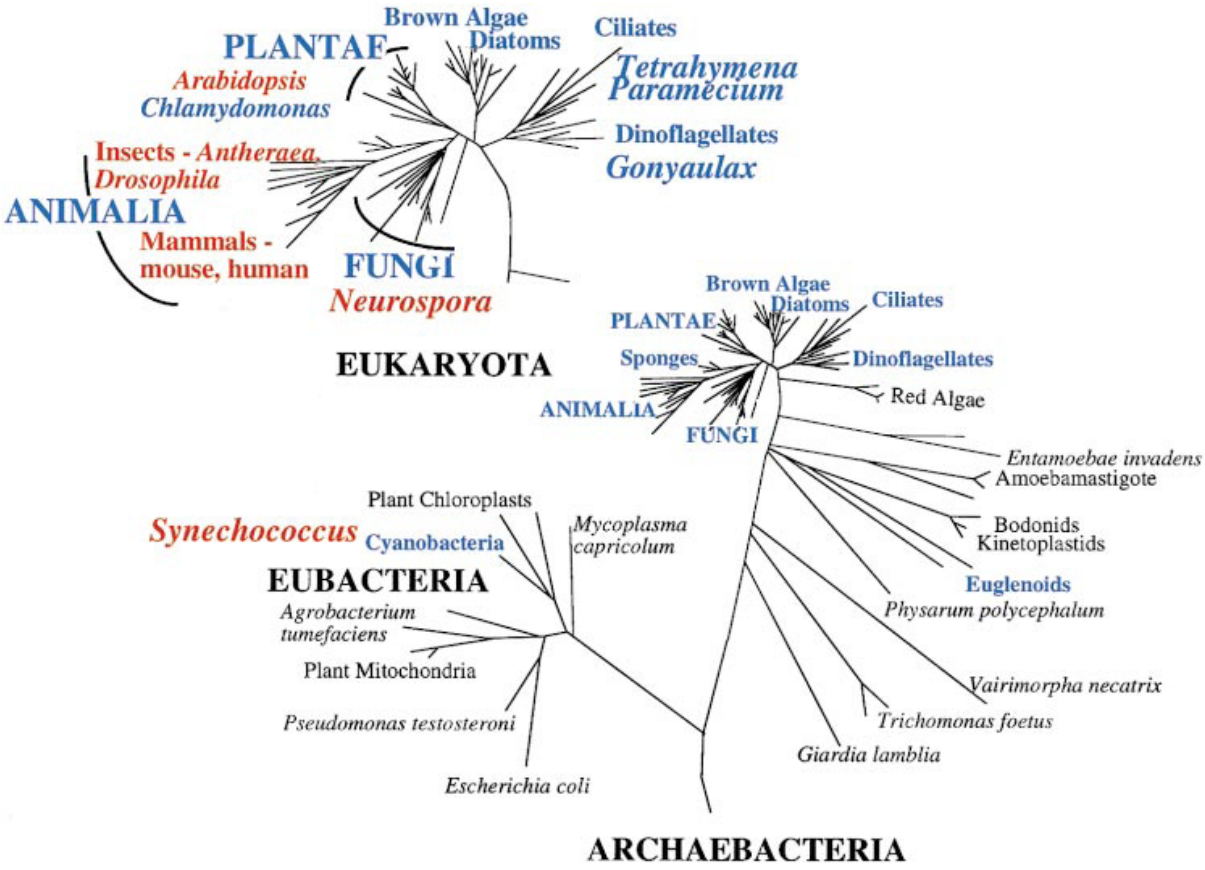


Figure 1.2. Phylogenetic tree demonstrating the existence of circadian rhythms across all phyla. Line lengths correspond to evolutionary distance. Circadian rhythms have been described for phylogenetic groups shown in blue and genetic characterisation or the clock mechanism has progressed significantly for groups shown in red (as of 1999). From Dunlap, (1999).

1.2. The circadian clock

1.2.1 The suprachiasmatic nuclei as a master clock

Daily physiological rhythms are co-ordinated by a 'master' pacemaker, the suprachiasmatic nuclei (SCN), located in the anterior hypothalamus (Klein DC, 1991). The SCN are composed of around 10,000 GABAergic neurons subdivided into a ventral 'core' region that receives direct signals from the retina and brain stem, and a dorsal 'shell' region, which appears to govern output from the SCN pacemaker, driving behavioural and physiological rhythms (Hastings *et al.*, 2007). It is the detection of photic input by the retina and signalling to the SCN via a direct neural projection (the retinohypothalamic tract) that synchronises the central clock to a 24-hour cycle (Moore, 1997). Even in the absence of external stimuli however, the SCN maintain rhythmicity demonstrating the robustness of this central clock: *in vitro* isolated SCN neurons sustain circadian cycles of electrical firing (Liu and Reppert, 2000), cytosolic Ca^{2+} concentrations (Ikeda *et al.*, 2003) and gene expression

(Liu *et al.*, 2007) and circadian gene expression rhythms persist in organotypical SCN slices for many weeks (Maywood *et al.*, 2007). Output signals from the SCN are relayed by neural projections to either endocrine neurons, autonomic neurons of the paraventricular nucleus of the hypothalamus (PVN), other hypothalamic structures or areas outside the hypothalamus (Buijs and Kalsbeek, 2001) (Figure 1.3). Through these pathways the SCN can control endocrine cycles and metabolic pathways by either controlling the sleep-wake cycle, and thus nocturnal secretions of hormones such as prolactin and growth hormones, or by directly driving rhythmic hormone secretion independently of sleep, such as melatonin secretion from the pineal gland and cortisol secretion from the adrenal gland (Hastings *et al.*, 2007). The network of connections with the SCN also enables integration of signals from the periphery, allowing zeitgebers such as feeding cues to modify the central clock (Buijs and Kalsbeek, 2001) (Figure 1.3). A schematic summary of the central role of the SCN in circadian rhythm generation can be seen in Figure 1.4.

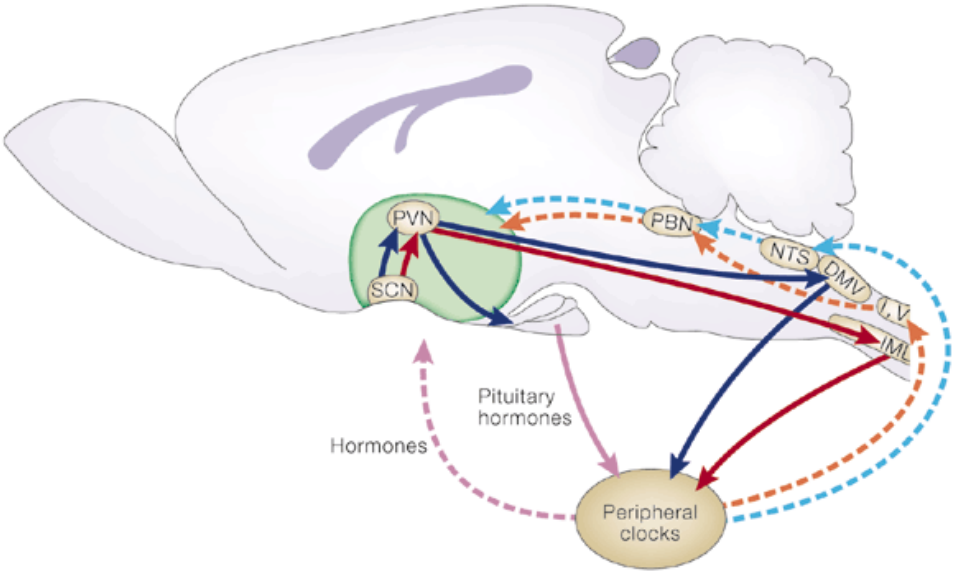


Figure 1.3. Pathways by which the SCN and peripheral clocks might communicate. The hypothalamus (green) is the chief target of both the SCN and the central nervous system. Information from the SCN is translated mainly by the paraventricular nucleus (PVN) into hormonal and autonomic signals (via the dorsal motor nucleus of the vagus (DMV), intermedolateral columns (IML) and pituitary gland) and these parasympathetic and sympathetic signals reach peripheral organs. Sympathetic sensory information from the periphery signals back to the SCN via layers I and V or the dorsal horn, nucleus of the solitary tract (NTS) and parabrachial nucleus (PBN), and vagal sensory information directly via the NTS and PBN. These connections provide the hypothalamus with information to integrate light-dark signals from the visual system and metabolic signals from peripheral organs. Figure from Buijs and Kalsbeek (2001).

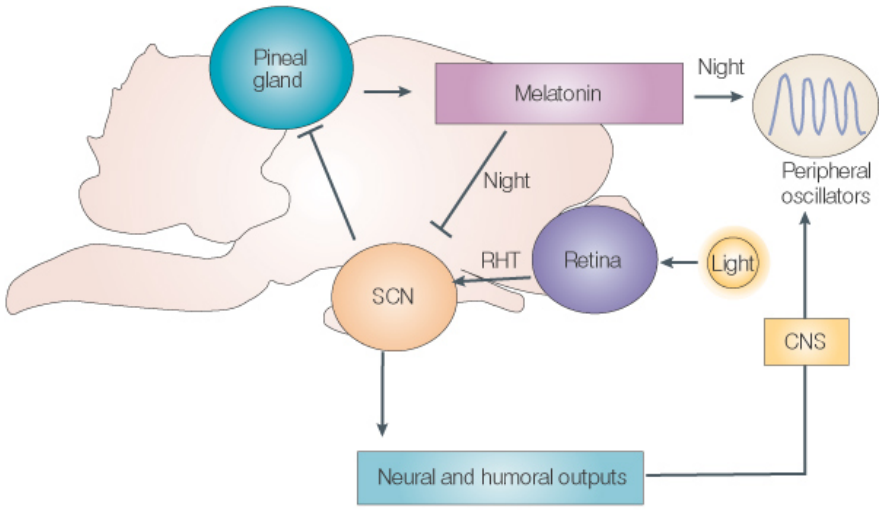


Figure 1.4. A summary of the co-ordinating role of the SCN. The SCN receives light signals from the retina through the retinohypothalamic tract (RHT) and transduces these signals into neural and hormonal outputs via other brain areas and hormone-secreting organs (see text). Circadian rhythms result from the effects of these outputs on the central nervous system (CNS) and direct effects on peripheral tissues. For example, the rhythmic secretion of melatonin from the pineal gland is controlled by signals from the SCN and regulates the sleep-wake cycle. Melatonin also feeds back to inhibit night-time SCN activity through the melatonin receptors expressed in the SCN. Figure from Bell-Pedersen *et al*., (2005).

1.2.2 Circadian transcriptional feedback loops

The SCN neurons can maintain a rhythm because they each possess a set of genes involved in positive and negative feedback loops that drive rhythms in the RNA and protein levels of key clock components (Reppert and Weaver, 2001). Two transcription factors of the basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Singleminded) domain family, Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-Like protein 1 (BMAL1/ARNTL/MOP3) drive the system. CLOCK:BMAL1 heterodimers activate transcription by binding to E-box enhancer elements and are highly selective for those containing the canonical nucleotide sequence *CACGTG*. Particularly, these CLOCK:BMAL1 heterodimers activate the rhythmic transcription of the *Period* genes (*Per1-Per3*) and *Cryptochrome* genes (*Cry1* and *Cry2*) (Figure 1.5). The resultant proteins peak approximately four hours after the mRNA and oligomerise and translocate back into the nucleus where they accumulate and interact with CLOCK:BMAL1 and repress their own transcription (along with other E-box containing genes). The importance of BMAL1 in this loop can be seen by the severe disruption of behavioural and molecular rhythms in *Bmal1*$^{-/-}$ knockout mice (Bunger *et al.*, 2000). PER1 and

PER2, and CRY1 and CRY2 on the other hand appear to compensate for each other to some degree as mutation of both *Per* or *Cry* genes is required to cause arrhythmicity. The individual mutants do display some changes in rhythm period however so the PERs and CRYs cannot completely compensate for each other (Ko and Takahashi, 2006).

The core molecular loop is augmented and stabilised by ancillary loops (Figure 1.5), the most prominent involving two orphan nuclear receptor proteins, REV-ERBα (NR1D1) and RORα (retinoic acid receptor-related orphan receptor α). CLOCK:BMAL1 activates the rhythmic transcription of these genes in phase with the *Per* and *Cry* genes and the resulting products antagonistically regulate the *Bmal1* promoter by repression (REV-ERBα) or activation (RORα) via two REV-ERB/ROR response elements (RORE) (Preitner *et al.*, 2002; Sato *et al.*, 2004c; Triqueneaux *et al.*, 2004; Akashi and Takumi, 2005; Guillaumond *et al.*, 2005). This antagonistic effect where activation by RORα is coupled to lack of repression when REV-ERBα levels are low maintains a robust circadian rhythm of *Bmal1* expression and provides a link between the positive and negative limbs of the clock.

Additional factors that are regulated by the core clock loop and may feedback into the system are the antagonistic transcription factors DBP and E4BP4 and the bHLH factors DEC1 (*Stra13*/*Sharp2*/*BHLHB2*) and DEC2 (*Sharp1*/*BHLHB3*). DBP (D-element binding protein) and E4BP4 (adenovirus E4 promoter ATF site binding protein 4), are basic leucine zipper transcription factors that are rhythmically expressed in anti-phase at the mRNA and protein level and activate or repress the same D-box element respectively (Mueller *et al.*, 1990; Mitsui *et al.*, 2001). *Dbp* mutant mice have shortened activity cycles (Lopez-Molina *et al.*, 1997) and DBP can activate the mouse *Per1* promoter (Yamaguchi *et al.*, 2000) so may influence the core feedback loop. *Per1*-3 and *Rorα* promoters contain D-boxes (Ueda *et al.*, 2005) and the antagonistic effect of DBP and E4BP4 on these promoters may help maintain the robust and high-amplitude periodicity of the clock. *Dbp* has a robust circadian expression rhythm in the SCN and peripheral tissues (Lopez-Molina *et al.*, 1997) and, like DEC1 and 2, is likely to play a more important role in transducing circadian regulation to genes that may not possess proximal E-boxes. DBP has been shown to regulates the rhythmic expression of key enzymes in the liver (Lavery *et al.*, 1999) but no DBP targets have yet been identified in the heart.

DEC1 (Differentially Expressed in Chondrocytes 1) and DEC2 can inhibit CLOCK:BMAL1-induced activation through competitive E-box-binding and can bind BMAL1 directly (Honma *et al.*, 2002; Sato *et al.*, 2004a), although this interaction is not required for repression (Li *et al.*, 2004). The evidence that DEC1 plays a role in strengthening and fine-tuning the core feedback loop is supported by recent findings that *Dec1* over-expression or depletion can delay or advance the phase of E-box-containing clock genes respectively (Nakashima *et al.*, 2008). Interestingly *Per2* and *Cry1* expression was unaffected as these genes do not contain canonical E-boxes (and are instead believed to be activated via non-canonical E-box elements, see Chapter 6 (section 6.1), demonstrating that the

targets of DEC1 are limited to canonical E-box-containing genes. Although DEC1 and DEC2 may play a role in stabilising the core feedback loop their primary role is likely to be as transducers of clock output as a number of genes are regulated by DEC1 and *Dec1*$^{-/-}$ knock-out mice retain normal circadian function in the liver (Grechez-Cassiau *et al.*, 2004). *Dec1* is up-regulated by multiple stimuli such as retinoic acid (Boudjelal *et al.*, 1997), transforming growth factor-β (Shen *et al.*, 2001) and hypoxia (Miyazaki *et al.*, 2002), and may therefore add a circadian aspect to gene regulation by these stimuli.

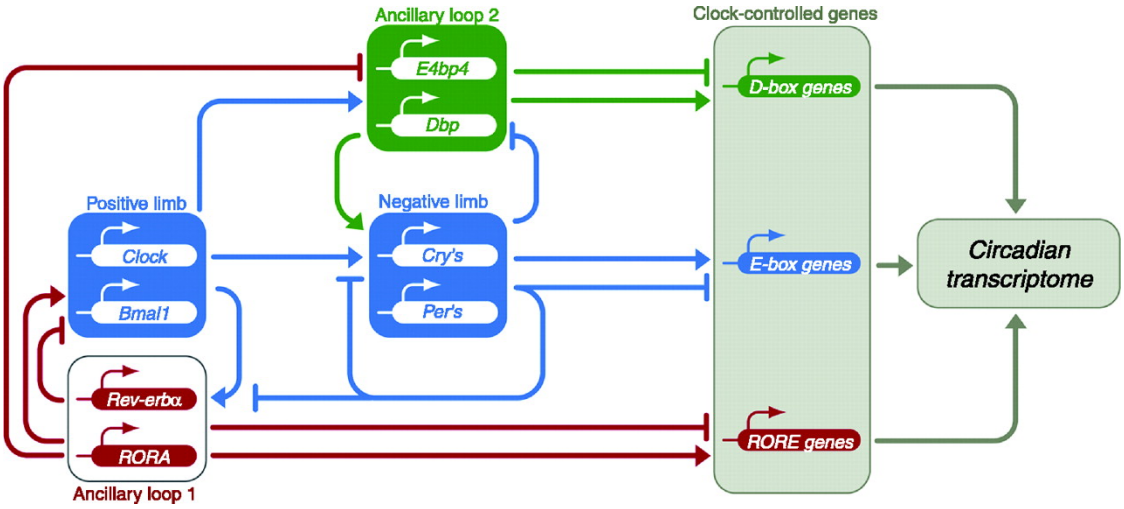


Figure 1.5. The molecular feedback loops of the mammalian circadian system. Schematic representation of the feedback loops that make up the molecular clock. The core loop (blue lines) consists of a positive limb driven by CLOCK and BMAL1 dependent activation of the *Per* and *Cry* genes, via E-boxes in their promoters, and a negative limb where PER and CRY proteins translocate back into the nucleus and inhibit their own expression, and activation of other E-box-containing genes. This core loop is stabilised by an ancillary loop involving the antagonistic factors REV-ERBα and RORα (red lines), which are rhythmically expressed through CLOCK/BMAL1 and PER/CRY complex interaction with their E-boxes. REV-ERBα and RORα in turn regulate the rhythmic expression of *Bmal1* through binding to the RRE sequence. A second loop that is regulated by the core loop and feeds back into the system involves DBP and E4BP4 (green lines). *Dbp* and *E4bp4* are also regulated by CLOCK/BMAL1 and REV-ERBα and RORα and can go on to regulated the *Per* genes and *Rev-erbα* via binding to the D-box sequence. These loops give rise to different expression phases of clock genes and maintain robustness. The rhythmically expressed clock factors proceed to regulate the rhythmic expression of genes that carry E-box, D-box or RRE sequences. These primary clock-controlled genes (CCGs) will in turn regulated the rhythmic expression of secondary CCGs, ultimately orchestrating a circadian transcriptome that gives rise to physiological rhythms. Figure from Hastings *et al.*, (2007).

The amplitude, phase and period of the core circadian feedback loop may be further stabilised by adenosine 3',5'-monophosphate (cAMP) signalling in the SCN, which is itself regulated by the clock, representing a feed-forward mechanism (O'Neill *et al.*, 2008). The clock is also regulated by post-transcriptional and post-translational mechanisms that significantly contribute to the precision and stability of the feedback loop (Reppert and Weaver, 2002; Harms *et al.*, 2004; Dardente and Cermakian, 2007). Phosphorylation may be an important regulatory mechanism as CLOCK, BMAL1, PER1 and PER2 undergo temporal changes in phosphorylation *in vivo*. Two of the kinases involved in phosphorylating PER1-3, CRY1-2 and BMAL1 have been identified as Casein Kinase I δ (CKIδ) and CKIε (Akashi *et al.*, 2002; Eide *et al.*, 2002; Eide *et al.*, 2005). Phosphorylation of the PER proteins by CKIδ/ε promotes their degradation via the ubiquitin proteasome pathway and regulates their translocation to the nucleus (Vielhaber *et al.*, 2000; Akashi *et al.*, 2002; Takano *et al.*, 2004). PER2 and CRY2 are also phosphorylated by glycogen synthase kinase-3 (GSK-3) (Harada *et al.*, 2005; Iitaka *et al.*, 2005), which again promotes nuclear entry/nuclear retention of PER2 and CRY2 and degradation of CRY2 (Harada *et al.*, 2005). CRY1, CRY2 and BMAL1 can also be phosphorylated by mitogen-activated protein kinase (Sanada *et al.*, 2002; Sanada *et al.*, 2004). The importance of phosphorylation status in transactivational ability or nuclear localisation is implied by the observation that transcriptional activation by mCLOCK and mBMAL1 occurs when they are least abundant in the nucleus (Lee *et al.*, 2001). Mutations in *CKIδ* and *CKIε* also alter the circadian period in mammals (Lowrey *et al.*, 2000; Gallego *et al.*, 2006) and have been implicated in familial advanced sleep phase syndrome (FASPS) in humans (Toh *et al.*, 2001; Xu *et al.*, 2005), further demonstrating the importance of phosphorylation in maintaining 24-hour rhythms.

Further modifications of the core clock proteins are also likely to regulate the feedback loops as BMAL1 undergoes rhythmic SUMOylation – the covalent linking of a small ubiquitin-related modifier protein (SUMO) to lysine residues (Cardone *et al.*, 2005). This modification may regulate the nuclear localisation and degradation of BMAL1 via the ubiquitin proteasome pathway (Lee *et al.*, 2008).

The importance of chromatin remodelling in circadian regulation is also becoming increasingly apparent, particularly following the discovery that CLOCK is a histone acetyltransferase (HAT) (Doi *et al.*, 2006) and that *Per1*, *Per2*, *Cry1* and *Dbp* promoters are acetylated at histone H3 when being actively transcribed (Etchegaray *et al.*, 2003; Curtis *et al.*, 2004; Naruse *et al.*, 2004). BMAL1 and PER2 proteins are also rhythmically acetylated (Hirayama *et al.*, 2007; Asher *et al.*, 2008). The HAT activity of CLOCK may be counteracted by the histone deacetylase (HDAC), SIRT1 (Asher *et al.*, 2008; Nakahata *et al.*, 2008a). SIRT1 is dependent on the energy stored in nicotinamide adenine dinucleotide (NAD^+) to catalyse the removal of the acetyl group from histones and may present a link between the core clock loop and cellular metabolism. The current understanding of circadian rhythms in histone acetylation and deacetylation is summarised in Figure 1.6.

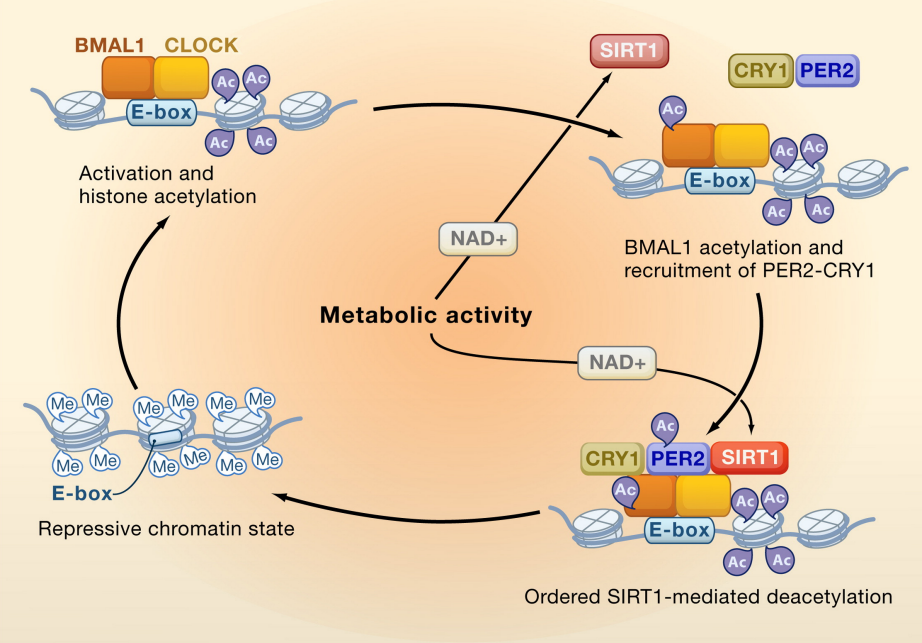


Figure 1.6. Circadian rhythms in histone acetylation and deacetylation. During activation of CCGs CLOCK:BMAL1 heterodimers are associated with the E-box element and CLOCK mediates acetylation of the tails of histones H3 and H4 (Doi *et al.*, 2006). BMAL1 also becomes acetylated, which may stabilise it (Nakahata *et al.*, 2008a) and aid recruitment of PER2 (Hirayama *et al.*, 2007) (which also becomes acetylated) and CRY1, ending the activation phase. NAD+-dependent deacetylation by SIRT1 of histones, BMAL1 and PER2 facilitates the re-establishment of a repressive chromatin state. Figure from Belden and Dunlap, (2008).

While post-translational mechanisms affecting the clock are increasingly coming to light, post-transcriptional mechanisms are also likely to play a role and the balance between mRNA synthesis and degradation appears to be important in the generation of circadian rhythms (Shu and Hong-Hui, 2004). In particular, inhibition of gene expression by microRNAs (miRNAs) may fine-tune the period of the clock and be involved in light-induced phase-resetting (O'Neill and Hastings, 2007). This has been proposed following the discovery of two brain-specific miRNAs, *miR-132* and *miR-219*, that are rhythmically expressed in the SCN, one of which is also induced by light (Cheng *et al.*, 2007). These miRNAs augmented *Per1* transactivation *in vitro* and have a number of predicted targets that may regulate the circadian expression of novel target genes as well as the core clock.

1.3. Intrinsic clocks in peripheral organs

As well as being expressed in the SCN, clock genes exhibit robust cyclic expression in many peripheral tissues (Reppert and Weaver, 2001; Balsalobre, 2002). This was initially thought to be passively driven by signals from the SCN (e.g. behavioural and endocrine rhythms) but the

demonstration of persistent circadian expression of clock genes in cultured mammalian fibroblasts following a serum shock established the existence of an intracellular clock mechanism (Balsalobre *et al.*, 1998). The existence of intrinsic clocks in peripheral tissues *in vivo* was demonstrated by real-time monitoring of cultured tissues (liver, lung, kidney, skeletal muscle and extra-SCN brain regions) from bioluminescent reporter animals [the *Per1 promoter:luciferase* rat (Yamazaki *et al.*, 2000) and mouse (Wilsbacher *et al.*, 2002) and PER2::LUCIFERASE fusion protein mouse (Yoo *et al.*, 2004)]. In such experiments the bioluminescence rhythms in peripheral tissue cultures dampen over time, whereas rhythms from SCN cultures are sustained indefinitely, implying that the peripheral clockwork is less robust (Hastings *et al.*, 2007). However, single cell imaging of fibroblasts in culture has shown that the apparent loss in bioluminescence results from cells drifting out of phase with one another, although maintaining high-amplitude rhythms (Nagoshi *et al.*, 2004; Welsh *et al.*, 2004). SCN neurons on the other hand are able to communicate with one another and maintain synchrony (see section 1.2.1), leading to the view that the SCN clock is the master synchroniser of peripheral oscillators. In peripheral tissues clock genes expression rhythms tend to peak 4-8 hours later than in the SCN (Hastings *et al.*, 2003), which reflects this relationship.

In addition to the distinct phase distribution of CCG expression in peripheral oscillators, experiments with PER2::LUCIFERASE transgenic mice also showed tissue specific differences in phase and period of bioluminescence (Yoo *et al.*, 2004). This may be due to the utilisation of different clock genes, such as the *Bmal1* paralogue *Bmal2*. Like BMAL1, BMAL2 (ARNTL2/MOP9/CLIF) can bind to CLOCK and form transcriptionally active heterodimers (Hogenesch *et al.*, 2000; Maemura *et al.*, 2000). The two factors are likely to play different roles in clock regulation as their expression patterns differ. *Bmal1* appears to be more abundantly and ubiquitously expressed throughout the body, with high levels in the human and murine brain, skeletal muscle and heart (Ikeda and Nomura, 1997; Yu *et al.*, 1999) as well as in the human liver and kidney and most tissues to some extent (Maemura *et al.*, 2000). The expression profile of *Bmal2* is less defined but appears to be less abundant and more specific with highest levels being detected in murine and human brain and placenta (Hogenesch *et al.*, 2000; Maemura *et al.*, 2000) and varying levels in other human tissues, such as the heart, lungs, kidneys, liver, skeletal muscle and endothelial cells (Maemura *et al.*, 2000; Schoenhard *et al.*, 2002). Whether *Bmal2* expression cycles is unclear but it has been observed that activation of an E-box-containing reporter by BMAL2:CLOCK was almost four times higher than BMAL1:CLOCK (Hogenesch *et al.*, 2000; Chong *et al.*, 2006), suggesting that BMAL1 and BMAL2 fulfil separate temporal-spatial roles in circadian regulation.

Peripheral clocks may be further regulated by tissue-specific expression of clock-regulated genes such as *Dec1* and *Dec2*. *Dec1* appears to be expressed in most tissues whereas *Dec2* expression is most

abundant in the rodent central nervous system and skeletal muscle, also detectable in the heart and lung tissue, but absent in the liver and kidney (Rossner *et al.*, 1997; Fujimoto *et al.*, 2001).

In mammals peripheral clocks are unable to detect light and are reliant on signals from the SCN to entrain them to external day-night rhythms and each other. The role of the SCN as a phase co-ordinator is evident in SCN-ablated mice where *Per2* expression rhythms in peripheral tissues become progressively desynchronised (Sakamoto *et al.*, 1998; Yoo *et al.*, 2004). However, how the SCN signals to the peripheral clocks is not fully understood and a combination of neural and hormonal mechanisms is believed to be involved (see section 1.2.1). In particular, the importance of hormonal mechanisms was demonstrated in an experiment where encapsulated intact SCN (with neuronal contacts eliminated) transplanted into SCN-ablated hamsters were able to rescue circadian rhythms via humoral signals (Silver *et al.*, 1996). Circadian gene expression has also been observed in circulating mononuclear leukocytes, confirming that peripheral clocks must be synchronised by hormonal mechanisms (Oishi *et al.*, 1998). A slower hormonal mechanism also explains the time-lag in clock gene expression between the SCN and peripheral clocks (Hastings *et al.*, 2003).

The identity of the humoral synchronising factors involved is not currently known but several observations suggest that glucocorticoid signalling is important. In particular, the glucocorticoid analogue dexamethasone (DEX) can induce circadian gene expression in rat-1 fibroblasts and phase-shifted expression in liver, kidney and heart, without affecting SCN neurones, which lack the glucocorticoid receptor (Balsalobre *et al.*, 2000a). Some other tissues were unaffected, adding a degree of tissue-specific control. Glucocorticoids are themselves secreted rhythmically by the adrenal gland (Nelson, 1972) and appear to be able to counteract other resetting signals, such as feeding, possibly preventing inappropriate uncoupling of organ clocks (Le Minh *et al.*, 2001). *Rev-erbα* expression is inhibited by glucocorticoids (Torra *et al.*, 2000) representing a possible mechanism whereby glucocorticoids can signal to the core clock mechanism. In fact, treatment of SCN-ablated mice with DEX activated approximately 60% of all cycling genes in the liver, including genes that do not contain the glucocorticoid response element (GRE) (Reddy *et al.*, 2007).

Glucocorticoids are likely to be important but cannot be the only hormones involved in synchronising peripheral clocks since disruption of the glucocorticoid receptor in mice did not affect circadian gene expression in the periphery (Balsalobre *et al.*, 2000a). Other compounds have been shown to activate the expression of some circadian genes, including forskolin, adenylate cyclase agonists (which increase cAMP production), the phorbol ester TPA (12-o-tetradecanoylphorbol 13-acetate), growth factors (that activate protein kinase C and MAP kinases), calcimycin (which increases the intracytoplasmic Ca^{2+} concentration), and retinoic acid, which is also a critical regulator of cardiac growth (Olson and Schneider, 2003). Nuclear hormones may also have a role in entrainment since the retinoid X receptor alpha (RXRα) and retinoic acid receptor alpha (RARα), have been shown to

negatively regulate CLOCK:BMAL1 and MOP4:BMAL1-mediated clock gene activation in the vasculature in a hormone-dependent manner (MOP4/NPAS2 is a paralogue of CLOCK that also binds to BMAL1) (McNamara *et al.*, 2001). Retinoic acid phase-delayed *Per2* oscillations, whereas DEX phased-advanced *Per2* in the vasculature, suggesting that glucocorticoids and retinoids may have opposing effects and use different mechanisms to reset peripheral clocks.

External signals can also entrain some peripheral clocks independently of the SCN. For example, using a restricted feeding pattern, Stokkan and colleagues (2001) reported a shift of the liver clock by 10 hours within 2 days, whereas the SCN rhythm remained phase-locked to the light-dark cycle. Some resetting also occurred in the lung clock but at a slower rate, implying that resetting is autonomous among peripheral tissue. However, feeding is not essential for phase resetting in the mouse heart clock (Sakamoto *et al.*, 2004). There are likely to be different types of signals by which feeding can reset peripheral clocks, such as food metabolites, food-induced metabolites, and hormones whose secretion is controlled by feeding (Schibler *et al.*, 2003). Recent work has shown that the redox state of two electron carriers, nicotinamide adenine dinucleotide (NAD) and phosphorylated NAD (NADP) influences the DNA-binding activity of CLOCK:BMAL1 and MOP4:BMAL1 heterodimers (Rutter *et al.*, 2001). Since the NAD/NADH and NADP/NADPH ratios are affected by cellular metabolism, metabolic changes caused by feeding may reset peripheral clocks. Feeding can also alter core body temperature patterns, as can the SCN and locomotor activity in rodents, and these small temperature changes can also phase shift peripheral clocks (Schibler *et al.*, 2003).

1.4. The circadian transcriptome

1.4.1 Clock controlled genes

Dbp, *Rev-erbα* and other E-box-containing genes are primary clock-controlled genes (CCG), genes that are directly regulated by the core clock transcription factors resulting in rhythmic expression. Many primary CCGs, such as these, are also transcription factors or other signalling molecules that can rhythmically control the expression and/or function of other factors, which can then go on to influence others in a 'circadian cascade'. An example of such a primary CCG is *HNF-4α* in the liver, which regulates many metabolic pathways and a large proportion of the liver transcriptome and is also strongly rhythmically expressed (Reddy *et al.*, 2007). The primary CCGs are likely to exhibit the most robust circadian rhythms, being under direct control from the core clock, whereas the further downstream CCGs will probably be under the influence of more factors and pathways and may have altered expression rhythms under different conditions (Figure 1.7). Certain rhythmic stimuli could however augment expression rhythms, depending on the phase of stimuli and gene expression. Such a regulatory cascade enables the transduction of co-ordination signals from the SCN into local intracellular rhythms influencing a variety of processes, via the core clock mechanism in peripheral

tissues. It also allows the possibility of input from other signals and pathways, increasing adaptability of the system.

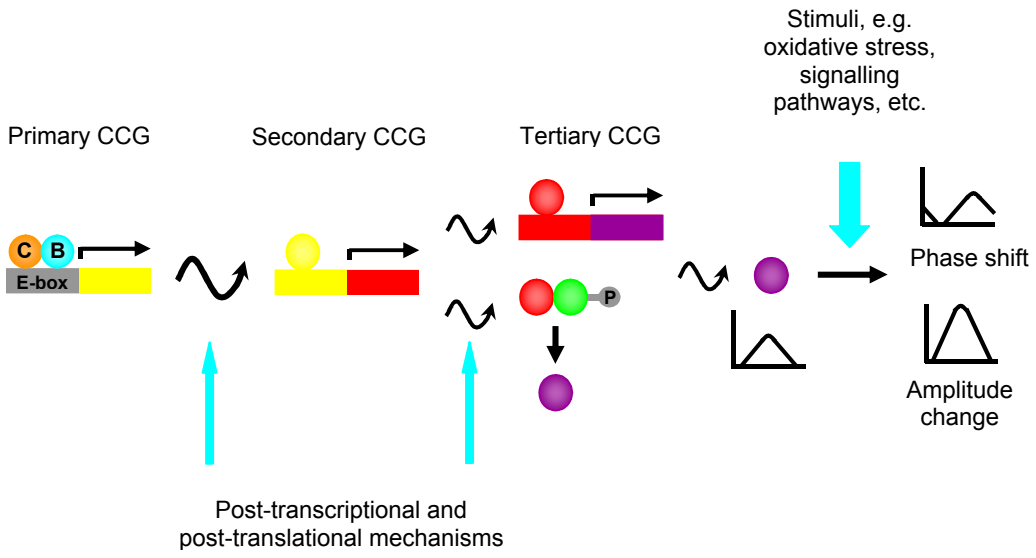


Figure 1.7. An example of circadian rhythm transduction through a hierarchy of CCGs. Binding of the clock factors CLOCK (C) and BMAL1 (B) to E-box element-containing gene promoters (grey) initiates the rhythmic expression of primary clock controlled genes (CCG; yellow). If the rhythmically expressed products of the primary CCGs are transcription factors (yellow oval), protein modifiers (e.g. kinases) or influence gene expression or protein function in other ways the initial circadian expression rhythm can be passed on to secondary and tertiary CCGs, etc. This hierarchy results in the transduction of rhythmic expression and/or function to non-E-box-containing genes (red, purple) and their products. Each stage of the hierarchy can potentially be regulated by additional circadian and non-circadian post-transcriptional and post-translational mechanisms (thin aqua arrows) and be influenced by an array of signalling pathways and stimuli (thick aqua arrow), possibly resulting in altered output expression or physiological rhythms.

1.4.2 Diverse roles of tissue-specific CCGs

Recent microarray studies have identified genes that are rhythmically expressed in numerous rodent tissues (SCN, liver, heart, kidney, skeletal muscle and adipose tissue) (Akhtar *et al.*, 2002; Panda *et al.*, 2002; Storch *et al.*, 2002; Oishi *et al.*, 2003; Ptitsyn *et al.*, 2006; Zvonic *et al.*, 2006; McCarthy *et al.*, 2007; Miller *et al.*, 2007) and immortalised fibroblasts (Grundschober *et al.*, 2001; Duffield *et al.*, 2002). Between 2 and 10% of the genes that where probed in the earlier microarrays exhibited cycling patterns, although due to the sensitivity of the microarrays and instability of circadian transcripts this number was likely an underestimate and recent analyses suggest that over 20% of the transcriptome (Ptitsyn *et al.*, 2006) and proteome (Reddy *et al.*, 2006) is clock-regulated. Circadian transcriptome

profiles may be tissue-specific as shown by the study of the heart and liver where only 37 transcripts, approximately 10% of the total cycling transcripts, were common to the two tissues, the majority of these being part of the core transcriptional loops (Storch *et al.*, 2002) (Figure 1.8). However, even though the genes differ, some are involved in regulating similar pathways in the heart and liver and the different profiles may reflect response to different synchronising factors. The newly identified CCGs belonged to a broad spectrum of functional groups, demonstrating that the clock influences a variety of cellular processes. In serum stimulated rat-1 fibroblasts the identified CCGs are predominantly associated with transcriptional control, intracellular signalling, protein turnover, cell movement and cell cycle/apoptosis, or were heat shock proteins, chaperones and enzymes (Duffield, 2003). In the liver a number CCGs are involved in metabolism and biosynthesis. Many of these gene products are rate-limiting enzymes thus whole biochemical pathways may be under circadian control, further demonstrating the importance of the clock.

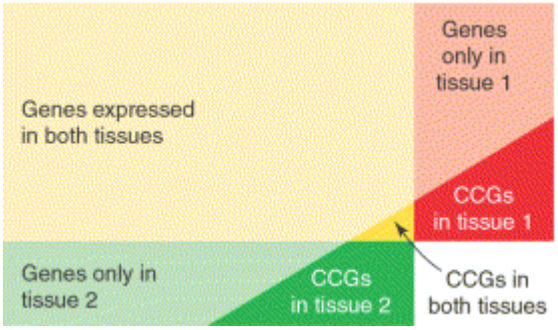


Figure 1.8. A Venn diagram of gene expression in two different tissues. Approximately 80% of genes expressed in tissue 1 (red) are also expressed in tissue 2 (green), shown by the yellow overlap. At least 10% of the genes in tissue 1 or tissue 2 are likely to be clock-controlled genes (CCGs; darker colours) but most of these appear to be tissue specific with only a 10% overlap of CCGs, based on data from microarray studies of the liver (Panda *et al.*, 2002) and heart (Storch *et al.*, 2002). Figure from Morse and Sassone-Corsi (2002).

The microarray studies have also shown that the CCG transcripts accumulate at many different times throughout the 24 hour cycle (Akhtar *et al.*, 2002; Duffield *et al.*, 2002; Panda *et al.*, 2002; Storch *et al.*, 2002) and each tissue had a distinct phase distribution pattern. In the liver all phases were represented but almost all the CCGs peaked in the morning in the heart and preceding dawn and dusk in the SCN, with some peaking immediately after the day/night transition. This pattern in SCN gene expression may enable the clock to anticipate day and night, one of the key characteristics of the clock. The variation in phases of CCG expression suggests that different cellular functions must be performed at different times and the existence of peripheral clocks enables this to happen without requiring a very large number of signals from the SCN. Sequestering chemically incompatible reactions to different time windows may also optimise cellular physiology (Schibler *et al.*, 2003).

1.5. The importance of the clock in physiology

The most obvious circadian rhythm in humans and other mammals is the cycle of sleep and wakefulness. During the circadian day the body functions catabolically to cope with various events and physical demands whereas during the night anabolic functions such as growth and repair take over (Hastings *et al.*, 2003). As the evening approaches the preparation for sleep can be seen by a decrease in body temperature and an increase in the synthesis of the pineal hormone melatonin (Figure 1.9). Circulating mediators such as growth hormones are secreted during the night and before dawn the secretion of adrenocorticoids, such as serotonin, is activated to prepare the body for waking. The changes associated with this 'biological clock' have evolved over time to allow organisms to better adapt to their environment and prosper.

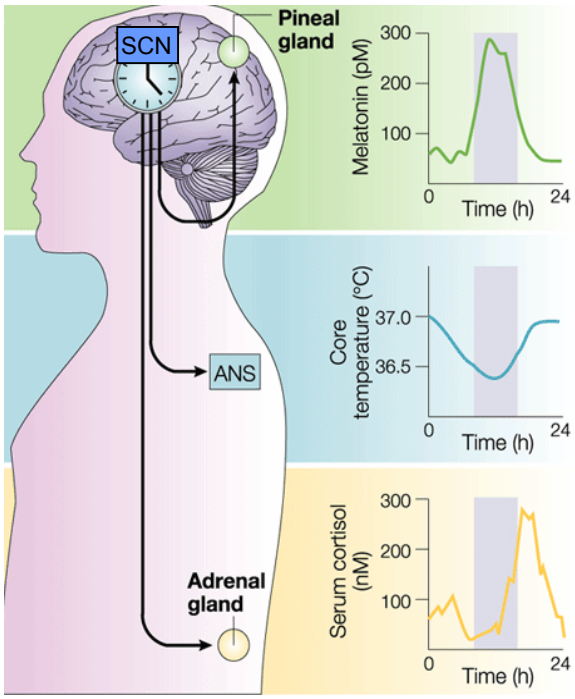


Figure 1.9. Physiological circadian rhythms. Co-ordination of physiological rhythms, such as body temperature and hormone levels, through neural and endocrine links from the suprachiasmatic nuclei (SCN) ensures that the sleep-wake cycle and metabolism is adjusted accordingly to anticipate the demands of a 24-hours day. ANS, autonomic nervous system (adapted from Hastings *et al.*, 2003).

The importance of the biological clock becomes more apparent when the sleep-wake cycle is no longer synchronised with the internal rhythms. This is an increasingly common occurrence among humans in the emerging 24-hour society as the sleep-wake pattern is altered by social factors such as shift work and jet lag. The immediate effects of such 'circadian stress' on fatigue and alertness have obvious implications for safety in shift-workers but there are also long-term problems including an increased risk of chronic illness such as cardiovascular disease (CVD), hypertension, metabolic

syndrome, gastrointestinal disturbances and cancer (Knutsson, 2003; Schernhammer *et al.*, 2003; Oishi *et al.*, 2005d; Schernhammer *et al.*, 2006; Sookoian *et al.*, 2007) and poor sleep patterns can lead to metabolic problems, increasing the risk of obesity and diabetes (Knutson *et al.*, 2007; Van Cauter *et al.*, 2007).

In humans, mutations in the *Per2/3*, *CK1δ/ε* and *Clock* genes have been associated with altered sleep patterns, such as Familial Advanced Sleep Phase Syndrome (FASPS) (Ebisawa, 2007). Individuals suffering from FASPS display advances in sleep onset and wakening, core body temperature and melatonin secretion rhythms (Jones *et al.*, 1999). Disrupted circadian rhythms are also strongly associated with mood disorders and diseases such as major depressive disorder and bipolar disorder, which display abnormal sleep/wake, appetite and social rhythms (Boivin, 2000; Bunney and Bunney, 2000; McClung, 2007). In addition, impaired circadian function is associated with poor prognosis in cancer patients (Levi, 2001) and increased tumour progression in mice (Filipski *et al.*, 2002; Filipski *et al.*, 2004). In fact, mutation of *Per2* renders mice more susceptible to spontaneous and irradiation-induced tumours (Fu *et al.*, 2002) whilst alterations in *Per1* expression levels can alter the rate of apoptosis in human cancer cells (Gery *et al.*, 2006). These mutations demonstrate direct physiological effects of clock disruption and it will be interesting to investigate whether humans with such disorders display other pathologies, such as CVD.

1.5.1 Circadian variations in cardiovascular function and dysfunction

The discovery that 10% or more of the transcriptome may be clock regulated and that expression of most cardiac CCGs peaks in the morning (Storch *et al.*, 2002) implies that circadian orchestration plays an important role in maintaining optimal cardiac function in response to the external environment. In the cardiovascular system (CVS), circadian variations in blood pressure, heart rate and cardiac output have been documented, with increased cardiac activity in the morning in preparation for activity. Blood pressure shows a strong circadian rhythm with a drop at night and peak pressure in the morning, with the difference between peak and trough pressure being higher hypertensive patients (Millar-Craig *et al.*, 1978; Panza *et al.*, 1991). The morning peak in blood pressure depends on several factors, including changes in cardiac output and vascular smooth muscle (VSM) tone (McNamara *et al.*, 2001). Heart rate also show circadian variations and studies in rats showed a peak in heart rate during the subjective day (darkness) that was not caused by activity and was lost with SCN-ablation and reduced by light exposure (Scheer *et al.*, 2001). A circadian rhythm in resting heart rate in humans has also been shown in darkness, which was increased by light depending on the phase of the day-night cycle and the intensity of the light (Scheer *et al.*, 1999). Clock gene mutant mice models show that both the circadian variations in blood pressure and heart rate are dependent on *Bmal1* and *Clock* expression (Curtis *et al.*, 2007).

Whilst these rhythms have evolved to maintain optimal function they can also render the CVS more susceptible to cardiovascular events, the occurrence of which has increased in modern society. The relative risks of myocardial infarction (MI) and sudden cardiac death are increased by 40% and 30% respectively in the morning and about 9% and 7% of the total cases are due to this circadian surge (Cohen *et al.*, 1997). Myocardial ischemia and haemorrhagic stroke (Casetta *et al.*, 2002) also show pronounced morning peaks. Figure 1.10 summarises how local tissue clocks can combine through the use of different clock components and regulation of CCGs to influence cardiovascular physiology and pathology.

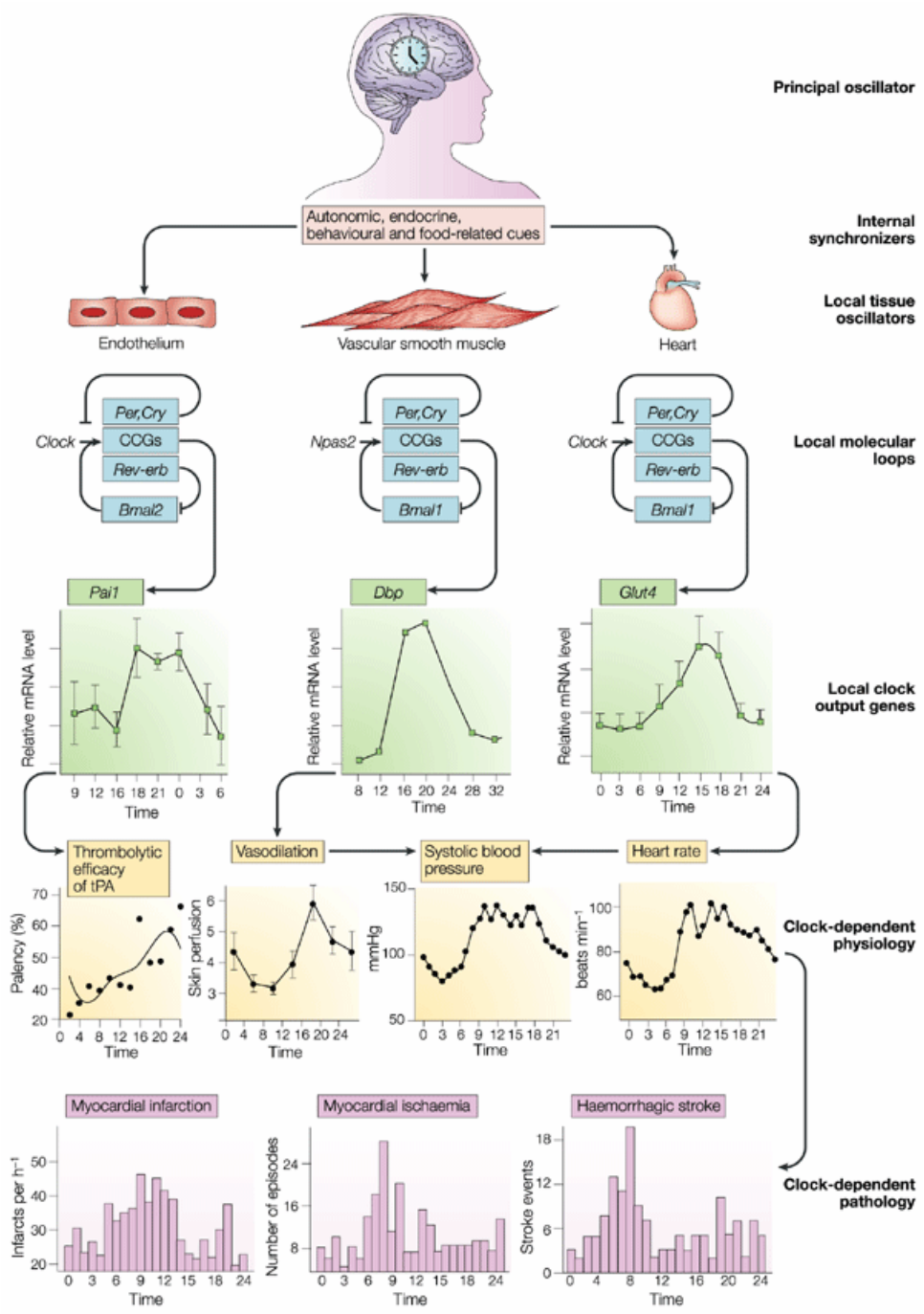


Figure 1.10. A summary of the role of peripheral circadian clocks in cardiovascular disease. Local peripheral oscillators, based on clock gene feedback loops, are synchronised by signals from the principle oscillator in the SCN and internal cues. They drive tissue-specific circadian patterns of CCG expression and in turn these molecular cycles sustain local clock-dependent physiologies. In combination these factors influence pathological events in the cardiovascular system. Figure from Hastings *et al.*, (2003).

1.5.2 Clock disruption in cardiovascular disease

Animal models have been very useful in demonstrating the effects of clock dysfunction, in particular the disruption of normal metabolic function. *Clock* mutant mice exhibit disrupted rhythms in locomotion and feeding and become obese by 6 weeks of age (Turek *et al.*, 2005). By 6-7 months of age these animals went on to develop a spectrum of biochemical abnormalities, such as hypercholesterolemia, hyperglyceridemia and hyperglycemia, hallmarks of the metabolic syndrome. Disrupted *Clock* and *Bmal1* expression also alters glucose homeostasis (Rudic *et al.*, 2004) and over-expression of a dominant negative CLOCK mutant specifically in the rat heart attenuated myocardial triglyceride metabolism and fatty acid oxidation, cardiac power and oxygen consumption (Durgan *et al.*, 2006; Bray *et al.*, 2008).

Models of disrupted physiology have also demonstrated the importance of the clock in cardiac function and disease. Diurnal variations in cardiac metabolism and contractile function have been demonstrated in an isolated working rat heart model together with the rhythmic expression of genes involved in these processes (Young *et al.*, 2001b). The introduction of pressure-overload induced hypertrophy to this *ex vivo* model by aortic constriction abolished these gene expression rhythms. Rhythmic clock gene expression was also attenuated in the same model of hypertrophy, implying that the loss of rhythmic metabolic gene expression may result from disruption of the clock (Young *et al.*, 2001c). Conversely, disruption of circadian rhythms by entrainment of mice to a shorten light-dark cycle (10 hours light followed by 10 hours dark) attenuated the compensatory hypertrophic response pathway in the heart following aortic constriction (Martino *et al.*, 2007). Heart failure caused by hypertrophy may therefore result from the loss of the heart's intrinsic capacity to anticipate, respond, and adapt to changes in the environment through its circadian clock.

Rodent models of diabetes also demonstrate the importance of correct circadian and metabolic regulation as streptozotocin-induced diabetes has been shown to advance the phase of clock gene expression in the heart (Young *et al.*, 2002; Herichova *et al.*, 2005). Additionally, diabetes mellitus causes diurnal variations of many neurohumoural factors to change, such as insulin, glucocorticoids (Velasco *et al.*, 1988), growth hormone and thyroid hormone (Ortiz-Caro *et al.*, 1984) as well as glucose and fatty acid levels and sympathetic activity (Bernardi *et al.*, 1992). Diabetes is associated with changes in morphology, gene expression, metabolism and contractile performance in the heart and these symptoms may therefore result in part from disruption of clock gene expression and the associated diurnal variations.

These rodent models describe both physiological disorders following clock impairment, and alterations in clock function in response to disorder. Whichever mechanism is more important, these experiments and others demonstrate a clear link between the clock and metabolism. This link may be of particular relevance to cardiovascular disorders, either directly through influencing cardiomyocyte

and vascular metabolism and function or indirectly through altering the risk factors of CVD. The ability of the heart to cope with diurnal variations in workload and stress and the vulnerability to MI is therefore likely to involve the circadian regulation of a host of genes within the heart as well as vasculature and other tissues that contribute to CVD and restoring circadian control of gene expression may therefore be a novel intervention in cardiovascular dysfunction. The identification of peripheral clocks now allows the link between the clock and cardiovascular function and dysfunction, and the phenomenon of increased cardiovascular events after waking, to be investigated in a new perspective. Local tissue-specific events, clock components, CCGs and factors involved in entrainment may all play a role.

1.5.3 Potential mechanisms linking the clock and cardiovascular dysfunction

1.5.3.1 Local cardiovascular events – hypoxia and oxidative stress

The heart is one of the most metabolically active organs in the body and as such has a high demand for oxygen. When oxygen delivery cannot meet the metabolic requirements of the heart, due to variations in workload and oxygen demand or changes in coronary blood flow for example, hypoxia can develop (Shohet and Garcia, 2007). Recent studies in rats and humans indicate that prolonged hypoxia can dampen the amplitude of circadian rhythms in variables such as body temperature and metabolic rate (Mortola, 2007) and may therefore interfere with circadian rhythms in heart function. In the mouse brain exposure to hypoxia up-regulated *Per1* expression and PER1 and CLOCK protein levels (Chilov *et al.*, 2001) so hypoxia may influence clock function at the molecular level, possibly via the transcription factor Hypoxia-Inducible Factor 1α (HIF-1α), which was found to interact with PER1 in the same study.

In response to hypoxia the activation of HIF-1α and Endothelial PAS domain protein 1 (EPAS1/HIF-2α) regulates transcription of several genes involved in oxygen homeostasis by binding to hypoxia response elements (HREs) in the promoters/enhancers of hypoxia sensitive genes such as vascular endothelial growth factor (VEGF) and erythropoietin (Ladoux and Frelin, 1997; Semenza *et al.*, 1999). Both HIF proteins are members of the bHLH-PAS family of transcription factors. They usually heterodimerise with a constitutively expressed β subunit, aryl hydrocarbon receptor nuclear translocator (ARNT) but can also form functionally active heterodimers with BMAL1 and BMAL2 (Hogenesch *et al.*, 1998; Takahata *et al.*, 1998; Hogenesch *et al.*, 2000). HIF-1α is expressed in the heart but there is some disagreement regarding whether EPAS1 expression is limited to endothelial cells (Ladoux and Frelin, 1997) or is more widely expressed, including in myocytes and stromal cells of the rat heart (Wiesener *et al.*, 2003). It does appear though that EPAS1 is most abundantly expressed in the endothelial cells of various tissues, whereas HIF-1α is expressed in a wider range of cells (Takahashi *et al.*, 2004). Therefore the hypoxia-responsive factors HIF-1α and EPAS1 may play an important role in linking the clock and tissue-specific hypoxia-related cardiovascular pathologies.

Severe hypoxia caused by coronary occlusion (by thrombosis or growth/rupture of atherosclerotic plaques) can lead to ischemia in the heart, resulting in MI (Buja, 2005) and subsequent restoration of blood flow and oxygen (reperfusion) is a major cause of oxidative stress (Ferrari *et al.*, 1990). Oxidative stress refers to a disturbance in the oxidation-reduction state of a cell, resulting in excessive generation of reactive oxygen species (ROS) during oxidative metabolism. High levels of ROS are thought to alter gene expression and protein structure and function and may play a major role in heart physiopathology, such as atherogenesis, congestive heart failure and vascular remodelling (angiogenesis) (Molavi and Mehta, 2004). In a rat model of ischemia/reperfusion oscillations in clock genes were rapidly attenuated within the ischemic region of the heart but not the non-ischemic regions, implying that loss of synchronisation between different regions of the heart as well as between the heart and environment, may contribute to the deleterious effects of reperfusion and pathogenesis of MI (Kung *et al.*, 2007). The local oxidation state in the heart may therefore link the circadian clock to cardiovascular dysfunction.

1.5.3.2 Dual role of clock factors in the periphery

BMAL1 and BMAL2 do not just bind to CLOCK but can form functionally active heterodimers with other members of the bHLH-PAS protein family, such as HIF-1α and EPAS1 (see above) and Member Of PAS family 4 (MOP4) (Hogenesch *et al.*, 1998; Hogenesch *et al.*, 2000). MOP4 (aka Neuronal PAS domain protein 2, NPAS2) is a paralogue of CLOCK, sharing 50% identity in amino acid sequence (Hogenesch *et al.*, 1997; Zhou *et al.*, 1997). MOP4:BMAL1 activates E-box containing genes such as *Per1* and *Vasopressin* and is negatively regulated by CRY1 and CRY2 (Kume *et al.*, 1999). MOP4 is less abundant than BMAL1 with highest expression levels in the mouse brain (Hogenesch *et al.*, 1998) and in the forebrain, circadian expression of *Per2* is dependent on MOP4 (Reick *et al.*, 2001). In the vasculature, *Mop4* expression has a robust rhythm, cycling in phase with *Bmal1* in murine aortic cells and human vascular smooth muscle cells (McNamara *et al.*, 2001). It is therefore possible that the MOP4:BMAL1 heterodimer drives *Per2* expression and is part of the core clock feedback loop in the vasculature. The ability of MOP4:BMAL1 heterodimers to bind DNA has been shown to be regulated by the redox state of NAD cofactors in a purified system (Rutter *et al.*, 2001) and MOP4:BMAL1-mediated clock gene activation is affected by the nuclear hormones RXRα and RARα in the vasculature. MOP4 may therefore be an important clock component in the vasculature, particularly in response to hormones and oxidative stress.

As well as being a key component of the molecular circadian clock, REV-ERBα and RORα have been implicated in the regulation of a number of processes linked with the metabolic syndrome (Fontaine and Staels, 2007). These two transcription factors are members of the nuclear receptor superfamily with unknown ligands and are referred to as 'orphan' nuclear receptors. *Rev-erbα* is highly expressed in adipose tissue, skeletal muscle, brain and liver and is induced during adipose differentiation

(Chawla and Lazar, 1993), promoting enhanced adipocyte production and lipid storage (Fontaine *et al.*, 2003). REV-ERBα also down-regulated the expression of triglyceride-rich lipoproteins (apoA-I and apoC-III) (Vu-Dac *et al.*, 1998; Raspe *et al.*, 2002) and may thus act as a link between the clock and development of obesity. Additionally, REV-ERBα has been implicated in vascular inflammation as it is expressed in different cell types of the vascular wall (endothelial cells and vascular smooth muscle cells) and the immune system (macrophages and human peripheral blood mononuclear cells) and up-regulated the expression of inflammatory cytokine markers (Fontaine and Staels, 2007). REV-ERBα may therefore be involved in the development of atherosclerosis and the onset of acute cardiovascular events. RORα may also work with REV-ERBα in regulating lipid metabolism as the RORα-deficient 'staggerer' mutant mice have lower plasma triglyceride and apoC-III lipoprotein levels (Raspe *et al.*, 2001), whereas levels are higher in REV-ERBα null mice (Raspe *et al.*, 2002). 'Staggerer' mutant mice also have altered circadian rhythms (Sato *et al.*, 2004c; Akashi and Takumi, 2005), develop severe atherosclerosis and hypoalphalipoproteinemia (Mamontova *et al.*, 1998) and may have an altered immune inflammatory response (Boukhtouche *et al.*, 2004). REV-ERBα and RORα may therefore be integrators of circadian and metabolic regulation and thus be important modulators of CVD risk factors.

1.5.3.3 Clock-controlled genes in the heart

As mentioned earlier, up to 90% of the circadian transcriptome in peripheral tissues may be tissue specific. The genes and products that are regulated by the clock in the heart and CVS are therefore likely to play key roles in mediating temporal variations in cardiac function and linking the clock and cardiovascular dysfunction.

One enzyme that is believed to be instrumental in the morning excess of cardiac events is Plasminogen Activator Inhibitor 1 (PAI-1). PAI-1 is produced by diverse tissues and cell types such as the vascular endothelium, adipose tissue, heart, liver, monocytes and platelets (Loskutoff *et al.*, 1986; Sawdey and Loskutoff, 1991; Chomiki *et al.*, 1994; Samad and Loskutoff, 1996). It is the principal inhibitor of fibrinolysis, preventing intravascular plasminogen activation by tissue-type plasminogen activator (tPA) and thus the breakdown of blood clots in vessels. PAI-1 is also involved in many diverse processes under normal and pathological conditions such as fibrosis, extracellular matrix remodelling, tumour angiogenesis, pre-thrombotic events, haemorrhage and thrombus formation, obesity and insulin resistance (Kohler and Grant, 2000; Binder *et al.*, 2002; De Taeye *et al.*, 2005; Lijnen, 2005; Vaughan, 2005).

Circulating PAI-1 exhibits a clear circadian rhythm in humans with peak activity, and thus higher clotting potential, early in the morning (Angleton *et al.*, 1989). This increased morning inhibition of fibrinolysis coincides with, and is possibly associated with, the peak risk of MI (Muller *et al.*, 1989; Cohen *et al.*, 1997). In fact, increased plasma PAI-1 levels are believed to contribute to the

development of CVD and are now considered to be a risk factor (Nordt *et al.*, 1999). Circadian rhythms of *Pai-1* expression have also been observed in the murine heart, brain, kidney and lungs with a peak at the onset of night (ZT18) in the heart, corresponding with the onset of activity in these nocturnal animals. In the hearts of *Clock* mutant mice the circadian peak in *Pai-1* expression is lost (Minami *et al.*, 2002) and expression is down-regulated in the liver (Panda *et al.*, (2002), Supplementary table S5). *Pai-1* expression is likely to be directly controlled by the clock as CLOCK:BMAL1 and CLOCK:BMAL2 can activate the human *Pai-1* promoter (Maemura *et al.*, 2000; Schoenhard *et al.*, 2003).

The activity of PAI-1 is tightly regulated at the transcriptional level and induced by a range of stimuli, including growth factors and cytokines (e.g. endotoxins, TNF-α and TGF-β), hormones (e.g. insulin and glucocorticoids), proteinases, fatty acids and lipoproteins, and hypoxia (Bruzdzinski *et al.*, 1993; Kohler and Grant, 2000; Huber *et al.*, 2001). The human *Pai-1* proximal promoter also contains a deletion/insertion polymorphism (four or five guanine bases, 4G/5G), with the 4G genotype being associated with higher plasma PAI-1 levels (Dawson *et al.*, 1993; Eriksson *et al.*, 1995). This is believed to be due to the binding of an additional repressor protein to the promoter containing the 5G allele. The 4G allele has also been associated with CVD and MI (Dawson *et al.*, 1993; Mansfield *et al.*, 1995; Ossei-Gerning *et al.*, 1997; Margaglione *et al.*, 1998), although the strength of this association is relatively weak and other studies have found no relationship between genotypes and MI (Ye *et al.*, 1995; Ridker *et al.*, 1997). Interestingly, the diurnal variation in plasma PAI-1 activity is predominantly confined to the 4G allele (Hoekstra *et al.*, 2002; van der Bom *et al.*, 2003) and this may be due to greater activation of the 4G allele by CLOCK:BMAL1 (the 4G/5G polymorphism overlaps a canonical E-box) (Chong *et al.*, 2006). The strong circadian regulation of *Pai-1* expression coupled with the regulation of PAI-1 by factors associated with increased CVD risk and the role of PAI-1 in a number of processes that contribute to CVD and MI suggests that the correct temporal expression of *Pai-1* is very important for maintaining correct cardiovascular function.

1.5.3.4 Pleiotropic roles of endogenous synchronisers

The factors that entrain peripheral clocks to signals from the central SCN clock may also play a role in linking the clock and CVD. Melatonin is rhythmically produced and secreted by the pineal gland in response to signals from the SCN and is able to synchronise all tissues expressing the melatonin receptor (Simonneaux and Ribelayga, 2003; Pevet *et al.*, 2006). It may transmit rhythmic timing cue to peripheral clocks by directly modifying the transcriptional feedback loop, as the rhythm of *Rev-erbα* and *Bmal1* expression was advanced by melatonin treatment in the rat SCN (Agez *et al.*, 2007). Melatonin can also regulate important processes linked to CVD, such as glucose metabolism, oxidative stress and blood pressure (Derlacz *et al.*, 2005; Grossman *et al.*, 2006; Winiarska *et al.*, 2006).

Glucocorticoids are rhythmically secreted by the adrenal gland (Buijs *et al.*, 2003) and also believed to be synchronisers of peripheral tissues via binding to the glucocorticoid receptor (that is expressed in most tissues except the SCN) and subsequent activation of genes containing the glucocorticoid response element (GRE) (Rosenfeld *et al.*, 1993; Tronche *et al.*, 1998). Like melatonin, glucocorticoids can regulate CVD risk factors and excessive activation of the glucocorticoid receptor induces obesity, insulin resistance, glucose intolerance, dyslipidemia and hypertension (Walker, 2007). Glucocorticoids may also exert a direct effect on the heart and blood vessels and influence vascular function and remodelling following MI and atherogenesis, and are believed to be involved in recovery post-MI (Alisky, 2006; Walker, 2007). Dysynchrony with the external environment and disrupted secretion of endogenous synchronisers may therefore contribute to increased CVD risk and manipulation of the circulating levels of these hormones may provide therapeutic avenues to treating or preventing CVD.

1.6. Aims

The existence of daily 24 hour rhythms in physiology is becoming clearer following the discovery of the molecular circadian clock. Progress in understanding the workings of this rhythm-generating clockwork and the mechanisms by which it transduces rhythms to peripheral tissues via autonomous clocks will shed some light on the molecular basis of physiological rhythms. However, how the clock orchestrates rhythmic expression of physiological processes at a cellular level is not fully understood. As the clock is primarily a transcriptionally based mechanism, identifying target genes of the molecular clock in peripheral tissues will provide further information on the temporal functioning of organs, such as the heart, and in particular why the CVS is more prone to pathological events at certain times of the day. Delineation of the cardiac circadian transcriptome may give an understanding of how the ability of the heart to cope with its environment varies over the course of 24 hours and provide new avenues for therapeutic intervention.

This study has used four approaches to identify novel cardiac CCGs and further investigate the mechanism of their circadian expression in the heart. Specific aims were to:

1. Confirm the existence of rhythms in clock gene expression in the mouse heart and identify cardiac genes that are rhythmically expressed.
2. Investigate whether these putative CCGs are regulated by the independent cardiac clock or synchronising factors using SCN-ablated mice *in vivo*.
3. Determine whether the putative CCGs are downstream targets of the cellular circadian clock *in vitro*.
4. Investigate the mechanisms of circadian transcriptional regulation of putative CCGs *in vitro* and the interplay between the circadian clock and other regulatory mechanisms such as hypoxia.

2. General methods and materials

The general laboratory methods and materials used are described in this chapter. Specific methods are described in the individual results chapters where relevant.

All chemicals were obtained from Sigma or Fisher unless otherwise stated.

2.1 RNA extraction and quantification

2.1.1 Tissue homogenisation and cell lysis

Hearts were homogenised on wet ice for approximately 10 minutes in 1ml TRIzol reagent (modified acid guanidium thiocynate phenol/chloroform method; Invitrogen) with a T8 Ultra Turrax hand-held rotor-stator homogeniser and 5mm dispersing element (IKA). Samples were left on ice for 5 minutes until sediment had separated and supernatant was removed for RNA extraction.

Medium was removed from cells in culture and cells lysed by adding 1ml TRIzol reagent per well of a 6 well culture plate and leaving at room temperature for 5 minutes. Samples were then transferred to Eppendorf tubes for RNA extraction.

2.1.2 RNA extraction from tissue and cell homogenates

RNA was extracted from homogenates by phase separation with 200µl chloroform, incubation at room temperature for 2 to 3 minutes and centrifugation at 13000rpm for 15 minutes at 4°C. The aqueous phase was transferred to a new tube and RNA precipitated with 500µl isopropanol. RNA was recovered by centrifugation at 13000rpm for 10 minutes at 4°C and washed with 75% ethanol followed by centrifugation at 10000rpm for 5 minutes at 4°C. The RNA pellet was air dried and resuspended in 200µl dH_2O per heart sample of 20µl dH_2O per culture plate well.

RNA quality was checked by non-denaturing agarose (Melford Laboratories) gel electrophoresis. 1µg RNA was diluted to 10µl volume in dH_2O and heated at 70°C for 10 minutes. Samples were transferred to ice for 2 minutes and pulse centrifuged at 13000rpm. 3µl RNA loading buffer (50% v/v glycerol, 50% v/v formamide) was added and samples were loaded onto a 1% w/v agarose gel made with 1X TBE (892mM Tris base, 890mM Boric acid, 20mM EDTA [ethylene diamine tetraacetic acid]) alongside a molecular weight marker. Ethidium bromide was added to gels at a concentration of 0.035µg/ml. Samples were electrophoresed in 1X TBE buffer at 50V for up to 1 hour and RNA was visualised under ultraviolet light.

2.1.3 RNA quantification

RNA concentration was determined by diluting RNA 1:50 with dH_2O and measuring absorption in a spectrophotometer at wavelengths of 260nm and 280nm. Absorption at 260nm (A_{260}) was equivalent to 40µg RNA/ml. The concentration of RNA in ng/µl was calculated as follows:

Concentration (ng/µl) = 40 x A_{260} x dilution factor

2.1.4 Elimination of genomic/plasmid DNA and cDNA synthesis

Genomic and plasmid DNA that could be undesirably amplified in subsequent PCR reactions was removed. 1µg total RNA was digested by treatment with amplification grade DNase I (Sigma, final concentration of 0.1U/µl) and 1X Reaction Buffer (20 mM Tris-HCl, pH 8.3, 2mM MgCl2) in a volume of 10µl at room temperature for 15 minutes. The reaction was stopped and RNA denatured ready for reverse transcription by adding 1µl Stop Solution (50mM EDTA) and heating at 70°C for 10 minutes.

cDNA synthesis was performed by reverse transcription (RT) using SuperScript II Reverse Transcriptase (Invitrogen). Reagents for reverse transcription were added directly to the DNase-treated RNA mix: 25µg/ml oligo(dT) primer, 0.5mM each dNTP mix, 10mM DTT (dithiothreitol), 1X First-Strand Buffer and 10U/µl SuperScript II Reverse Transcriptase. The 20µl reaction was incubated at 42°C for 55 minutes followed by 70°C for 15 minutes in a thermocycler. The end product was diluted 5x with dH_2O for use in polymerase chain reaction (PCR) amplification.

2.1.5 Semi-quantitative Polymerase Chain Reaction (PCR)

Products ranging in size from approximately 150-500bp were amplified from typically 2µl of cDNA template in a 20µl reaction containing 1X Reaction Buffer IV (ABgene; 75mM Tris-HCl pH 8.8, 20mM (NH4)2SO4, 0.01% v/v Tween 20), 1.5mM $MgCl_2$, 0.2mM each dNTP, 0.5µM each primer and 0.025U/µl Taq DNA polymerase (ABgene). Mineral oil was added to each sample and the amplification reaction carried out in a thermocycler. The same programme was used for each set of primers since the primers were designed within a narrow range of melting temperatures. An initial denaturation step of 2 minutes at 94°C was followed by cycles of 94°C for 45 seconds, 59°C for 45 seconds and 72°C for 45 seconds. The optimal cycle number for each specific primer pair was found by plotting the amount of product produced from pooled cDNA samples against cycle number and identifying the cycle number where amplification was in the exponential phase (see Chapter 3, section 3.2). Primers were designed based on cDNA sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and using the Primer 3 program (http://primer3.sourceforge.net/, (Rozen and Skaletsky, 2000). Primers were designed to either across an exon-exon boundary to avoid amplifying any remaining genomic DNA or to bind to two different

exons and give a larger intron-containing product visible on an agarose gel if genomic DNA was amplified. PCR products were viewed and quantified by agarose gel electrophoresis.

2.1.6 Agarose gel elctrophoresis

6X loading buffer (40% v/v glycerol, 60% v/v TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0), and very small amount of bromophenol blue) was added to samples to a final concentration of 1X. Samples were loaded onto a 2% w/v agarose gel made with 1X TBE (892mM Tris base, 890mM Boric acid, 20mM EDTA) alongside a DNA size marker (PCRsizer 100bp DNA ladder, Norgen Biotek). Ethidium bromide was added to gels at a concentration of 0.035µg/ml. Samples were electrophoresed in 1X TBE buffer at 80V for up to 1 hour and DNA bands were visualised under ultraviolet light. Band intensity was quantified from electronic images using Gene Tool software (Syngene).

2.1.7 Quantitative real-time PCR

Quantitative real-time PCR was performed using either TaqMan® Gene Expression Assays (Applied Biosystems) with absolute abundance calculated by comparison with a standard curve of dilutions, or using SYBR® Green dye with custom designed primers and relative abundance calculated using the Pfaffl method.

2.1.7.1 Quantitative real-time PCR using TaqMan® Gene Expression Assays

PCR reactions were carried out using TaqMan® probes designed by Applied Biosystems (Table 3.2, Chapter 3) in 20µl reactions containing 1X TaqMan Universal PCR Master Mix (Applied Biosystems; AmpliTaq Gold® DNA polymerase, dNTPs, passive reference dye (ROX), optimised buffer components), 1X TaqMan Gene Expression Assay mix (Applied Biosystems; 900nM each gene-specific primer, 250nM gene-specific TaqMan MGB 6-FAM dye-labelled probe) and between 0.05µl and 3µl cDNA template depending on the gene being amplified. Reactions were run in triplicate to 40 cycles (95°C for 15 seconds, 60°C for 1 minute) following enzyme activation at 95°C for 10 minutes on the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems). Quantities were measured and normalised by plotting threshold cycle (Ct) values on a standard curve derived from the Ct values of the endogenous control gene *mTbp* (TATA box binding protein) with serial dilutions of cDNA.

2.1.7.2 Quantitative real-time PCR using SYBR® Green and analysed using the Pfaffl method

PCR reactions were carried out using custom primers designed as described in section 2.1.5 and listed in Table 3.2, Chapter 3. 25µl reactions contained 1X SYBR® Green PCR Master Mix (Applied Biosystems; AmpliTaq Gold® DNA polymerase, dNTPs, PCR buffer, $MgCl_2$, passive reference dye (ROX) and SYBR® Green I dye), 0.2µM forward primer, 0.2µM reverse primer and between 0.05µl and 3µl cDNA template depending on the gene being amplified. Reactions were run in triplicate to 40 cycles (95°C for 45 seconds, 59°C for 45 seconds, 72°C for 45 seconds) following enzyme activation

at 95°C for 10 minutes on the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems). A final step of 95°C for 15 seconds and 60°C for 1 minute followed during which a dissociation curve was generated to confirm the presence of specific products.

In order to perform the Pfaffl method (Pfaffl, 2001) a series of cDNA dilutions were first made and used to generate standard curves of Ct values for each primer pair and the reference gene primers. By plotting log of input cDNA verse Ct the efficiency of each PCR reaction could be calculated according to the equation:

$$\text{Efficiency} = 10^{(-1/\text{slope})}$$

The efficiency of the reference gene and gene of interest do not have to be equal, but are incorporated into the analysis, resulting in increased accuracy. The relative expression ratio (fold change) was then calculated as follows:

1. Mean Ct values from triplicate wells were determined for calibrator and samples for the genes of interest and reference gene.

   Normally a calibrator is a non-treated control sample so for this study of temporal expression analysis the calibrator was a pool of all cDNA samples, with each sample representing one heart from one time-point.

2. Change in Ct (ΔCt) was calculated for the genes of interest and reference gene:

   Gene of interest ΔCt = mean gene of interest Ct (calibrator) - mean gene of interest Ct (sample)

   Reference gene ΔCt = mean reference gene Ct (calibrator) - mean reference gene Ct (sample)

3. The values for efficiency of each gene is then raised to the power of the respective ΔCt , and the ratio of gene of interest:reference gene is calculated using the formula:

$$\text{Ratio (fold change)} = \frac{\text{Efficiency (gene of interest)}^{\Delta\text{Ct (gene of interest)}}}{\text{Efficiency (reference gene)}^{\Delta\text{Ct (reference gene)}}}$$

   The formula makes the assumption that while the efficiencies of the reference gene and gene of interest may differ, the efficiency of amplification for each sample within a PCR reaction is the same.

2.2 DNA cloning and sub-cloning

2.2.1 High fidelity PCR

Gene promoter fragments were amplified from 50ng mouse genomic DNA by PCR using specific primers with restriction sites added in for later sub-cloning (see Chapter 6) and Expand PCR High Fidelity Taq blend (Roche; a mixture of Taq DNA Polymerase and Tgo polymerase with 3'-5' exonuclease 'proofreading' activity). 40µl reactions were carried out containing 1X Expand High Fidelity Buffer 2 (Roche), 1.5mM $MgCl_2$, 0.2mM each dNTP, 0.5µM each primer and 0.09U/µl enzyme mix. An initial denaturation step of 2 minutes at 94°C was followed by 30 cycles of 94°C for 45 seconds, 59°C for 45 seconds and 72°C for 1 minute per kb, followed by a final extension of 72°C for 5 minutes. The PCR product was run on a 2% w/v agarose gel (see section 2.1.6) and excised from the gel.

2.2.2 Extraction and purification of DNA from agarose gels

Gel slices were placed in dialysis membrane tubing that had previously been boiled in 1mM EDTA and 2% sodium bicarbonate and stored in 100% ethanol at 4°C. 400µl 1X TBE was added to the gel slice and the tube was clamped and placed in a gel tank containing 1X TBE. A potential difference of 80V was applied for 15 minutes or longer until the DNA has passed from the gel slice to the buffer (as determined by viewing under ultraviolet light). The DNA-containing buffer was removed and DNA was purified by phenol:chloroform extraction. 1 volume phenol:chloroform (1:1) was added followed by mixing by vortex and phase separation by centrifugation at 13000rpm for 4 minutes. DNA was precipitated from the aqueous phase with 2 volumes 100% ethanol at -80°C for 30 minutes and centrifugation at 13000rpm for 15 minutes. The pellet was washed with 80% ethanol and resuspended in 20µl TE buffer containing RNase (10mM Tris-HCl ph8.0, 1mM EDTA, 1% v/v RNase mix (Sigma)).

2.2.3 DNA quantification

DNA concentration was determined by diluting DNA 1:50 with dH_2O and measuring absorption in a spectrophotometer at wavelengths of 260nm and 280nm. Absorption at 260nm (A_{260}) was equivalent to 50µg DNA/ml. The concentration of DNA in ng/µl was calculated as follows:

$$\text{Concentration (ng/µl)} = 50 \times A_{260} \times \text{dilution factor}$$

2.2.4 Ligation of PCR products into vector

PCR products generated with Expand DNA polymerase have a 3' single A overhang which allows cloning into the pGEM-T Easy vector (Promega) with a 3' single T overhang at each end of the insertion site. A 3:1 ratio of insert (PCR product) to vector (50ng pGEM-T Easy) was used in a 20µl reaction containing 1X Rapid Ligation Buffer (Promega; 30mM Tris-HCl pH 7.8, 10mM $MgCl_2$,

10mM DTT, 1mM ATP, 5% w/v polyethylene glycol) and 0.15U/µl T4 DNA Ligase (Promega). Reactions were incubated at 4°C overnight and stopped by the addition of 2µl 3M NaAc (pH 5.2), 2µl tRNA (10ng/µl) and 70µl 100% ethanol. DNA was precipitated at -80°C for 30 minutes and centrifugation at 13000rpm for 15 minutes. The resulting pellet was washed with 80% ethanol and resuspended in 20µl 10mM Tris-HCl (pH 8.5).

2.2.5 Transformation of bacteria with plasmids

100µl of competent cells (*E.coli* DH5α, Invitrogen) were transformed with 10µl of the ligation by heat shock. The reaction was placed on ice for 30 minutes, heat shocked at 37°C for 45 seconds and returned to ice for 2 minutes. 400µl pre-warmed SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 10mM NaCl, 2.5mM KCl, 20mM Mg^{2+} stock, 20mM glucose) was added followed by incubation at 37°C for 1.5 hours with shaking. 100µl of transformed cells were plated on Luria Bertani medium (LB) agar plates (2% w/v LB (0.5% w/v yeast extract, 0.5% w/v NaCl, 1% w/v tryptone in dH_2O), 1.5% w/v agar) containing 100µg/ml Ampicillin (Sigma) and incubated at 37°C overnight. Where blue-white selection was required (as with cloning into pGEM-T Easy) 40µl X-Gal (Melford Laboratories; 5% w/v 5-bromo-4-chloro-3-indolyl-β-D-galactoside in N,N'-dimethylformamide) was added to LB agar plates and allowed to soak in for 30 minutes before spreading of cells.

2.2.6 Inoculation of transformants and amplification of plasmids

X-Gal is broken down to a blue metabolite in bacteria expressing β-galactosidase, the product of the *lacZ* gene. pGEM-T Easy contains this gene and successful cloning of an insert interrupts this gene, resulting in white colonies. pGEM-T Easy also confers ampicillin resistance so only transformed bacteria can grow on an ampicillin containing plate. Ampicillin resistant white transformants were picked and grown in 5ml LB containing ampicillin (0.1% v/v) at 37°C with shaking at 225rpm overnight.

2.2.7 Speed plasmid preparation and identification of positive clones

To prepare DNA for analysis by restriction digest alkaline lysis speed mini-preparations were performed. 3ml bacterial culture was centrifuged at 13000 rpm for 1 minute. The pellet was resuspended in 100µl (1 volume) Solution A (50mM Tris-HCl pH8.0, 4% v/v Triton X-100, 2.5M LiCl, 62.5mM EDTA). 1 volume phenol:chloroform (1:1) was added followed by mixing by vortex and phase separation by centrifugation at 13000rpm for 4 minutes. DNA was precipitated from the aqueous phase with 2 volumes 100% ethanol at -80°C for 30 minutes and centrifugation at 13000rpm for 15 minutes. The pellet was washed with 80% ethanol and resuspended in 20µl TE buffer containing RNase (10mM Tris-HCl ph8.0, 1mM EDTA, 1% v/v RNase mix (Sigma))

2.2.8 Restriction enzyme digestion

To identify colonies containing plasmids successfully ligated with PCR fragment inserts the prepared plasmid DNA was digested with restriction enzymes to excise the PCR fragment insert if present. The restriction enzyme sites were introduced into the PCR fragments by adding them to the amplification primers (see Chapter 6, section 6.2). Restriction enzyme digest was also used to transfer inserts from one vector to another (sub-cloning, see section 2.2.11). The required amount of DNA was digested with 0.02U/µl of each enzyme in a 20µl reaction (or 50µl reaction for sub-cloning) with 1X of the appropriate buffer depending on the enzymes used (REACT 2, 3 or 4 (Invitrogen) and incubated at 37°C for 2 hours or over night. The total reaction volume was then run on a 0.5-1% agarose gel, depending on the size of the excised fragments (as described in section 2.1.6). If the fragment was required for sub-cloning it was extracted from the gel and purified as described in section 2.2.2).

2.2.9 Purification of plasmid DNA from bacterial culture (mini-prep.)

The remaining culture from positive colonies was used to inoculate 10ml of fresh LB containing ampicillin and grown overnight at 37°C with shaking at 225rpm. Plasmid DNA was purified from 5ml overnight culture using the GenElute™ Plasmid Miniprep Kit (Sigma) according to the manufacturer's protocol (spin column based). Eluted plasmid DNA was used for sub-cloning or sequencing.

2.2.10 DNA sequencing

All cloned PCR fragments were sequenced to verify that no errors were introduced during amplification or subsequent steps. Sequencing reactions were performed using a dye terminator method with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to manufacture's protocol. 300ng plasmid was used for amplification and dye incorporation. Labelled amplification products were separated through POP-7™ Polymer and sequences read using the ABI 3130xl genetic analyser (Applied Biosystems).

2.2.11 Sub cloning of promoter fragments into pGL3 luciferase reporter vector

To quantitatively assess the degree of activation by trans-activating factors in eukaryotic cells promoter fragments were cloned from pGEM-T Easy into the pGL3-Basic (for proximal promoter fragments) or SV40 minimal promoter-containing pGL3-Promoter (for distal fragments) vectors that contains a modified coding region for the firefly (*Photinus pyralis*) luciferase (Promega). Cloning the fragment (insert) into the multiple cloning site upstream of the *luciferase* gene and subsequent activation of the constructs results in the production of luciferase and subsequent measurable luminescence in the presence of luciferin substrate and ATP.

Inserts were excised from pGEM-T Easy and pGL3 and linearised by restriction enzyme digestion, as described in section 2.2.8. Excised inserts and linearised vector were separated by agarose gel

electrophoresis (section 2.1.6) and extracted from gel slices by electroelution, as described in section 2.2.2.

Ligation of inserts into linearised vector was performed with a 5:1 ratio insert:vector in a 20µl reaction containing 1X T4 DNA Ligase Buffer (Invitrogen; 50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 1mM DTT, 1mM ATP, 5% w/v polyethylene glycol-8000) and 0.25U/µl T4 DNA Ligase (Invitrogen). Reactions were then incubated at 4°C overnight and plasmid DNA precipitated as in section 2.2.4. Competent cells were transformed and plated as before (section 2.2.5). A control transformation with cut pGL3 vector (no insert) was also plated to indicate the degree of re-circularised plasmid. Ampicillin resistant white transformants were picked, and confirmed as carrying an insert-containing plasmid as before (sections 2.2.6 to 2.2.10).

2.2.12 Purification of plasmid DNA from bacterial culture (midi-prep.)

Positive colonies (and over-expression plasmids) were further grown for larger recovery of high quality plasmid DNA for transfection into cells (section 2.4.2). 5 µl of starter culture was used to inoculate a larger volume of LB containing ampicillin (e.g. 50ml). Midi-preparations were performed using the PureYield™ Plasmid Midiprep System (Promega) according to the manufacturer's protocol (spin column based). Plasmid DNA was concentrated by precipitation with 2 volumes 100% ethanol and 0.1 volume sodium acetate, recovered as described above in section 2.2.7 and resuspended in 50µl 10mM Tris-HCl (pH 8.5). The concentration was determined by measuring absorption as described in section 2.2.3.

2.2.13 Site-directed mutagenesis

Transcription factor binding sites in promoter-reporter vectors were mutated by site-directed mutagenesis using the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. Mutagenic primers were designed to mutate E-box and E-box-like sequences to *Sal I* restriction sites (GTCGAC) and verified for use with the kit using Stratagene's online QuikChange T_m calculator (at www.stratagene.com). Authenticity of all mutated constructs was checked by restriction digest and sequencing.

2.3 Protein Manipulation

2.3.1 Extraction of proteins from cultured mammalian cells

Protein was extracted from transfected H9c2 cells using M-PER Mammalian Protein Extraction reagent (Pierce) according to the manufacturer's protocol. 1X Complete Mini Protease Inhibitor Cocktail (Roche) was added to the reagent prior to cell lysis to inhibit proteases and maintain protein integrity.

2.3.2 Protein quantification

Protein was quantified using the Bio-Rad Protein Assay (Bio-Rad) (a colourmetric assay) according to the manufacture's protocol. Bovine serum albumin (BSA) was used as a protein standard at concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0µg/µl. 20µl of sample or standard was assayed with the addition of 1ml diluted dye reagent and absorbency detected at 595nm in a spectrophotomoter.

2.3.3 Western blotting

Western blotting was performed using a method based on that described by Bollag *et al*. (1996) and Sambrook *et al.* (1989). 1X loading buffer (188mM Tris-HCl (pH 6.8), 6% v/v Sodium dodecyl sulfate (SDS), 30% v/v Glycerol, 15% v/v β-mercaptoethanol, 0.03% w/v bromophenol blue) was added to 15µg protein. Samples were then boiled (100°C) for 5 minutes prior to loading onto an 8% SDS-polyacrylamide gel. Samples were run alongside a SeeBlue Plus2 pre-stained standard (Invitogen) in gel running buffer (25mM Tris, 250mM Glycine, 0.1% v/v SDS) with a constant current of 10mA overnight. When the SDS-polyacrylamide gel had run to completion the proteins were transferred from the gel to a Protran nitrocellulose transfer membrane (Schleicher and Schuell BioScience) with a constant current of 400mA for 3 hours using a wet module system (Bio-Rad trans-blot cell) containing transfer buffer (48mM Tris, 39mM Glycine, 0.0375% v/v SDS, 20% methanol) surrounded by ice.

The presence of protein on the nitrocellulose membrane was confirmed by staining with Ponceau S solution (Sigma). Ponceau S was washed off with three 5 minute washes in TBS-T pH 7.6 (20mM Tris, 137mM NaCl, 0.1% v/v Tween-20). The membrane was then blocked for 1 hour at room temperature with shaking in TBS-MT (TBS-T, 5% w/v skimmed milk powder) prior to incubation with the primary antibody diluted in TBS-MT at 4°C overnight with shaking. Excess primary antibody was removed with three 5 minute washes in TBS-T at room temperature with shaking prior to incubation with the secondary antibody for 1 hour at 4°C with shaking. Excess secondary antibody was also removed with three 5 minute washes in TBS-T. Bound secondary antibody detection was performed by enhanced chemiluminescence (ECL) using the EZ-ECL kit (Geneflow) according to the manufacturer's protocol. The luminescent signal created was detected on Hyperfilm ECL (Amersham). Membranes were partially stripped to allow for probing with further primary antibodies by washing in TBS-T with rocking for 4 hours.

2.3.4 Electromobility shift assay (EMSA)

2.3.4.1 Preparation of whole cell protein extracts.

CelLytic™-M cell lysis extraction reagent (Sigma) was used to extract whole cell protein extracts from NIH-3T3 cells according to the manufacturer's protocol. In order to inhibit endogenous protease

activity 1X Complete Mini Protease Inhibitor Cocktail (Roche) was added to the reagent before continuing with cell lysis. Protein was quantified as described in section 2.3.2.

2.3.4.2 Preparation of double stranded probes for use in EMSA

The dehydrated sense/antisense oligonucleotides were re-suspended in TEN buffer (50mM Tris-HCL pH8.0, 150mM NaCl, 5mM EDTA) to a final concentration of 300ng/µl. Equal amounts of complimentary sense and anti-sense oligonucleotides were incubated for 10 minutes at 95°C, to disrupt any secondary coiling of the oligonucleotides that may impede annealing, and were then allowed to anneal by slow cooling at 15-25°C. Prior to the digoxigenin (DIG) end-labelling reaction the annealed oligonucleotides were further diluted with TEN buffer to a final concentration of 25ng/µl. Labelling reactions contained 25ng/µl annealed probe, 1X labelling buffer (Roche), 5mM CoCl2, 0.05mM DIG-ddUTP and 2.5 U terminal transferase (Roche). Reaction was then mixed briefly by pulse spin centrifugation and incubated at 37°C for 15 minutes. The reaction was terminated on ice with the addition of 0.02M EDTA (pH 8).

The efficiency of the labelling reaction was determined by comparison of a spotted dilution serious of labelling reaction with one of a labelled control-oligonucleotide (supplied by Roche) in a direct detection assay (section 2.3.4.3). The dilution series was 0ng/µl, 0.004ng/µl, 0.04ng/µl, 0.4ng/µl, and 4ng/µl. These dilutions were directly spotted onto Hybond+ nylon membrane and visualised by chemiluminescent detection and autoradiography (section 2.3.4.3).

The sequences of complimentary sense and anti-sense oligonucleotides used as labelled probes or unlabelled competitors in EMSA's are listed in Table 10.

2.3.4.3 EMSA reaction

DIG-labelled probes (25ng/µl) were incubated with 5-10µg of total whole cell protein extract for 15 minutes at 15-25°C in a reaction containing 1X binding buffer (Roche), 1µg poly [d(I-C)], 0.1µg poly L-lysine and either double distilled H_20 or 200X molar excess of the appropriate competitor probe. 1X loading dye (Roche) was added to the reactions, and protein-DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel (5% acrylamide, 0.06% bis-acrylamide, 40mM TrisHCL, 90mM boric acid, 2.5mM EDTA, 0.04% TEMED, 0.1% APS) in TBE buffer (40mM TrisHCL, 90mM boric acid, 2.5mM EDTA) at 150V for 1-2 hours at 4°C.

Following electrophoresis, migrated DNA-protein complexes were transferred, via contact blotting, to a positively charged nylon membrane (Hybond+, Amersham Biosciences). Post transfer the membrane was placed on Whatmann 3MM paper pre-soaked with 2X SCC buffer (Roche) and cross-linked at 120mJ for two minutes in a stratalinker. Following cross-linking DIG-labelled oligonucleotides were visualised by incubation with alkaline phosphatase-labelled F(ab)2 anti-DIG

antibody, followed by chemiluminescence reaction with 100µg/ml CPSD substrate (Roche) and auto-radiographic visualisation, following the manufacturers instructions for the direct detection assay (Roche).

2.4 Mammalian cell culture

2.4.1 Cell maintenance

NIH-3T3 (murine embryonic fibroblasts) and H9c2 (rat ventricular cardiomyocytes) cell lines were maintained in Complete Dulbecco's Modified Eagle Medium (DMEM) (31966; Invitrogen) supplemented with 10% v/v foetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passaged by trypsinisation with 3ml trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; BioWhittaker) (37°C, 5 minutes) and maintained in T-75 flasks.

The NIH-3T3 cell line was obtained from the American Type Culture Collection and H9c2 cell line from the European Collection of Cell Cultures.

2.4.2 Cell synchronisation

100% confluent cells were treated with either dexamethasone or horse serum ('serum shock') to synchronise. Dexamethasone (Sigma) was added directly to the cell culture medium to a final concentration of 100nM. After 2 hours the medium was removed, cells washed with serum-free medium and normal culture medium replaced. For 'serum shock' confluent cells were first serum starved by replacing medium containing 10% FBS with medium containing 0.5% FBS (after washing with serum-free medium). After 24 hours medium was removed and replaced with medium containing 50% horse serum (Invitrogen) for 2 hours. This medium was then removed and cells washed twice with serum-free medium before replenishment with 0.5% FBS medium. With both treatments cells samples were taken before treatment (T0), 2 hours after treatment (T4) and for every 4 hours for 50 hours and RNA extracted, as described in section 2.1.2.

2.4.3 Transfection

An appropriate number of cells were seeded into 24-well plates (end-point luciferase reporter assays), 6-well plates (over-expression assays) or 96-well plates (real-time luciferase reporter assays) to give approximately 70% confluency when transfected using jetPEI cationic polymer transfection reagent (Q-BIOgene). Co-transfections of promoter-reporter plasmids and over-expression plasmids were carried out according to the manufacturer's protocol with a ratio of 2µl jetPEI:1µg DNA, which was found to be optimal. DNA amounts used were kept constant by adding empty pcDNA3.1(+) vector (Invitrogen) which was also used as a negative control. 10ng Renilla reporter plasmid per well

(Promega) was co-transfected in end-point luciferase assay experiments to correct for variations in transfection efficiency. Media was changed 24 hours post-transfection.

For over-expression assays cells were lysed with 1ml TRIzol (Invitrogen) per well (6 well plate) and RNA was extracted (as described in section 2.1.2).

2.5 Transcriptional activation assays

2.5.1 End-point luciferase assays

48 hours post-transfection cells were washed with phosphate-buffered saline (PBS) and lysed with 100µl 1X Passive Lysis Buffer (Promega) on a rocking platform for 15 minutes. Lysates were transferred to microcentrifuge tubes and insoluble material was removed by centrifugation at 10000rpm for 30 seconds. Firefly and *Renilla* luciferase activity was measured using a Dual Luciferase Reporter Assay Kit (Promega). 20µl cell lysate was added to 100µl Luciferase Assay Reagent II and the luminescence produced measured by a luminometer (Berthold). *Renilla* luciferase activity was measured by adding 100µl 1X Stop & Glo Reagent (Promega).

2.5.2 Real-time luciferase assays

Cells were grown in 6-well plates and transfected with 1.5µg destabilised luciferase reporter plasmids per well following the protocol outlined in section 2.4.3. After 16 hours cells were trypsinised with 0.5ml trypsin per well, pelleted by centrifugation for 5 minutes at 1500rpm and resuspended in 300-600µl cell culture medium. Following counting cells were plated at a density of 20000 cells/well into multiple wells of a white 96-well plate and grown to 100% confluency (24 hours). Cells were then treated with dexamethasone (as described above) for 2 hours. After removal of the medium and dexamethasone cells were washed twice with DMEM that did not contain phenol red (31053; Invitrogen) prior to the addition of assay medium (DMEM without phenol red, 4mM GlutaMAX-I supplement (Invitrogen), 1M sodium pyruvate (Invitrogen), 0.1mM beetle luciferin). After incubation for 15 minutes at 37°C bioluminescence was measured every 2-4 hours for 74 hours in a BMG NOVOstar plate reader at 37°C.

3. Circadian profiling of gene expression in the mouse heart

3.1 Introduction

Circadian clocks have been identified in key components of the CVS. A number of studies have reported daily oscillations in the expression of circadian clock genes in extracts from whole rodent tissues, such as heart and aorta, and cardiovascular cell types, such as cardiomyocytes and vascular smooth muscle cells (VSMCs) (Young, 2006). It has also been noted that the intrinsic properties of the heart and vasculature oscillate significantly over the course of a day. Physiological circadian variations in blood pressure, heart rate and cardiac output have been documented and disturbance of the diurnal blood pressure rhythm has been associated with hypertension (Lemmer, 1996) and an increased risk of cardiovascular events and left ventricle remodelling (Izzedine *et al.*, 2006; Seo *et al.*, 2006).

Diurnal changes in CVS functions are mediated by the interaction of both extracellular (i.e. neurohormonal factors) and intracellular (i.e. circadian clock) influences. The extent to which diurnal variations in cardiac physiology are mediated through neurohormonal mechanisms and the environment or by intrinsic properties of the heart is unknown. However, variations in heart function have been observed in *ex vivo* experiments where the influence of external endocrine cues is removed so the changes seen are likely mediated by changes in gene and protein expression. *Ex vivo* the rat heart shows pronounced diurnal variations in contractile function and oxidative metabolism (Young *et al.*, 2001b) and tolerance to oxidative stress (Lapenna *et al.*, 1992), and diurnal variations of the electrical properties of cardiomyocytes have been reported (Yamashita *et al.*, 2003).

Circadian clocks likely evolved over time to allow an organism to better adapt to the external 24-hour environment. Rhythmic changes in gene expression and thus the physical properties of the heart will therefore allow optimal function and enable the heart to respond to external stimuli in synchrony with the environment. Impairment of this mechanism and the subsequent inability to anticipate and respond to the environment in an appropriate way may lead to myocardial dysfunction. The identification of genes that are regulated by the clock (clock-controlled genes, CCGs) will therefore point to clock-regulated processes within the heart that can become dysregulated when the internal clock is disturbed.

Previous studies suggest that at least 8% of the cardiac transcriptome cycles and cycling transcripts are involved in a wide range of processes (Storch *et al.*, 2002). Due to the limitations of microarrays this number is likely to be an underestimate (see section 3.4.3) and important targets of the cardiac circadian clock may have been missed. Other studies have also suggested the circadian expression of cardiac genes that were not identified in the microarray study by Storch and colleagues (see below).

The aim of this chapter was to examine the temporal expression of selected key genes involved in heart function (such as metabolism, electrophysiology and transcription) and dysfunction (such as hypertrophy). Genes that have been suggested to cycle in previous studies were selected as well as those which have not been linked to the clock in order to validate the results of those studies. The circadian expression of core clock genes and outputs was also examined to validate the techniques and samples used.

Microarray studies have identified groups of genes with variable functions that exhibit altered expression in hypertrophy and heart failure (Wagner *et al.*, 2004; Wellner *et al.*, 2005). Cycling of these genes may therefore enable the heart to better respond to hypertrophic stimuli and stress at different times of the day. Hypertrophy is characterised by the up-regulation of genes involved in foetal development, including *Brain (*or *B-type) Natriuretic Peptide (Bnp)* and *Atrial (*or *A-type) Natriuretic Peptide (Anp)* in the ventricles (Gardner, 2003; Rajabi *et al.*, 2007). BNP and ANP are cardiac hormones predominantly produced in the ventricles and atria of the heart respectively and may limit the myocardial hypertrophic response by lowering blood pressure and inhibiting myocyte proliferation and fibrosis (Rosenkranz *et al.*, 2003; Franco *et al.*, 2004).

Connective Tissue Growth Factor (CTGF) is a secreted growth factor that is expressed in a number of tissues and cell types and is involved in extracellular matrix remodelling, fibrosis and the hypertrophic response (Moussad and Brigstock, 2000; Matsui and Sadoshima, 2004). It is also expressed in fibrotic lesions in many tissues and after cardiac ischemia in human and rodent hearts (Chen *et al.*, 2000; Ahmed *et al.*, 2004; Chuva de Sousa Lopes *et al.*, 2004; Gabrielsen *et al.*, 2007). Circadian regulation of *Ctgf* has not been previously described in the heart but strong cycling has been observed in the mouse aorta and kidney in a transcriptome microarray study (Su *et al.*, 2002). The actions of *Ctgf* and *Bnp* in cardiac fibrosis and remodelling are opposed. CTGF is profibrotic whereas BNP has antifibrotic properties and TGF-β-induced regulation of *Ctgf* is abrogated by BNP (Kapoun *et al.*, 2004; Kemp *et al.*, 2004; Koitabashi *et al.*, 2007). Structural remodelling is a key determinant of clinical outcome in heart disease and cardiac fibrosis (disproportionate accumulation of fibrillar collagen) is a major aspect of this process. Fibrosis is generally detrimental as the accumulation of collagen stiffens the ventricles, impeding contraction and relaxation, can impair the electrical coupling of cardiomyocytes, and can decrease oxygen diffusion and so lead to hypoxia (Manabe *et al.*, 2002). The zinc-finger transcription factor Krüpple-like Factor 5 (KLF5/BTEB2) is also an essential factor in cardiovascular remodelling, activating many genes involved in this process, thus linking external stress to remodelling (Nagai *et al.*, 2005). The balance between levels of these factors is likely to be important in myocardial fibrosis and heart failure (Koitabashi *et al.*, 2007) and circadian regulation of these genes could provide multiple targets for the clock to fine-tune the fibrotic response and remodelling over the course of a day. A temporal fibrotic response may also govern compensatory

remodelling following stress and damage caused by diurnal variation in workload, suggesting a protective physiological role of the cardiac clock.

Myocyte Stress 1 (*Ms1/Abra; actin-binding Rho activating protein*) is predominately expressed in cardiac and skeletal muscle and was identified as a gene up-regulated in the early stages of pressure overload-induced left ventricular hypertrophy in the rat, where it may be involved in the initial signalling of the hypertrophic response (Mahadeva *et al.*, 2002). Whether the heart enters into hypertrophy may therefore depend on the circadian regulation of *Ms1*. Expression of *Ms1* can be regulated by the stress-responsive cardiac transcription factors Myocyte Enhancer Factor 2c (MEF2C) (Kuwahara *et al.*, 2007) and GATA4 (S. Ounzain, personal communication). Both of these transcription factors are involved in the hypertrophic response (Molkentin, 2000; Ren *et al.*, 2007), and MS1 may play an intermediary role. It has been recently proposed that GATA4 may be involved in circadian regulation by activating the *Bmal1* promoter, which is attenuated by a single nucleotide polymorphism (SNP) that is associated with hypertension and type 2 diabetes (Woon *et al.*, 2007).

The extent of ischemia, followed by reperfusion, can also contribute to the clinical outcome following MI. A recent study has identified that clock gene oscillations (peak-to-trough fold differences) were rapidly attenuated in the ischemic regions of rat hearts following ischemia-reperfusion (IR) (Kung *et al.*, 2007). Disruption of circadian expression of the gene *Apoptosis Repressor with CARD domain* (*Arc/Nol3; Nucleolar protein 3*) may be one mechanism that contributes to myocardial damage following IR. ARC is an inhibitor of apoptosis that is expressed in skeletal muscle and the heart and interacts selectively with caspases (Koseki *et al.*, 1998). It is believed to have a protective role against cardiomyocyte death following ischemia and mechanical stress and is found at lower levels in the failing human heart (Donath *et al.*, 2006). Myocardial dysfunction following IR may therefore be a consequence of loss of circadian regulation and there may be a temporal window when the heart can better recover. Apoptosis induced by oxidative stress has also been implicated in IR (Fliss and Gattinger, 1996) and ARC may provide protection against this (Zhang and Herman, 2006). Oxidative stress occurs when there is excessive production of reactive oxygen species (ROS), primarily produced by mitochondrial metabolism. Several peripheral tissues, including the heart, show diurnal variations in their antioxidant capacity (Lapenna *et al.*, 1992; Young, 2006), which may also be in part mediated by cyclic expression of *Arc*.

Genes involved in enabling the heart to adapt to different metabolic states are also important in preventing dysfunction. The normal heart under resting conditions obtains about 70% of its energy from fatty acid oxidation and the majority of the remaining 30% from glucose oxidation (Abel *et al.*, 1999). The heart also possesses the ability to switch between the use of fatty acids and glucose according to the nutritional state, physical activity and diurnal substrate availability. For instance, by switching from utilising fatty acids to carbohydrates, which is more efficient in terms of the amount of

oxygen consumed in ATP generation, the risk of ischemia can be minimised (Young, 2006). This substrate flexibility is lost in various myocardial disease states, which is believed to contribute to observed heart failure and sensitivity to ischemic injury (Taegtmeyer, 1994; Burkart *et al.*, 2007).

Key regulators of fatty acid metabolism that may confer circadian regulation are Peroxisome Proliferator-Activated Receptors (PPAR) α and γ. PPARs activate many genes involved in several aspects of fatty acid metabolism via the Peroxisome Proliferator Response Element (PPRE) (Wahli *et al.*, 1995; van Bilsen *et al.*, 2002). Expression of rodent *Pparα* displays circadian rhythmicity in the *ex vivo* heart (Young *et al.*, 2001b), aorta (Rudic *et al.*, 2005) and liver (Lemberger *et al.*, 1996) and CLOCK:BMAL1 transactivate *Pparα* (Oishi *et al.*, 2005c), providing a mechanism by which the clock is able to regulated rhythmic fatty acid metabolism.

The PPRE and REV-ERBα/RORα Response Element (RRE) sequences overlap and it has been proposed that REV-ERBα can attenuate PPARα-mediated transcription (Kassam *et al.*, 1999) so PPARα and REV-ERBα could operate antagonistically to control circadian regulation of PPAR target genes. PPARα and PPARγ may also affect clock output since they can induce REV-ERBα expression (Fontaine *et al.*, 2003) and PPARα is required to maintain rhythmic expression of *Bmal1* in the mouse liver, via binding to a PPRE (Canaple *et al.*, 2006). In addition, the rhythmic expression of various genes involved in lipid metabolism is attenuated or abolished in *Pparα* knock-out mice (Patel *et al.*, 2001; Gibbons *et al.*, 2002). These mechanisms of interaction indicate that PPAR, particularly PPARα, and REV-ERBα likely represent a mode of cross-talk between circadian and fatty-acid-responsive signalling pathways in the liver (Fontaine and Staels, 2007). A similar mechanism in the heart would enable response to, and anticipation of, daily variations in fatty acid availability. In addition to being involved in cardiac metabolism PPAR have been implicated in hypertrophy (Finck, 2007) and are also protective against oxidative stress (Diep *et al.*, 2002; Tao *et al.*, 2003). Therefore, PPAR are important molecules in the physiological and pathological regulatory processes of the heart.

The ability to adapt to diurnal variations in fatty acid availability may also be mediated by Uncoupling Protein 2 and 3 (UCP2 and UCP3), two mitochondrial proteins involved in the metabolism of fatty acids as an energy source (through β-oxidation) (Young *et al.*, 2001a; Murray *et al.*, 2005). Circadian expression of *Ucp2* and *Ucp3* has previously been observed in the whole rat heart *ex vivo* (Young *et al.*, 2001b) and in isolated, synchronised rat cardiomyocytes (*Ucp3*) (Durgan *et al.*, 2005; Durgan *et al.*, 2006). Expression of *Ucp2* and *3* is regulated by PPARα in the heart, potentially making them second-order CCGs. Cyclic expression of UCP2 may be important in protecting the heart against ischemia as it helps prevent the accumulation of ROS and mitochondrial Ca^{2+} overload in cardiomyocytes (Teshima *et al.*, 2003; McLeod *et al.*, 2005).

While glucose is not the main fuel source in the resting heart it becomes more important during states of increased workload, ischemia and pressure overload-induced hypertrophy. In these states the use of the glucose transporter GLUT4 increases over its isoform, GLUT1 (Abel, 2004). *Glut4* has been shown to be rhythmically expressed in the heart in parallel with glucose metabolism (Young *et al.*, 2001b), and may be involved in the adaptation of the heart in pathological conditions.

The ability of the heart to adapt to pathological events may also be modulated by diurnal variations in the intrinsic electrical properties of the heart, which have been linked to the rhythmic expression of the potassium channels Kv1.5 (KCNA5) and Kv4.2 (KCND2) (Yamashita *et al.*, 2003). Like many proteins mentioned above, Kv4.2 may be involved in the hypertrophic response since Kv4.2 over-expression in cultured rat neonatal cardiomyocytes prevented phenylephrine-induced cell hypertrophy (Zobel *et al.*, 2002) and over-expression of a dominant-negative form of Kv4.2 induced cardiomyocyte hypertrophy (Kassiri *et al.*, 2002).

Many of these important genes are transcription factors and regulation by the clock could be transmitted to wider gene networks through them. The core clock factors CLOCK and BMAL belong to a large family of bHLH factors that can heterodimerise and bind E-box and E-box-like sequences (Massari and Murre, 2000). This presents the intriguing possibility of co-operation and cross-talk between the circadian clock and a number of other pathways and the potential for the clock to be influenced by the internal environment and vice-versa. Depending on E-box-binding strength, bHLH proteins may out-compete clock factors until a specific level is reached, providing a rheostat mechanism of control. Of particular interest in the CVS is the potential link between the clock and hypoxia. In humans and rodents, hypoxia can interfere with diurnal variations in physiological properties, such as body temperature, hormone levels and cardiovascular parameters, therefore the alteration of circadian control may cause many of the physiological changes seen in hypoxia (Mortola, 2007). Hypoxia may influence circadian rhythms at a molecular level (see Chapter 6). HIF-1α and EPAS1 (HIF-2α) are two oxygen-responsive proteins that are important in heart function and disease states and can form functional heterodimers with BMAL1 (Hogenesch *et al.*, 1998; Takahata *et al.*, 1998). Circadian expression of *Hif-1α* has been shown in a microarray study of the mouse heart (Storch *et al.*, 2002).

Epas1 knock-out mice display a severe phenotype with multiple-organ pathology, including cardiac hypertrophy, metabolic abnormalities and altered gene expression patterns (Scortegagna *et al.*, 2003) and EPAS1 may play a role in the adaptation of cardiac myocytes during heart failure (Tanaka *et al.*, 2002). Circadian regulation of *Epas1* may therefore be important in enabling the heart to respond to hypoxic stress during the active phase and allow the core clock mechanism to adapt to hypoxia.

Constitutive and abundant E-box-binding factors, such as Upstream Stimulatory Factors (USF) 1 and 2, might also interfere with the binding of clock factors to E-boxes. USF1 and USF2 are ubiquitously expressed and are key regulators of a number of gene regulation networks through binding the consensus E-box element CANNGT, with a higher affinity for the canonical E-box CACGTG (Lin *et al.*, 1994; Corre and Galibert, 2005). They are stress-responsive and DNA-binding activity of USF1 can be modulated by multiple signal transduction pathways (Corre and Galibert, 2005). USFs may therefore represent a pathway for the integration of the circadian clock and extra-cellular stress.

bHLH-PAS domain proteins such as CLOCK, BMAL1, EPAS1 and HIF-1α all belong to Class VII of the HLH super family (Massari and Murre, 2000). Class V of this family lack the basic DNA-binding domain and have been characterised as dominant-negative regulators of Class I and II HLH proteins. This class includes the ID (Inhibitor of DNA binding) proteins (ID1-4) (Massari and Murre, 2000). Since the ID proteins and Class VII factors all contain HLH dimerisation domains it is conceivable that the ID proteins may be involved in the regulation of the molecular clock or clock-controlled genes. ID proteins are involved in regulating differentiation, tumourigenesis and the cell cycle (Ruzinova and Benezra, 2003) but may also have important roles in the heart due to a possible role in cardiac myocytes apoptosis (Tanaka *et al.*, 1998) and the severe cardiac defects seen in *Id* knockout mice embryos (Fraidenraich *et al.*, 2004).

3.2 Methods

3.2.1 Gene expression analysis

Mouse heart samples were kindly obtained from Dr. E. Maywood, Division of Neurobiology, MRC Laboratory of Molecular Biology, Cambridge. Adult male CD-1 mice (Harlan-Olac) were caged in groups of eight, in sound-proofed, ventilated environmental chambers and entrained to a lighting schedule of 12 hr bright white light and 12 hr dim red light (12L:12DR). On release into continuous dim red light (DR:DR) entrainment was confirmed by monitoring spontaneous activity rhythms. During the second cycle of DR:DR hearts were harvested at Circadian Time (CT) 0, 4, 8, 12, 16 and 20. Between 4 and 6 mice were used per time-point, with each being sacrificed on a separate day. RNA was extracted from hearts by homogenisation with TRIzol reagent and relative transcript abundance determined by quantitative and semi-quantitative RT-PCR as described in Chapter 2. For semi-quantitative PCR, and SYBR Green quantitative PCR transcript analysis the protein folding enzyme *Cyclophilin A* (*Peptidylprolyl isomerase A*) was used as an endogenous control gene and constant expression levels were confirmed by the use of another control gene, the ribosomal protein *36b4* (*Acidic ribosomal phosphoprotein P0)*. The ubiquitous transcription factor *Tbp* was used as the control gene for Taqman quantitative PCR. The choice of endogenous template control gene is important since it may influence the observed rhythms if the control gene themselves cycle or are regulated by conditions that differ between mice (Thellin *et al.*, 1999; Bustin, 2000). For example, circadian expression of the transcript for the commonly used endogenous control gene *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) has been shown in the rat retina (Kamphuis *et al.*, 2005) and *Neurospora crassa* (Shinohara *et al.*, 1998) and transcript levels of *GAPDH*, *β-actin* and *Cyclophilin* varied widely with hypoxia (Zhong and Simons, 1999). The use of two control gene, as in this study, minimises the risk of spurious results. The use of single-copy genes, such as *Cyclophilin A*, over the use of genes encoding for ribosomal RNA, such as *18S* or *28S* (which are abundantly expressed and better controls for RNA detection, e.g. Northern Blotting) can also improve the accuracy of detection in RT-PCR where the target transcripts are expressed at a similar level to the controls (Thellin *et al.*, 1999; Zhong and Simons, 1999; Vandesompele *et al.*, 2002). See Table 3.1 and Table 3.2 for details of primers and probes used.

Table 3.1. Primer sequences used in semi-quantitative and SYBR green quantitative RT-PCR. m=mouse, r=rat, h=human.

Gene	Primer sequence	Cycle number
mrAnp	F: GGATTTCAAGAACCTGCTAGAC R: GGAAGCTGTTGCAGCCTAGT	20
mArc	F: CGGAGTTCGAAAAATGGGC R: CTTGCTCTGACGCTCTTGGC	22
mBmal1 *(Splice variants b, b' & g')*	F: AGAGGCGTCGGGACAAAATGA R: GGGTTGGTGGCACCTCTCAAA	33
mrBmal2 *(Splice variants a & b)*	F: TGGAAAAGCGTCGGAGAGACA R: AGCCTTCTGCTGCCTTGAGGA	36
mBnp	F: CAAGATGCAGAAGCTGCTGG R:CAACAACTTCAGTGCGTTACA	22
mClock	F: GAGCAGCGGACACGGATGATA R: GGGACAACCTGGCCTTGCATA	31
mCry1	F: ACAGCATCCGCTGCGTCTAT R: GCTGTCCGCCATTGAGTTCT	31
mCry2	F: TTCCCAAGGCTGTTCAAGG R: CAAGTCCCACAGGCGGTAGT	31
mCtgf	F: CCTAGCTGCCTACCGACTGG R: GCCCTTCTTAATGTTTTCCTCC	28
mCyclophilin A	F: CCACCGTGTTCTTCGACATC R: CTGGGAACCGTTTGTGTTTG	25
mDbp	F: CCACCGCGCAGGCTTGAC R: ACTTCTCATCCTTCTGTTCCTC	31
mDec1	F: TGGTGATTTGTCGGGAAGAA R: CCACTGTCTGTGTCCGTGTC	30
mDec2	F: GGGATTTTATAGGACTGGACT R: AGCGCTCCCCATTCTGTAAAG	32
mE4bp4	F: CTTGCAGGTCCATGGGTCC R: TCGGCGGGTGAAGAGAGTT	29
mEpas1	F: CCATGTTCAAGATGAGGTCTGC R: CACGCCTGACACCTTTTGAGC	27
mGata4	F: TCCTACTCCAGCCCCTACCC R: CGGCGCTGAGGCTTAATGA	32

mrGlut4	F: CGTTGGTCTCGGTGCTCTTAG R: GAAGCCAAGCAGGAGGACGG	22
mHif-1α	F: CACCGATTCGCCATGGAGGG R: CTCACTGGGCCATTTCTGTGT	32
mId1	F: CCTGGTCTGTCGGAGCAAAG R: GATCGTCGGCTGGAACACAT	29
mId2	F: AAAGCCTTCAGTCCGGTGAG R: GATGCTGATGTCCGTGTTC	29
mId3	F: GTGCTGCCTGTCGGAACGTA R: GCTCAGCTGTCTGGATCGGG	28
mKlf5	F: CGTCACCACCAAGCTCAGA R: ACCTCCAGTCGCAGCCCTC	34
mKv1.5	F: GTGCCCGTCATCGTCTCCA R: CCTTCGCAGGTCCACGTTG	30
mKv4.2	F: CAACACTGGGGTATGGCGACA R: CTCCGAGGACTGCAGCTGGT	29
mMef2c	F: AGATAGTGTCATGTTGCAGGTTCA R: CTCATGGCTTAGGGATGTGCTTTC	30
mrhMop4	F: ACTCCAACGTCCAGATGTTTC R: AGCCTGAGCTGCCGATCAT	32
mrMs1	F: TGACAGCATAGACACAGAGGAC R: CACTGCTGCCACCTGCCTT	28
mPai-1	F: GGCCAATGGAAGACCCCTTTC R: AGCCTGGTCATGTTGCCCTTC	29
mPer1	F: CTGGGGACCAGGTCATTAAG R: GTCGACACACGCCATCACAT	29
mPer2	F: ATTAGACGGTGCTCGGAAGA R: ATGGTCCAAACCACGTAAGG	29
mPer3	F: TCTGCTGCTGCCTCAGGAAG R: GGCGACACAGCTCTGGAGGT	29

mPparα	F: GGCTGTAAGGGCTTCTTTCG R: TGTATGACAAAAGGCGGGT	30
mPparγ	F: GAATACCAAAGTGCGATCAAAG R: ACCTGATGGCATTGTGAGAC	31
mhRev-erbα	F: AGACATGACGACCCTGGACTC R: CACCATGCCATTCAGCTTG	28
mRorα	F: TATGCGAGCTCCAGCCGAGG R: GGCGTACAAGCTGTCTCTCT	34
mUcp2	F: TCCGGCTGCAGATCCAAGG R: GTCTTGTAGGCTTCGACAGTG	27
mrUcp3	F: CTGTGGAAAGGGACTTGGC R: GGGCACAAATCCTTTGTAGA	28
mUsf1	F: CTATCGCCAGCATCCAGTC R: CCCCAGTAGTGCCTCTGAGC	30
mUsf2	F: TCAGCACAATGAAGTGGAACG R: GACAGCAGCTGAAGGGTGAG	28

Table 3.2. Taqman probes used in real-time quantitative RT-PCR.

Gene	Probe catalogue number	Transcripts bound	Exons bound (across boundary)
mBmal1 (Arntl)	Mm00500226_m1	*Arntl (NM_007489)* *mBmal1b (AB015203)* *mBmal1b' (AB012601)* *mBmal1g' (AB012602)*	11/12 7/8 8/9 7/8
mPer1	Mm00501813_m1	*mPer1 (NM_011065)*	18/19
mCry1	Mm00514392_m1	*mCry1 (NM_007771)*	1/2
mMop4 (Npas2)	Mm00500848_m1	*mMop4 (NM_008719)*	5/6
mDbp	Mm00497539_m1	*mDbp (NM_016974)*	1/2
mBmal2 (Arntl2)	Mm00549497_m1	*mArntl2 (NM_172309)* *mBmal2a (AY005163)* *mBmal2b (AY014836)*	7/8 7/8 7/8
mDec1 (Bhlhb2, Stra13)	Mm00478593_m1	*mDec1 (NM_011498)*	4/5
mPai-1 (Serpine1)	Mm00435860_m1	*mPai-1 (NM_008871)*	7/8
mBnp (Nppb)	Mm00435304_g1	*MBnp (NM_008726)*	1/2
mTbp	Mm00446973_m1	*mTbp (NM_016974)*	1/2

3.2.2 Rat ventricular cardiomyocyte isolation

Rat ventricular cardiomyocytes were kindly isolated by Dr. G. Rodrigo and Ms. H. Collins, Cardiology Group, Department of Cardiovascular Sciences, University of Leicester, using the method previously reported in Lawrence and Rodrigo (1999) and modified from the method described by Mitra and Morad (1985). Adult male Wistar rats were entrained to a 12 hours light:12 hours dark cycle for six weeks and sacrificed at Zeitgeber Time (ZT) 2 or ZT14. The chest cavity was opened by thorocotomy and the heart was rapidly excised with some aorta and transferred to calcium-free Tyrode solution (4-10ºC) (135mM NaCl, 6mM KCl, 0.33mM NaH_2PO_4, 5mM Na Pyruvate, 10mM Glucose, 10mM HEPES, 1mM $MgCl_2$, pH 7.4). For retrograde perfusion the aorta was cannulated with a flanged cannula secured distal to the coronary vessels. Pressure caused by perfusion was released by a small piece of tubing placed in the left ventricle if necessary. The heart was perfused with calcium-free Tyrode for six minutes followed by the addition of Type I Collagenase and Type XV Protease for 10 minutes to break down the heart's connective tissue and isolate single myocytes by enzymatic digestion. Enzyme-containing Tyrode was washed off with normal Tyrode (as calcium-free

Tyrode + 2mM $CaCl_2$) for three minutes and the atria were removed. During the final 30 seconds Tyrode was collected in a flat bottomed conical flask, into which the heart was placed. The heart was then coarsely minced with scissors and shaken at 37^{O}C in the flask. When the Tyrode solution became turbid with cells the supernatant was decanted and heart placed into a new flask with fresh Tyrode with shaking at 37ºC. The process was repeated until the heart was completely dissolved. Cells were passed through a 200μm^2 pore stainless steel sieve and left to settle for five to ten minutes. The supernatant was removed and cells washed three times in fresh Tyrode followed by resuspension in Tyrode. After transport and approximately two hours after rats were sacrificed the Tyrode solution was removed and approximately 2-3 x10^6 cells were lysed by the addition of 1ml TRIzol reagent with one minute of disruption with a hand-held rotor-stator homogeniser (IKA T8 Ultra Turrax hand held homogeniser with 5mm dispersing element). The homogenised cells were then centrifuged at 13,000rpm for 10min at 4ºC and the supernatant transferred to room temperature for 5 minutes. 0.2ml of chloroform was then added and RNA extraction was continued as described in Chapter 2.

3.2.3 Bioinformatics

Comparative sequence analysis was carried out using the Evolutionary Conserved Regions (ECR) Browser (Ovcharenko *et al.*, 2004) from NCBI DCODE.org Comparative Genomics Developments (http://www.dcode.org/). Conserved predicted transcription factor binding sites where identified from ECRs by rVISTA 2.0 (Loots and Ovcharenko, 2004), also available from DCODE, using the TRANSFAC professional library V10.2.

3.2.4 Statistical analysis

Statistical analysis was performed by one-way ANOVA with the null hypothesis that all data points were the same. The null hypothesis was rejected if $P<0.05$, indicating a significant difference. Significant results were further analysed using Newman-Keuls *post-hoc* multiple comparison tests.

3.3 Results

3.3.1 Circadian expression of clock genes in the mouse heart *in vivo*

To assess the functionality of the circadian clock in the mouse heart the mRNA levels of several mouse homologues of the molecular clock genes were examined. Semi-quantitative and quantitative real-time RT-PCR with Taqman probes was carried out on hearts taken at 4 hour intervals from circadian time (CT) 0 to CT20 as described in section 3.2.1. For semi-quantitative PCR the optimal cycle number for each specific primer pair was first identified by plotting the amount of product produced from pooled cDNA samples against cycle number. The cycle number where amplification was in the linear phase was used for subsequent PCR reactions. Two example optimisation graphs are shown in Figure 3.1 and cycle numbers used are listed in section 3.2.1. Good agreement was seen between quantitative and semi-quantitative PCR, confirming that semi-quantitative PCR is an appropriate method to look at circadian transcript cycling. Robust circadian rhythms in mRNA expression were observed for the core clock genes *Bmal1*, *Per1* and *Cry1* (Figure 3.3) and *Per2* and *Per3* (Figure 3.4) in the mouse heart, as summarised in Table 3.3. An example of a semi-quantitative RT-PCR gel picture is shown in Figure 3.2. The temporal expression of *Cry2* and *Clock* measured by semi-quantitative RT-PCR (Figure 3.4) was more variable and significant transcript cycling was not observed.

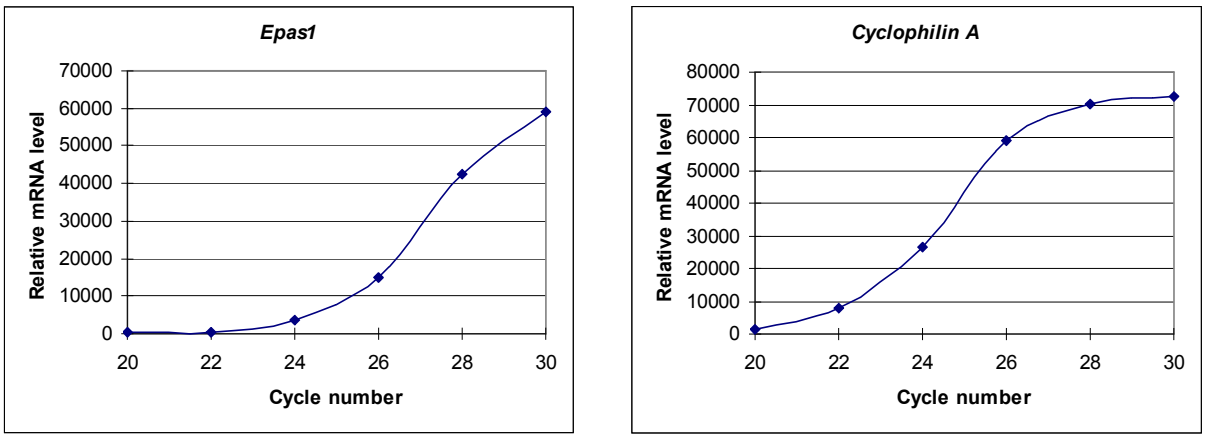


Figure 3.1. Examples for cycle number optimisation for semi-quantitative PCR. The *Epas1* and *Cyclophilin A* transcripts were amplified from pooled cDNA from mouse hearts in six identical reactions performed at different cycle numbers. Subsequent PCR reactions were carried out at 27 and 25 cycles respectively.

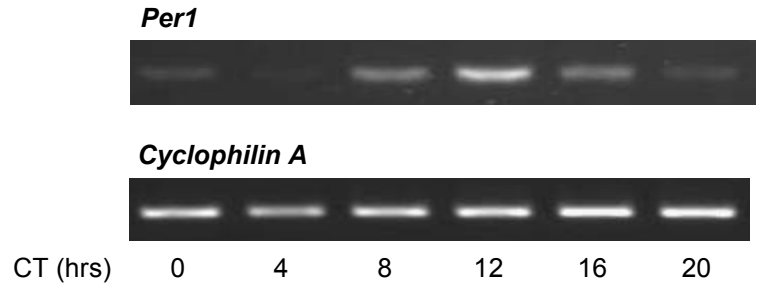


Figure 3.2. An example of an agarose gel image from semi-quantitative RT-PCR. End-point semi-quantitative PCR reactions performed with mouse heart cDNA template and specific primer pairs for *Per1* and *Cyclophilin A* were separated on an agarose gel by electrophoresis and the reaction products were visualised and quantified under ultra-violet light as described in Chapter 2.

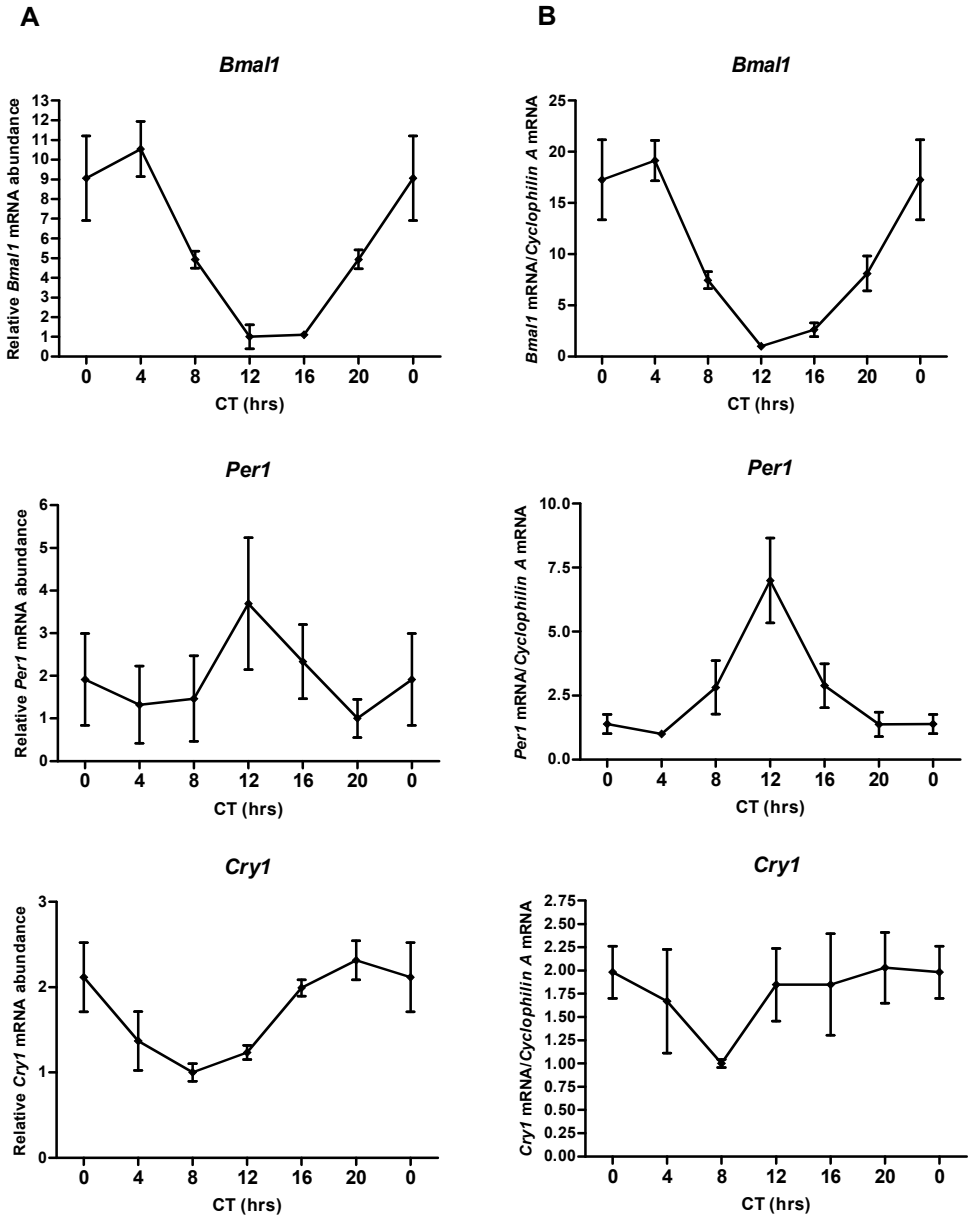


Figure 3.3. Circadian mRNA expression of clock genes in the mouse heart. (A) Taqman quantitative real-time RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *TATA box binding protein (mTbp)*. (B) Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for three hearts per point (two-three observations) and are standardised to the lowest expression point. CT0 is double-plotted.

ANOVA $P<0.01$ for all graphs except *Per1* Taqman (A) and *Cry1* semi-quantitative (B).

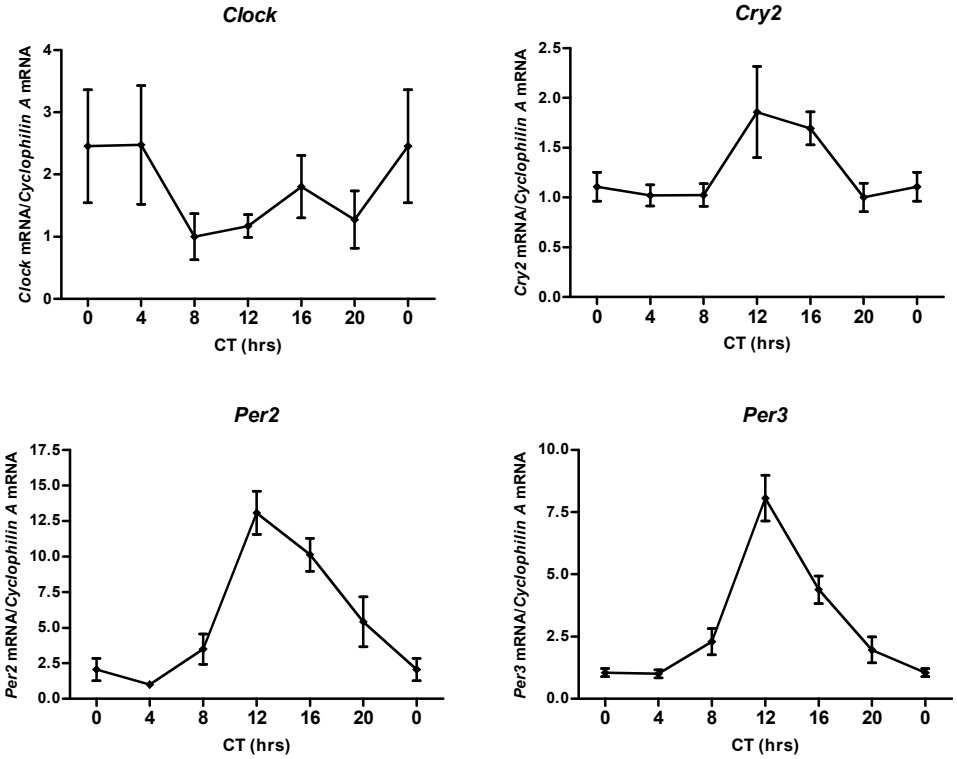


Figure 3.4. Circadian mRNA expression of clock genes in the mouse heart. Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for three hearts per point (two-three observations) and are standardised to the lowest expression point. CT0 is double-plotted. ANOVA $P<0.01$ for *Per2* and *Per3*, $P<0.1$ for *Cry2*, $P>0.1$ for *Clock*.

Table 3.3. Observed cycling of core clock genes in the mouse heart. Peaks and troughs significantly different from surrounding time-points were identified by *post-hoc* analysis with Newman-Keuls multiple comparison test, $P<0.05$. q=quantitative RT-PCR (Taqman), sq=semi-quantitative RT-PCR.

Gene	Peak (CT)	Trough (CT)	Fold difference (peak-trough)	$F_{5,12}$	P-value	Significance
Bmal1 (q&sq)	0-4	8-16	11 (q)-19 (sq)	12.6 (q) & 14.4 (sq)	0.0002 (q) & 0.0001 (sq)	**
Per1 (q&sq)	12	20-4	4 (q)-7 (sq)	1.0 (q) & 6.0 (sq)	0.463 (q) & 0.005 (sq)	**
Per2 (sq)	12	0-4	13	16.2	<0.0001	**
Per3 (sq)	12	0-4	8	25.46	<0.0001	**
Cry1 (q&sq)	20	8	2.5	4.8 (q) & 0.87 (sq)	0.012 (q) & 0.528 (sq)	**
Clock (sq)				1.1	0.422	NS
Cry2 (sq)				3.0	0.058	NS

REV-ERBα is a target of the CLOCK:BMAL1 heterodimer and together with RORα forms a subsidiary loop of the molecular clockwork. Strong circadian expression of *Rev-erbα* that is anti-phase to that of *Bmal1* was observed (Figure 3.5A and Table 3.4) as expected from the feedback model. *Rorα* expression did not show significant temporal variations and there was a high amount of disagreement between transcript levels at different time-points (Figure 3.5B). The bHLH factors *Dec1* and *Dec2* also form a subsidiary loop and appeared to be rhythmically expressed in phase with *Per1* (Figure 3.5C & D and Table 3.4). *Dec2* expression peaked slightly after *Dec1*, as previously observed in the rodent SCN (Honma *et al.*, 2002).

Expression of the BMAL1 binding partner *Mop4* was also significantly variable. Like *Bmal1*, higher transcript levels were observed at CT4, although due to variability at this time-point *post-hoc* analysis did not reveal this peak to be significant (Figure 3.6 and Table 3.4). The *Bmal2* transcript, a paralogue of *Bmal1*, appeared to exhibit some temporal changes in abundance but there was little agreement between samples and these results were not significant (Figure 3.6).

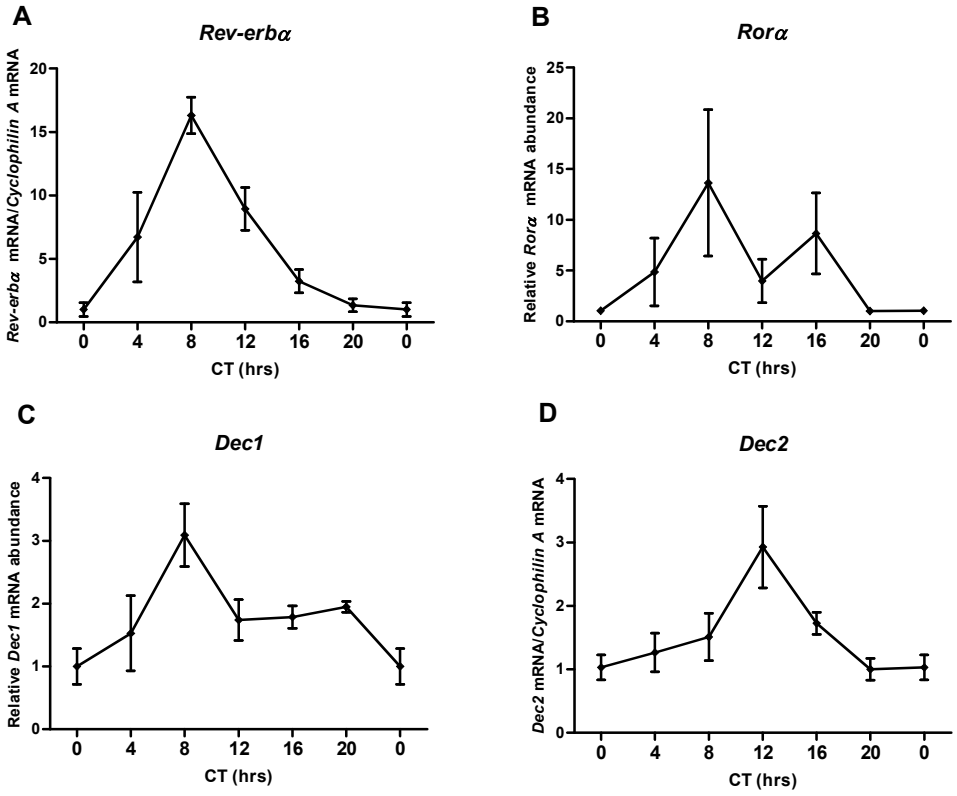


Figure 3.5. Circadian mRNA expression of clock genes in the mouse heart. Semi-quantitative (A and D) and SYBR green quantitative real-time (B) RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. (C) Taqman quantitative real-time RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *TATA box binding protein (mTbp)*. Values are the mean ±SEM for three hearts per point (two-three observations) (A, C and D) or 4-6 hearts per point (one observation) (B) and are standardised to the lowest expression point. CT0 is double-plotted. ANOVA $P<0.05$ for all graphs except *Rorα*.

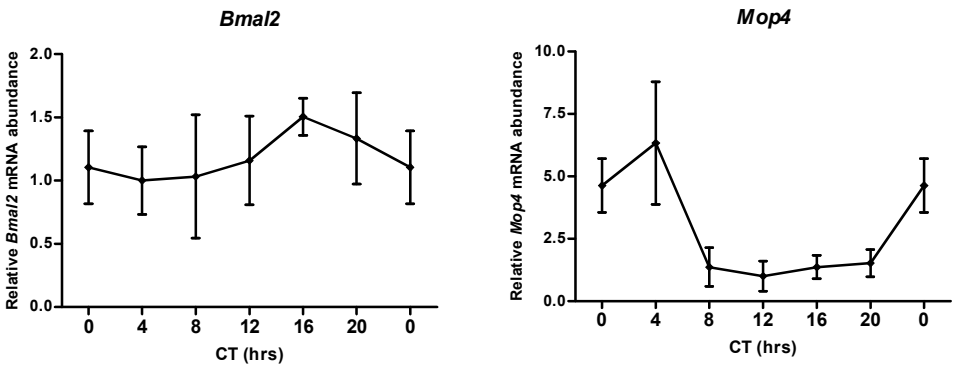


Figure 3.6. **Circadian/temporal mRNA expression of clock-related genes in the mouse heart.** Taqman quantitative real-time RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *TATA box binding protein (mTbp)*. Values are the mean ±SEM for 4-6 hearts per point (two-three observations) (*Bmal2*) or three hearts per point (two-three observations) (*Mop4*) and are standardised to the lowest expression point. CT0 is double-plotted.

ANOVA $P<0.05$ for *Mop4*, $P>0.05$ for *Bmal2*.

Table 3.4. Observed cycling of ancillary clock genes in the mouse heart. Peaks and troughs significantly different from surrounding time-points were identified by *post-hoc* analysis with Newman-Keuls multiple comparison test, $P<0.05$. q=quantitative RT-PCR (Taqman), s=semi-quantitative RT-PCR, # = SYBR green quantitative RT-PCR.

Gene	Peak (CT)	Trough (CT)	Fold difference (peak-trough)	$F_{df,df}$	P-value	Significance
Mop4 (q)	$0\text{-}4^{NS}$	$8\text{-}20^{NS}$	6	$F_{5,12} = 3.4$	0.038	*
Rev-erbα (sq)	8	20-0	16	$F_{5,12} = 10.9$	0.0004	**
Dec1 (q)	8	0	3	$F_{5,12} = 3.4$	0.036	*
Dec2 (sq)	12	20-0	3	$F_{5,12} = 4.2$	0.020	*
Rorα (q) #				$F_{5,26} = 2.2$	0.080	NS
Bmal2 (q)				$F_{5,27} = 0.4$	0.854	NS

3.3.2 Analysis of temporal expression of genes in the mouse heart

Having established the circadian profile of the core clock components, temporal expression of the known CCG *Dbp* was examined to assess clock output in the mouse heart. Robust circadian expression of *Dbp* was observed (Figure 3.7 and Table 3.5). Expression of *E4bp4*, the transcription factor that works antagonistically with DBP, also cycled with a rhythm in anti-phase to *Dbp* (Figure 3.7).

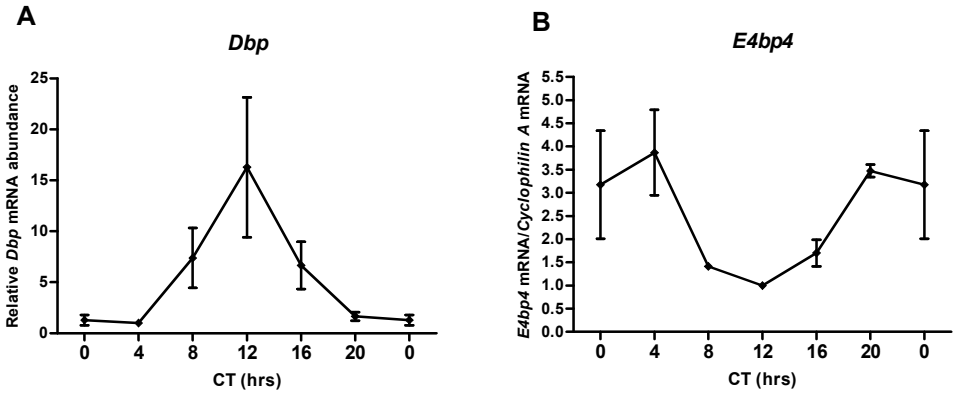


Figure 3.7. Circadian mRNA expression of clock-controlled genes in the mouse heart. (A) Taqman quantitative real-time RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *TATA box binding protein (mTbp)*. (B) Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for three hearts per point (two-three observations) and are standardised to the lowest expression point. CT0 is double-plotted.
ANOVA $P<0.05$ for both graphs.

To investigate how the clock might function in the mouse heart the temporal expression of genes involved in heart physiology was assessed. Significant transcript cycling was observed for three of the genes examined, *Pai-1*, *Bnp* and *Ms1* (Figure 3.8 and Table 3.5). *Post-hoc* analysis of transcript levels emphasised the significant peak in *Pai-1* expression at CT12-16 and also showed a significant difference between peak at CT4 and trough at CT12 for *Bnp* and peak at CT20 and trough at CT12 for *Ms1* (Figure 3.8 and Table 3.5).

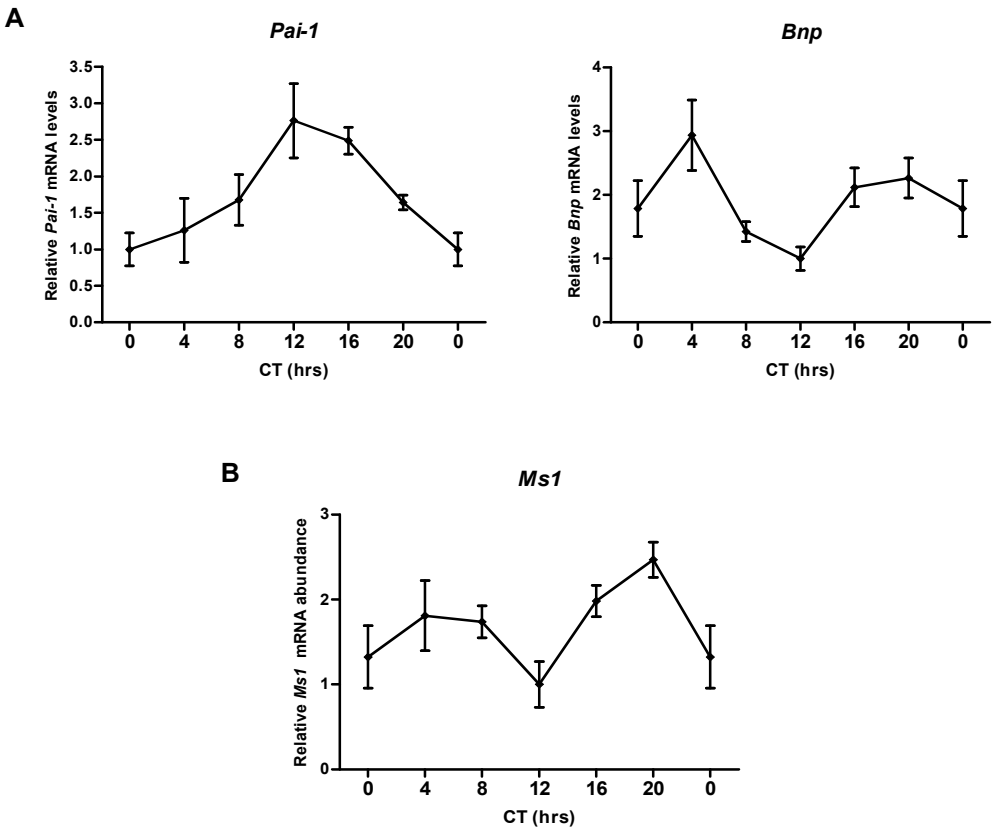


Figure 3.8. Circadian mRNA expression of clock-controlled genes in the mouse heart. (A) Taqman quantitative real-time RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *TATA box binding protein* (*mTbp*). (B) SYBR green quantitative real-time RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for four-six hearts per point (two-three observations) and are standardised to the lowest expression point. CT0 is double-plotted.

ANOVA $P<0.05$ for all graphs.

Table 3.5. Observed cycling of clock-controlled genes in the mouse heart. Peaks and troughs significantly different from surrounding time-points were identified by *post-hoc* analysis with Newman-Keuls multiple comparison test, $P<0.05$. q=quantitative RT-PCR (Taqman), s=semi-quantitative RT-PCR.

Gene	Function in heart	Peak (CT)	Trough (CT)	Fold difference (peak-trough)	$F_{df,df}$	P-value	Significance
Bnp (sq)	Vasoactive hormone, hypertrophic response	4	12	3	$F_{5,27} = 3.6$	0.013	*
Dbp (q)	Unknown	12	20-4	16	$F_{5,12} = 3.4$	0.039	*
Pai-1 (q)	Repair and remodelling (and clotting)	12-16	0-4	3	$F_{5,27} = 4.2$	0.006	**
E4bp4 (sq)	Inflammation, determination of cell fate	20-4	12	4	$F_{5,12} = 3.8$	0.027	*
Ms1 (q)	Intra-cellular signalling, hypertrophic response	20	12	2.4	$F_{5,21} = 3.3$	0.025	*

The genes investigated in this study are involved in a wide range of metabolic and physiological processes in the heart. Most transcripts showed wide variations in expression between samples but without significant expression patterns over the circadian time-course.

Two genes, *Anp* and *Ctgf*, appeared to have similar expression patterns to *Bnp* and *Ms1* (Figure 3.9). However, unlike *Bnp* and *Ms1* statistical testing showed these patterns not to be significant (Table 3.6).

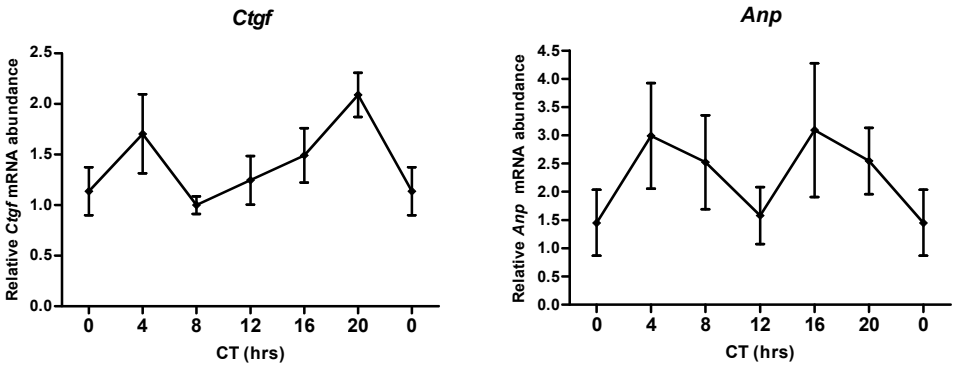


Figure 3.9. Temporal mRNA expression of growth factor and hormone genes in the mouse heart. SYBR green quantitative real-time RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for four-six hearts per point (one observation) and are standardised to the lowest expression point. CT0 is double-plotted.
ANOVA $P>0.05$ for both graphs.

Arc encodes for a protein that is associated with the cytoskeleton, like MS1. *Arc* transcript expression was variable and appeared to peak at CT0, but this result lacked statistical significance (Figure 3.10 and Table 3.6).

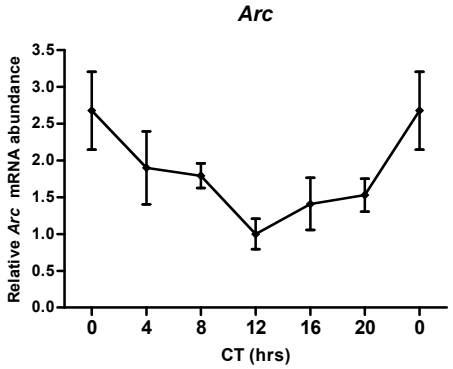


Figure 3.10. Temporal mRNA expression of a gene involved in intracellular signalling and cell survival in the mouse heart. SYBR green quantitative real-time RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for four-six hearts per point (one observation) and are standardised to the lowest expression point. CT0 is double-plotted.
ANOVA $P>0.05$.

Key genes involved in cardiac fatty acid and glucose metabolism were investigated (Figure 3.11 and Table 3.6) but no significant temporal variation was observed and transcript levels were highly variable.

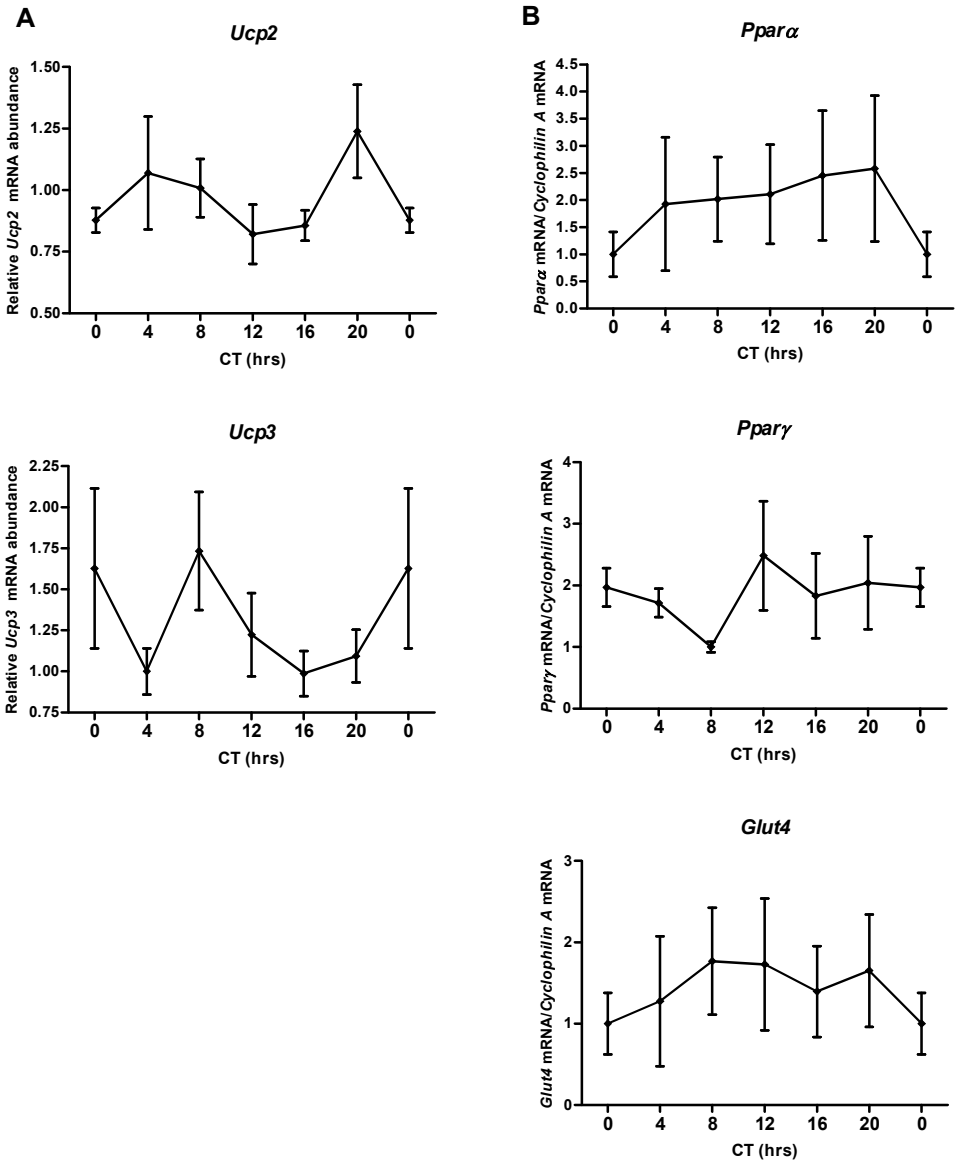


Figure 3.11. **Temporal mRNA expression of metabolism genes in the mouse heart.** (A) SYBR green quantitative real-time RT-PCR expression values and (B) Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for four-six hearts per point (one observation) (A) or three hearts per point (one-two observations) (B) and are standardised to the lowest expression point. CT0 is double-plotted.

ANOVA $P>0.05$ for all graphs.

The expression of important cardiac transcription factors and ion channels was also examined but again transcript expression was not significantly variable when all time-points were examined together (Figure 3.12 and Figure 3.13 and Table 3.6).

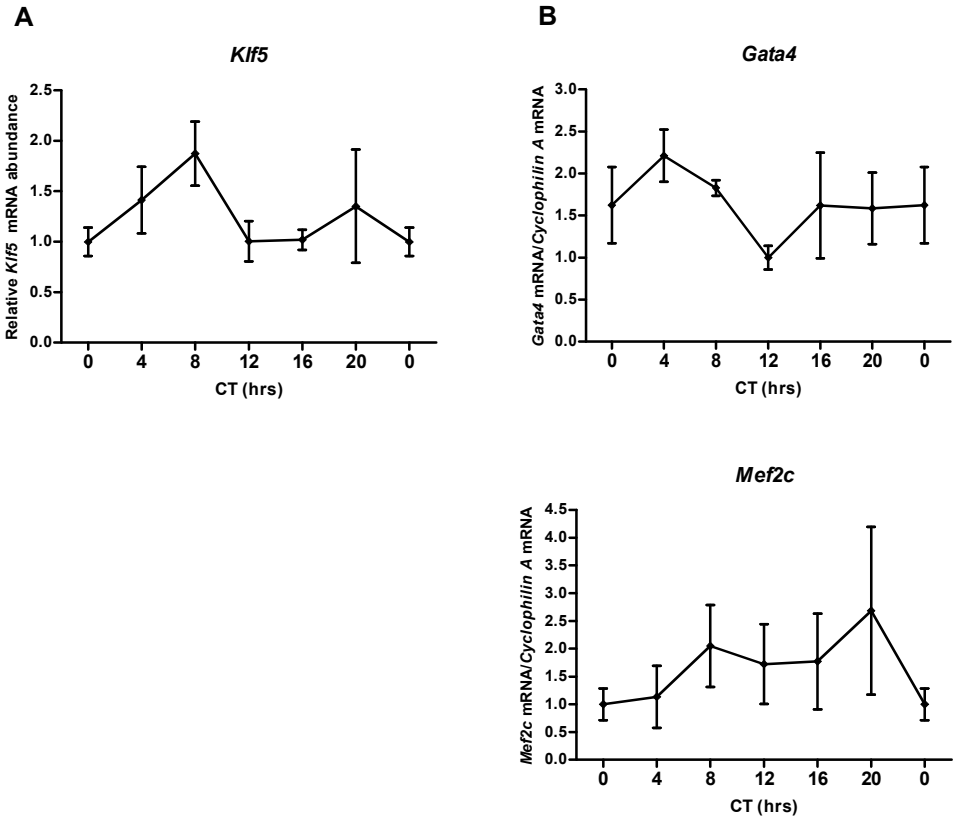


Figure 3.12. Temporal mRNA expression of cardiac transcription factor genes in the mouse heart. (A) SYBR green quantitative real-time RT-PCR expression values and (B) Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for four-six hearts per point (one observation) (A) or three hearts per point (one-two observations) (B) and are standardised to the lowest expression point. CT0 is double-plotted.
ANOVA $P>0.05$ for all graphs.

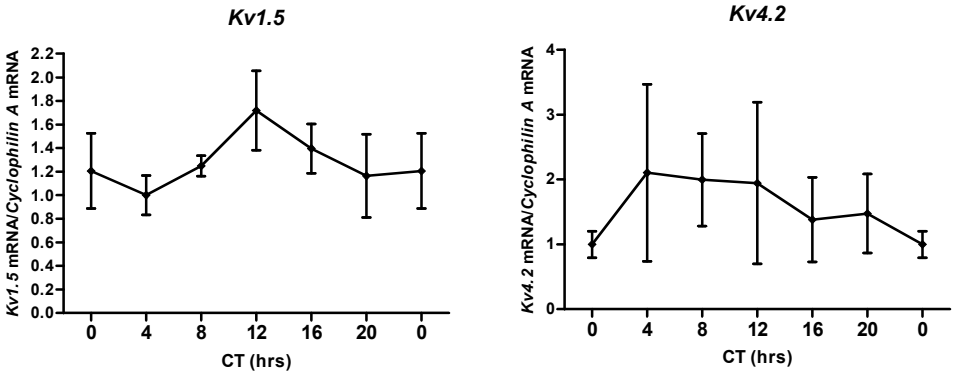


Figure 3.13. Temporal mRNA expression of cardiac-specific ion channel genes in the mouse heart. Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for three hearts per point (one-two observations) and are standardised to the lowest expression point. CT0 is double-plotted.

ANOVA $P>0.05$ for both graphs**.**

Table 3.6. Temporal expression of key cardiac genes in the mouse heart. All results were non-significant by ANOVA.

Gene	Function in the heart	$F_{df, df}$	P-value
Metabolism			
Ucp2	Mitrochrondrial and fatty acid metabolism	$F_{5,27} = 1.2$	0.34
Ucp3		$F_{5,27} = 1.4$	0.27
Pparα	Lipid and glucose metabolism	$F_{5,12} = 0.3$	0.91
Pparγ		$F_{5,12} = 0.7$	0.62
Glut4	Glucose metabolism	$F_{5,12} = 0.2$	0.95
Growth factors and hormones			
Anp	Vasoactive hormone, hypertrophic response	$F_{5,25} = 0.8$	0.55
Ctgf	Growth factor, remodelling, signalling	$F_{5,27} = 2.1$	0.094
Transcription factors			
Klf5	Cardiac development and disease	$F_{5,27} = 0.9$	0.47
Gata4	Cardiac development	$F_{5,12} = 1.0$	0.45
Mef2c	Activates structural genes, hypertrophic response	$F_{5,12} = 0.5$	0.76
Intracellular signalling and cell survival			
Arc	Inhibitor of apoptosis	$F_{5,27} = 2.3$	0.068
Ion channels			
Kv1.5	Voltage-gated potassium channels	$F_{5,12} = 0.9$	0.53
Kv4.2		$F_{5,12} = 0.2$	0.94

3.3.3 Temporal expression of bHLH factor genes in the mouse heart.

In addition to CLOCK and MOP4, BMAL1 can form functionally active heterodimers with other bHLH-PAS family members, such as HIF-1α and EPAS1 (HIF-2α), which can bind DNA elements similar to the canonical circadian E-box sequence. Therefore daily expression of different bHLH-PAS factors was measured.

The transcripts investigated appeared to fluctuate over the time-course but none of these expression patterns were statistically significant (Figure 3.14, Figure 3.15 and Table 3.7). The use of further samples may allow significant cycling patterns to be seen.

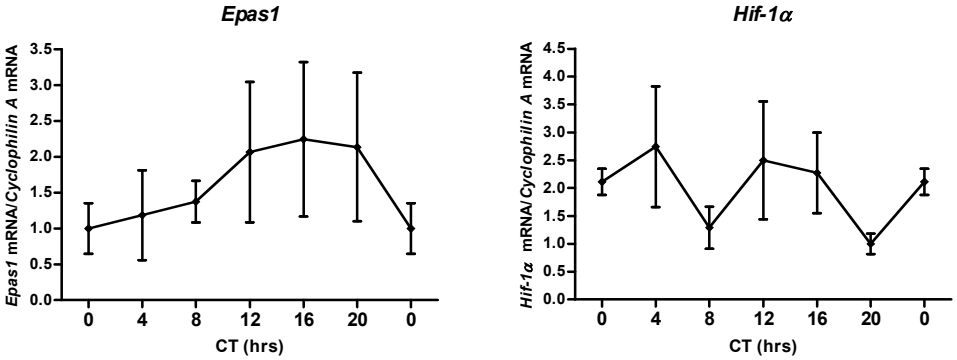


Figure 3.14. Temporal mRNA expression of hypoxia-responsive bHLH factor genes in the mouse heart. Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for three hearts per point (two-three observations) and are standardised to the lowest expression point. CT0 is double-plotted.
ANOVA $P>0.05$ for both graphs.

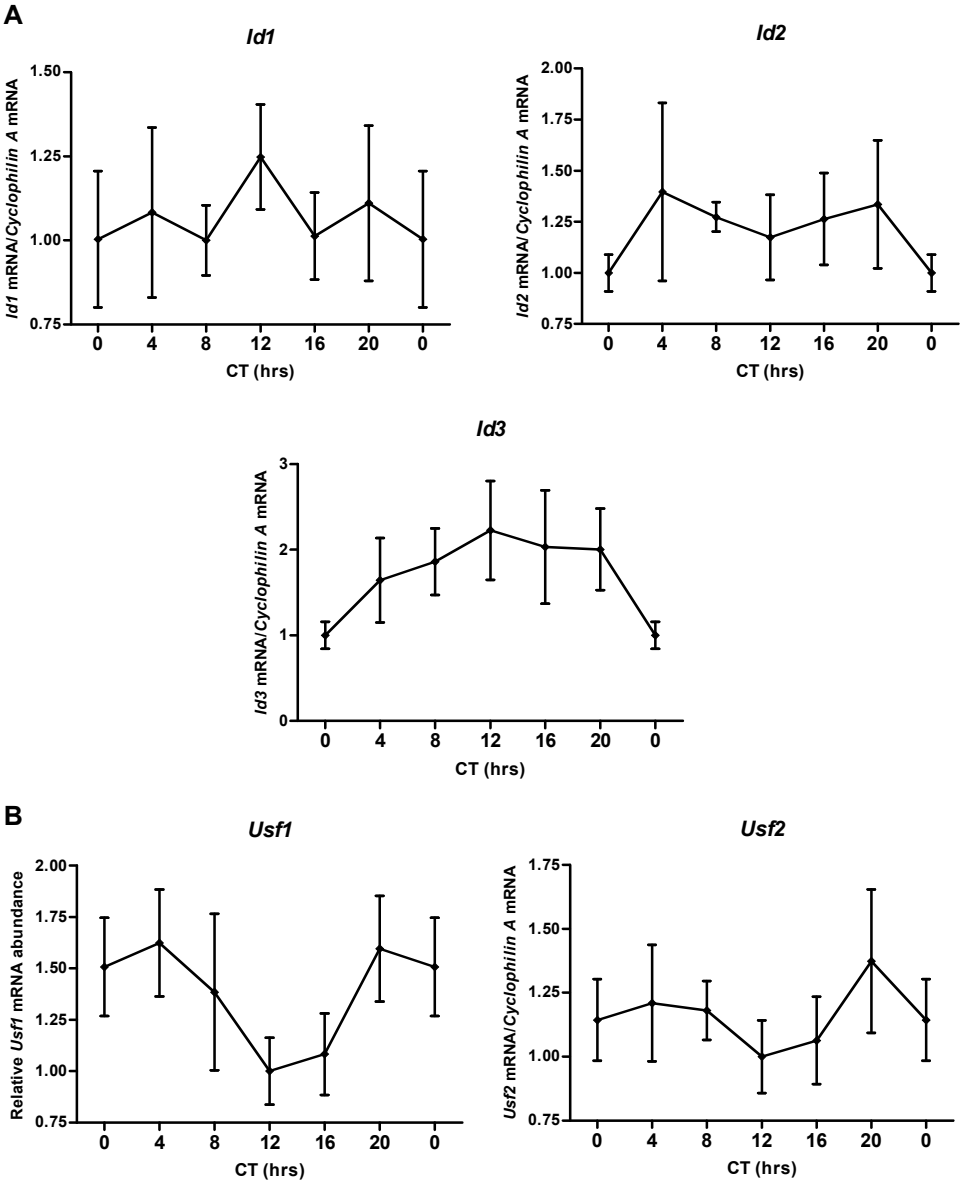


Figure 3.15. Temporal mRNA expression of bHLH factor genes in the mouse heart.
Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for three hearts per point (two-three observations) (A) or four-six hearts per point (one observation) (B) and are standardised to the lowest expression point. CT0 is double-plotted.
ANOVA $P>0.05$ for all graphs.

Table 3.7. Temporal expression of bHLH factor genes in the mouse heart. All results were non-significant by ANOVA.

Gene	$F_{df, df}$	P-value
Epas1	$F_{5,12} = 0.5$	0.47
Hif-1α	$F_{5,12} = 0.9$	0.48
Id1	$F_{5,12} = 0.3$	0.93
Id2	$F_{5,12} = 0.3$	0.91
Id3	$F_{5,12} = 0.3$	0.88
Usf1	$F_{5,27} = 1.3$	0.30
Usf2	$F_{5,27} = 0.4$	0.81

3.3.4 Bioinformatics

The presence of conserved canonical E-boxes in the proximal promoter of a gene would potentially enable the clock factors CLOCK:BMAL to bind to and activate that promoter in a circadian manner and regulatory elements for other cycling transcription factors may also potentially govern regulation by the clock. The presence of such sites in the promoters of key cardiac genes was investigated and putative conserved sites were identified (Table 3.8). Where sequence was available a minimum of 10kb upstream of the putative transcription start site was examined in order to identify as many sites as possible. However, it is important to note that further sites may lie outside these regions, either further upstream or in downstream non-coding regions such as introns.

Based on the lengths of the transcription factor binding sites (TFBS) investigated (see Table 3.8) one would expect a certain number of these sites to be found by chance in a region of DNA. This is calculated with the formula:

Frequency of TFBS being found by chance = once in every N^L base pairs (bp),

where N = the number of nucleotides permutations possible in a consensus site (i.e. A, C, G or T) and L is the length of the TFBS (in bases).

For example, the canonical E-box sequence CACGTG would be expected to occur by chance once in every 4^6 bp = once in every 4096 bp. In 10 kb one would therefore expect 2-3 (2.4) canonical E-boxes to be found by chance. The expected frequency of the longer D-box/E4BP4 and RRE binding sites is once every 16342 ($4^7 + 2^3$) respectively. This equates to 0.6 sites in 10 kb. For the consensus non-canonical E-box CANNGT the expected frequency increases to once every 256 bp (4^4), or 39 sites within 10kb. However, not all CANNGT sites will bind E-box-binding factors as the surrounding

bases are also important (see section 3.4.3). Therefore sites bound by known E-box-binding factors were identified using the TRANSFAC library of matrices, which have been experimentally validated. Canonical E-boxes, D-boxes and RRE were also identified using the TRANSFAC library (see Table 3.8 legend for matrices used) with the search settings optimised for each TFBS to reduce the identification of sites by chance.

Conservation of putative TFBS between species greatly increases the chance of that site being functional by eliminating the likelihood that the TFBS is present by chance. For this reason only putative TFBS that were conserved between one or more species were documented. The conservation of circadian response elements between divergent species such as mouse and human in a number of cardiac gene promoters suggests that many of these genes could be clock-controlled at a transcriptional level.

Table 3.8. Conserved potential circadian regulatory elements in cardiac promoters. Putative 5' transcription factor binding sites (TFBS) in the proximal promoters of mouse cardiac genes were identified using the Evolutionary Conserved Regions (ECR) browser (Ovcharenko *et al.*, 2004) and rVISTA2.0 (Loots and Ovcharenko, 2004) tools from NCBI dCODE (www.dcode.org). The following TRANSFAC matrices were used for identification of conserved TFBS by rVISTA2.0:- Canonical E-boxes: V$CLOCKBMAL_Q6, V$EBOX_Q6_01; Other E-boxes: V$ARNT_01, V$ARNT_02, V$MYCMAX_01, V$MAX_01, VUSF_01, VUSF_02, VUSF_Q6, VUSF_C, V$MYOD_01, V$MYOD_Q6; D-boxes/E4BP4: VDBP_Q6, VE4BP4_01; RRE: V$RORA1, V$RORA2. Conserved putative or verified TFBS identified by other groups in the Mammalian Promoter/Enhancer Database (PEDB) (Kumaki *et al.*, 2007), and the literature were also documented. r = conserved between mouse and rat, d = conserved between mouse and dog, h = conserved between mouse and human. TSS = Transcription Start Site. Canonical E-box = CACGTG, other E-boxes = CANNGT (with varying surrounding nucleotides), D-box/E4BP4 = RTTA[T/C]GTAAY, RRE = [A/T]A[A/T]NT[A/G]GGTCA.

Gene (distance analysed 5' of TSS)	**Conserved canonical E-boxes**			**Other conserved potential circadian TFBS**								
				Other E-boxes			**D-boxes /E4BP4**			**RRE**		
	r	d	h	r	d	h	r	d	h	r	d	h
Pai-1 (10kb)	2			8	3	1	2	2		1	1	
Bnp (10kb)	1			5		2	1			5		
Ucp2 (6kb)				5						1		
Ucp3 (5kb)	1		1	5	1	1				1	1	
Pparα (10kb + intron 2)				8		2	3		1			
Pparγ (15kb)			1	2			5		1	2		
Glut4 (13kb)			1	1		2						
Anp (10kb)	2			4			1			3		
Ctgf (10kb)				2		2	2					2
Klf5 (10kb)				2		2	2		1			
Gata4 (10kb)	1	1	2	3								
Mef2c (6.5kb)				6		1	1		2	1		
Kv1.5 (10kb)	1			5								
Kv4.2 (10kb)				6		5				1		
Ms1 (10kb)				5		1				1		
Arc (7kb)	1			5	1	2	2					
Usf1 (2kb)				3		2	2		1			
Usf2 (1kb)				3	2	1						

3.3.5 Temporal expression of core clock and output genes in isolated adult rat ventricular cardiomyocytes

In order to examine the cardiac clock *in vitro*, expression of core clock and output genes was measured in ventricular cardiomyocytes isolated from entrained adult rats at two different time-points. Expression of *Bmal1* was statistically significantly higher (3-fold) in cells isolated at ZT2 than in cells isolated at ZT14 (Figure 3.16). Expression of *Dbp*, a transcript that shows strong cycling anti-phase to *Bmal1*, was statistically significantly higher (2.5-fold) at ZT14 compared to ZT2. This is in agreement with the circadian profiles of *Bmal1* and *Dbp* expression seen in the whole heart (Figure 3.3 and Figure 3.7). *Pai-1* is highly expressed in vascular smooth muscle and endothelial cells but little is known about its role in myocytes. In the cardiomyocyte samples *Pai-1* expression was 2-fold higher at ZT14 compared to ZT2, although due to variation between samples this result did not have strong statistical significance (Figure 3.16). Peak *Pai-1* at ZT14 also agrees with the circadian expression of *Pai-1* seen in the whole heart (Figure 3.8). Therefore, it is likely that the circadian expression of clock genes and CCGs (such as *Pai-1*) in the cardiomyocyte is similar to and representative of the whole heart. Expression of another putative CCG, *Bnp*, was examined but only a non-significant 1.2-fold increase at ZT2 was seen (Figure 3.16). This is as expected since the expression profile of *Bnp* in the whole heart shows peak expression at CT4 and CT16, with CT4 levels being slightly higher (Figure 3.8). Cardiomyocytes isolated at more time-points may show a difference in expression.

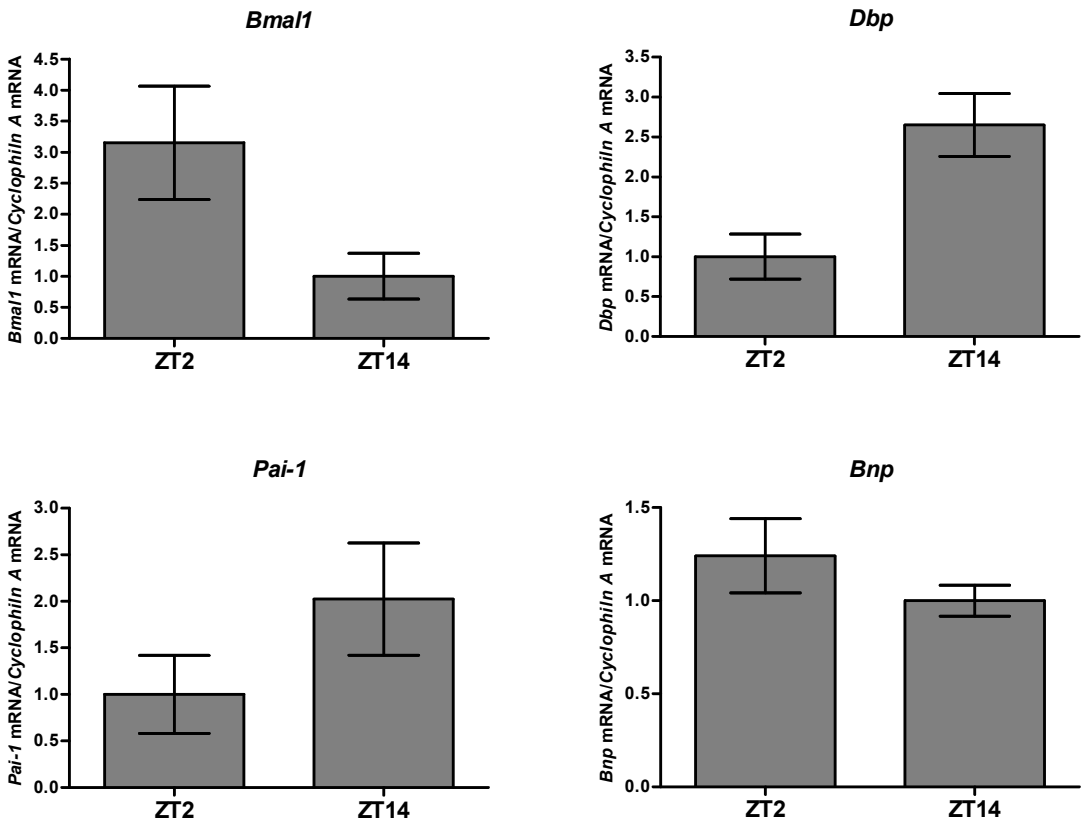


Figure 3.16. Temporal mRNA expression of core clock and output genes in isolated adult rat ventricular cardiomyocytes. Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SEM for five-six samples (two observations) per time-point and are standardised to the lowest expression point.

ANOVA *Bmal1* and *Dbp* $P<0.05$, *Pai-1* and *Bnp* $P>0.05$.

3.4 Discussion

3.4.1 The circadian expression of clock genes in the mouse heart

Prior to investigation of cardiac gene cycling the circadian expression of the key transcription factors involved in the molecular clock feedback loop was examined to confirm the clock was functioning in these samples. The generation of circadian expression rhythms and the transduction of these to genes outside the clock was confirmed by the rhythmic expression of various known clock output genes, such as *Dbp* and *E4bp4*. The core clock feedback loop is augmented and stabilised by subsidiary loops of RORα and REV-ERBα, and DEC1 and DEC2 and circadian expression of the latter three of these genes was also confirmed. Where circadian expression of clock genes has been demonstrated the phase (and in most cases amplitude) of the rhythms agrees with published data.

Whilst the core circadian transcriptional feedback loop found in the SCN exists in all tissues, tissue-specific factors, such as BMAL2, can provide a degree of autonomy and integration of internal and external signals. *Bmal2* expression in the mouse heart was confirmed here, albeit at very low levels. Whether *Bmal2* transcript levels cycle is unclear and in the current study it was not possible to discern a clear circadian expression rhythm (Figure 3.6), possibly due to the difficulty in amplifying this low abundant gene. Two splice variants of mouse *Bmal2* have been identified, full length *mBmal2a* and truncated *mBmal2b* (Okano *et al.*, 2001). The primers used here amplify both *mBmal2* isoforms and it is possible that the two isoforms are differentially expressed. Alternatively, *Bmal2* expression may be regulated by factors that are not clock-controlled and BMAL2 may still influence the rhythmic expression of E-box-containing genes through its heterodimerisation with other clock factors, such as CLOCK or MOP4, which are both rhythmically expressed in the heart (*Mop4* to a greater extent). BMAL2:CLOCK may be a stronger activator of E-box-containing genes than BMAL1:CLOCK (Hogenesch *et al.*, 2000) so BMAL1 and BMAL2 may fulfil separate spatial-temporal roles in circadian regulation.

3.4.2 Circadian expression of three genes suggests that the clock may influence cardiac physiology following myocardial infarction

The examination of temporal transcript cycling of key cardiac genes in this chapter has revealed conflicting results and disagreement with expression rhythms suggested in the literature. Transcript levels were highly variable for most genes but this variability was only significant for three genes, *Pai-1*, *Bnp* and *Ms1*. Interestingly, the regulation of these three genes has been linked to the common processes of cardiac hypertrophy and remodelling, as discussed below. Ventricular hypertrophy (cardiac myocyte growth, i.e. increase in size rather than number) and remodelling (structural changes in the myocardium to repair necrotic areas) are part of the adaptive response to the increased demands

and stress placed on the heart following MI, although in the long term can lead to maladaptation and heart failure.

Circadian expression of the *Pai-1* transcript has been previously observed in the mouse heart (Minami *et al.*, 2002) and was confirmed in this study (Figure 3.8). Increased *Pai-1* expression observed in rat cardiomyocytes at ZT14 compared to ZT2 (Figure 3.16) would agree with the rhythm in the mouse heart, however due to the variability between a limited number of samples this increase was not significant. The peak expression at CT12-16 corresponds to the onset of activity in nocturnal rodents and is in anti-phase to expression in the human circulatory system as would be expected. Studies in circulating blood mononuclear cells have confirmed that clock gene expression is anti-phasic between nocturnal rodents (Oishi *et al.*, 1998) and diurnal humans (Takata *et al.*, 2002) in peripheral clocks. PAI-1 is produced by a variety of cell types and while its principal role may be in regulating intravascular fibrinolysis, in cardiomyocytes it is involved in cell-associated proteolysis, repair and remodelling post-MI, through activation by inflammatory mediators, such as Transforming Growth Factor-β (TGF-β) and inhibition of urokinase-type plasminogen activator (uPA), the major PA on migrating cells (Creemers *et al.*, 2000; Macfelda *et al.*, 2002; Takeshita *et al.*, 2004). So while increased plasma PAI-1 levels in the morning may contribute to the risk of MI, increased levels in cardiomyocytes may augment remodelling in response to MI.

While *Pai-1* has been previously identified as a rhythmically expressed gene by a number of studies, rhythmic *Bnp* expression has not been previously investigated. Increased cardiac expression at CT22 was however shown in one microarray study (Storch *et al.*, 2002). This differs to the peak at CT4 observed in this study (Figure 3.8), although both studies show *Bnp* expression peaking in phase with *Bmal1* (CT4 in this study and CT22-23 in the microarray) and the differences in phase may be caused by different experimental conditions. Interestingly both *Bnp* and *Anp* were abundantly expressed in the adult whole heart throughout the day, despite the lack of hypertrophic stimuli. It is therefore possible that the apparent down-regulation of these genes post-development is actually an artefact of the time of sampling, or alteration in circadian expression (such as seen in the hypertrophied heart (Young *et al.*, 2001b; Young *et al.*, 2001c), rather than a permanent 'switching off'. *Bnp* and *Anp* promoters both contain conserved canonical E-boxes (Table 3.8) and *Anp* appeared to exhibit a similar expression pattern to *Bnp*, but this was not significant (Figure 3.10).

The suggestion of *Ms1* transcript cycling demonstrated by the significant peak in expression at CT20 (Figure 3.8) is a novel finding. As *Ms1* may be involved in the initial signalling of the hypertrophic response (Mahadeva *et al.*, 2002) whether the heart enters into hypertrophy may depend on the circadian regulation of *Ms1*. MS1 binds the actin cytoskeleton, linking it to gene transcription via Rho-activated pathways and Serum Response Factor (SRF) (Arai *et al.*, 2002), therefore the potential cycling of *Ms1* expression observed here may govern circadian regulation of these signalling

pathways. A recent experiment has also shown that CLOCK localises to the Z-disks of myofilaments in cardiac myocytes, where it may be involved in sensing muscle energy expenditure (Qi and Boateng, 2006). Clock factors and cytoskeleton-associated proteins may then represent another node in the network of signalling pathways and the clock that may enable the heart to respond and adapt to diurnal variations in workload.

An additional link between the clock and hypertrophy may be found in DBP. *Dbp* expression is highly rhythmic in the heart with a peak-to-trough ratio comparable to or greater than the core clock genes (Figure 3.3 - Figure 3.7). It is also rhythmically expressed in synchronised cardiac cells *in vitro* (see Chapter 5). A rodent model of pressure overload-induced hypertrophy, caused by surgical constriction of the aorta, resulted in the rhythmic expression of clock-output and cardiac genes being attenuated (Young *et al.*, 2001b; Young *et al.*, 2001c). One of the clock output genes that exhibited attenuation of peak expression was the transcription factor *Dbp*. Expression of *Dbp* was also found to be downregulated in an induced model of heart failure following hypertrophy in rat cardiomyocytes (Ueno *et al.*, 2003). Circadian regulation of *Dbp* expression (and DBP target genes) may therefore be important in the heart, disruption of which may contribute to the development of heart failure following hypertrophy. Bioinformatic analysis of the mouse *Pai-1* promoter suggested the presence of conserved DBP-binding sites (Table 3.8), therefore these two genes may co-operatively govern a circadian input into cardiac remodelling.

Like *Dbp*, *E4bp4* expression is also robustly circadian, cycling in anti-phase to *Dbp* (Figure 3.7). *E4bp4* may also play a role in coupling the clock to the response of the heart to stress such as MI. The extent of ischemia, followed by reperfusion, can contribute to the clinical outcome following MI and a recent study has identified that clock gene oscillations (peak-to-trough fold differences) were rapidly attenuated in the ischemic regions of rat hearts following ischemia-reperfusion (IR) (Kung *et al.*, 2007). This observation coincided with a rapid induction of the transcription factor E4BP4 at transcript and protein level. Disruption of *E4bp4* cycling by rapid induction would therefore attenuate cycling of DBP targets and, combined with attenuation of clock gene oscillations, may rapidly halt circadian expression rhythms in the heart

3.4.3 Temporal expression of cardiac genes in the mouse heart and the limitations of this study

Based on studies by other groups that suggest cycling of some of the transcripts examined in this chapter in cardiac and /or non-cardiac tissue (see section 3.1), including the microarray study by Charles Weitz's group (Storch *et al.*, 2002), it is worrying that significant temporal changes in expression were only seen for three out of 16 cardiac-relevant genes in this chapter, and that only one of these genes, *Bnp*, was identified in both this study and the microarray. It is possible that this may in part be due to the limitations of microarray studies. The microarray chip used in the study by Storch and colleagues (2002) represented 12,488 genes, which is 30-40% of the estimated total number of

mouse genes. To avoid the identification of false-positive cycling genes, Storch and colleagues employed a number of filtering steps. The most optimal parameters found identified only 67% of the guide genes (nine clock genes known to be rhythmically expressed). The identified CCGs were probably then an underestimate, demonstrating that microarrays cannot be used in isolation to identify all rhythmically expressed genes. Likewise, PCR is insufficient due to the inability to investigate genes that are not already candidates. Therefore multiple techniques are needed to effectively identify cycling transcripts and the identification of *Bnp* as a rhythmically expressed gene by two different techniques strengthens this finding.

The lack of agreement between this PCR-based study and others in the literature requires the accuracy of the data presented here to be questioned. A particular caveat of this study is the limited number of samples, which may not be sufficient to detect subtle cycling of transcript level. A solution to this would have been to cross-validate the results using additional techniques to detect transcript levels, such as Northern blotting and *in situ* hybridisation. The latter technique could also be used to determine which cell types express particular genes and where expression is rhythmic in all cell types. Increasing the number of samples to cover two 24-hour periods would also enable a degree of validation with the one technique as repetition of a peak would confirm it is real, and this repetition is needed to determine a truly circadian rhythm.

Both PCR and microarray-based techniques only examine changes in transcript abundance. As explained in Chapter 1, not all genes/proteins that are involved in relaying circadian rhythms are regulated at the transcriptional level and post-transcriptional and post-translational regulation is also important. Therefore, to more comprehensively identify cardiac factors that govern circadian regulation temporal variations in protein abundance should also be studied by Western blotting and/or immunohistochemistry.

A number of metabolic genes have been reported to cycle by Young and colleagues (2001) that were not identified as being rhythmic in this study. Many of these transcripts peak at ZT15 in the isolated rat heart (Young *et al.*, 2001b) and this common peak may reflect an induction of genes involved in metabolism caused by the experimental conditions at this time-point rather than clock regulation. Discrepancies may also be accounted for in part by the use of different time-points, such as 4 hour (as used here) or 6 hour intervals, where identification of the exact peak/trough times becomes more ambiguous. Differences between the experimental conditions and techniques used, such as the use of light-dark cycles or the transfer to constant darkness and the availability of food, and differences between the mouse and rat may also contribute. In the current study, mice were entrained to constant conditions of alternating 12 hours light and 12 hours dark (L:D) and then transferred to constant dim red light (DR:DR). The absence of white light as a zeitgeber ensures that the observed circadian rhythms result from control by the endogenous circadian clock rather than the external environment.

A study examining the expression of *Dec1* and *Dec2* in *Clock* mutant mice shows clear differences in the temporal expression profiles of *Dec1*, *Dec2* and *Dbp* in the hearts of mice harvested in L:D and D:D (constant darkness) (Noshiro *et al.*, 2005). The difference in the expression profile of *Per2* was less notable and another study exhibited similar profiles for *Rev-erbα* and *Rorα* transcripts in L:D and D:D (Akashi and Takumi, 2005). Therefore, it is conceivable that the light-dark conditions can affect the expression of peripheral clock genes and CCGs more than the core clock mechanism. Other studies of temporal expression have also used specific chambers of the heart, such as the left ventricle, which may present different results to the use of the whole heart in this study. The investigation of isolated cardiomyocytes presented here in part addresses this.

Whether genes that have been reported to be rhythmically expressed are direct targets of clock factors or are regulated by other rhythmic factors, such as nutritional status, diurnal variations in circulating hormones or changes in autonomic nervous function, may also effect how much their expression is determined by experimental conditions which could vary between experiments. This may be why no significant variations in *Kv1.5* or *Kv4.2* expression was observed in the current study, in contrast to a study by Yamashita and colleagues (2003), as the factors governing this regulation are unknown. (Yamashita *et al.*, 2003)

One gene that has been shown to be rhythmically expressed in the *ex vivo* heart (Young *et al.*, 2001b), aorta (Rudic *et al.*, 2005) and liver (Lemberger *et al.*, 1996) is *Pparα*. *Pparα* can also be transactivated by CLOCK:BMAL1 (Oishi *et al.*, 2005c) so it is surprising that rhythmic *Pparα* expression was not seen in this study. Despite the lack of transcript cycling, PPAR may still be involved in the circadian regulation of lipid metabolism due to interactions with REV-ERBα (see section 3.1) and its circadian regulation warrants further investigation in the following chapters.

The presence of conserved E-boxes and other circadian regulatory elements in the promoters of the genes investigated here (Table 3.8) implies that many of these genes may be clock-regulated. However, many of the genes with conserved upstream E-boxes did not exhibit any significant temporal variation in expression. E-boxes are versatile sequences that influence many pathways including proliferation, differentiation, tissue-specific responses, and cell death as well as circadian regulation (Kyriacou and Rosato, 2000; Munoz *et al.*, 2002). This knowledge combined with the lack of correlation between E-box presence and transcript cycling demonstrates that a bioinformatics approach to identifying candidate CCGs can not be used in isolation. Efforts to characterise the E-box and surrounding sequence required for circadian regulation have begun but have not yet enabled the exact sequence constraints to be identified and have produced conflicting results. There is however evidence that the sequence surrounding an E-box is important and it is possible that additional tissue-specific co-activators may be required for circadian transcription. The presence of binding sites for clock factors such as DBP and REV-ERBα, and non-canonical E-boxes are also likely to be

required for robust circadian expression (see Chapter 6), as has been shown for *Per2* (Akashi and Takumi, 2005; Yoo *et al.*, 2005; Ohno *et al.*, 2007). Circadian regulation is also evident for transcripts of second-order CCGs whose promoters do not possess an E-box but are regulated by primary CCGs, such as DBP

3.4.4 Temporal expression of bHLH factor genes in the mouse heart

No significant rhythms in transcript abundance were observed for any of the bHLH factors investigated. These factors may still be involved in circadian regulation however through competing with clock factors at E-boxes. For example, such a mechanism may involve repression by USFs or ID proteins until BMAL1 production reaches a critical level that can out-compete. The transcripts for *Id1, Id2* and *Id3* did not cycle in the present study (Figure 3.15) but *Id1* and *Id3* have been reported to cycle in cells from the SCN (Menger *et al.*, 2007) so they may have a role in the central clock mechanism. *Id1* expression is also rhythmic in the rat pineal gland, peaking at the end of the circadian night (Humphries *et al.*, 2002) and *Id1*, *2* and *3* may cycle significantly in the mouse aorta (Su *et al.*, 2002). Cycling of the *Usf* transcripts has not been reported before except in a mouse liver microarray study where a two-fold increase was observed at CT12 (Storch *et al.*, 2002).

Significant cycling of the *Hif-1α* transcript has been seen in the mouse heart with peak levels at CT2 (compared to *Bmal1* at CT23) (Storch *et al.*, 2002) but was not observed in the present study. HIF-1α may also influence clock output without itself cycling since it up-regulates the transcription factor *Rorα* (Chauvet *et al.*, 2004; Miki *et al.*, 2004). RORα is a transcriptional repressor that works antagonistically with REV-ERBα and together they regulate cyclic expression of *Bmal1* (Sato *et al.*, 2004c; Akashi and Takumi, 2005; Guillaumond *et al.*, 2005). The *Rorα* promoter contains four canonical E-boxes (Sato *et al.*, 2004c) but does not appear to cycle. These E-boxes may be more important for activation by HIF-1α and constant expression of RORα may influence circadian regulation through its antagonism of REV-ERBα activity. RORα activity may also be regulated by rhythmic binding of ligands such as cholesterol (Kallen *et al.*, 2002) following oscillations in dietary intake (controlled by the SCN clock) and liver cholesterol metabolism (regulated by DBP, (Wuarin *et al.*, 1992)). RORα may play a key role in the circadian regulation of an array of cardiovascular functions and allow physiology to feed back into the regulation of the clock since it has been implicated in many pathophysiological processes affecting the vascular system (Laitinen and Staels, 2003; Boukhtouche *et al.*, 2004).

3.4.5 Temporal expression of core clock and output genes in isolated adult rat ventricular cardiomyocytes

The whole heart is composed of many cell types such as cardiomyocytes, fibroblasts and endothelial cells therefore extracted heart RNA may contain molecules derived from different cell types. Some genes like *Pai-1* are highly expressed in non-myocyte cells such as vascular smooth muscle cells

(Binder *et al.*, 2002) therefore a high amount of vascular-derived transcripts in the RNA preparation may give results that are not representative of the cardiomyocyte.

Analysis of clock factor expression in isolated adult rat ventricular cardiomyocytes supports the presence of an independent molecular circadian clock in this cell type. The expression profiles seen at the two time-points also agree with the circadian expression profiles observed in the whole mouse heart (section 3.2.1), implying that the circadian expression rhythms in cardiomyocytes are representative of the whole heart, which is primarily composed of this cell type. The expression of putative CCGs *Pai-1* and *Bnp* was also examined but due to a large amount of variations between samples this result did not achieve statistical significance. Variation in gene expression between individual cell samples may be a consequence of the isolation technique used. Cells were isolated by enzymatic digestion and kept at room temperature for up to two hours post-isolation, a procedure that may induce the expression of stress-responsive genes or result in the halting of gene expression. However, the disruption may be minimal since Durgan and colleagues (2005) noted that isolated cardiomyocytes transferred to cell culture conditions maintained circadian expression from the parent rat prior to resynchronisation by serum shock. The results obtained here were also limited by the lack of time-points, which may account for the lack of variation in *Bnp* expression. Whether the observed cycling of transcripts is translated into rhythmic protein expression is unknown but the use of isolated cells would allow this to be assessed using an immunohistochemical approach. It should also be noted that the rats in this experiment were kept in 12 hours light:12 hour dark conditions and not transferred to constant darkness or dim red light so diurnal difference in gene expression could have been caused by the lighting cycle. The clock transcripts examined are not known to be involved in peripheral clock synchronisation in response to humoral signals (unlike *Per2*, which may be involved in resetting, see Chapter 4) so diurnal expression is likely to be clock-dependent.

3.4.6 Conclusions

The cycling and phase of core clock gene expression and of primary output genes, such as *Dbp*, confirms the presence of a functional molecular circadian clock in the mouse heart. To investigate how the rhythmic expression of core clock genes is translated into physiological rhythms in the heart and CVS the temporal expression of key cardiac genes was also examined. This approach did not reveal significant cycling of many transcripts but the novel temporal expression of *Bnp* and *Ms1* is of particular interest. Many of the genes investigated are involved in maintaining cardiac function and/or have been identified as regulators of pathological processes, such as the hypertrophic response to stress and remodelling. The potential involvement of the circadian clock in the regulation of these genes may have far reaching implications in the causes, timings and treatment of cardiac pathologies through enabling the heart to best adapt to its chronological environment. Therefore, following this 'hypothesis generating' approach of screening for temporal transcript cycling and circadian regulatory

promoter sites, further experiments were carried out to investigate the circadian regulation of these candidate genes (Chapters 4-6).

4. Suprachiasmatic nuclei (SCN) ablation and circadian gene expression in the heart: *in vivo* synchronisation by glucocorticoid

4.1 Introduction

Peripheral circadian clocks, such as that of the heart, are important for orchestrating circadian rhythms at a local tissue level, thus enabling adaptation to the environment. However, whilst peripheral clocks contain all the necessary molecular machinery to maintain rhythmic expression, adaptation to the external environment requires entraining signals from a light-sensing organ. This is achieved through the circadian clock in the SCN located in the hypothalamus. The cells of the SCN can maintain a rhythm in the absence of external stimuli and are entrained to a 24-hour cycle by photic input from the retina, via the retinohypothalamic tract (Moore, 1997). Selective destruction of the SCN led to the discovery that the SCN is required for circadian rhythms in locomotor activity, behaviour and levels of the glucocorticoid corticosterone (Moore and Eichler, 1972; Stephan and Zucker, 1972). The SCN, commonly referred to as the master clock, orchestrates circadian rhythms by integrating signals from peripheral organs with those from the visual system (Buijs and Kalsbeek, 2001). Studies using tissue explants have shown that peripheral clocks in isolation rapidly lose synchrony with other tissues and the environment and are reliant on entraining signals from the SCN (Yoo *et al.*, 2004). It is still unclear how the SCN communicates with peripheral clocks but evidence suggests that hormonal mechanisms play a prominent role (see Chapter 1, section 1.3).

Several observations suggest that glucocorticoid signalling is an important hormonal synchronising mechanism. Glucocorticoids are secreted by the adrenal glands and regulate gene transcription by binding to the glucocorticoid receptor, which is expressed in most cell types, but not the SCN neurons (Rosenfeld *et al.*, 1993; Tronche *et al.*, 1998). They are also secreted in a circadian pattern in humans (Nelson, 1972) and in mice rhythmic expression is dependent on a functional adrenal clock (Oster *et al.*, 2006). Glucocorticoids are therefore attractive candidates for mediating the circadian synchronisation of peripheral clocks. Treatment with the glucocorticoid analogue dexamethasone (DEX) induced circadian gene expression of core clock and output genes in rat-1 fibroblasts and phase-delayed the temporal expression of *Dbp* in the liver, kidney and heart of wild-type mice (Balsalobre *et al.*, 2000a). Mice with a hepatocyte-specific mutation of the glucocorticoid receptor gene also showed a phase-delaying response to DEX injection in the heart and kidney but not in the liver, demonstrating that the DEX-dependent phase-shifting of expression in these peripheral clocks is a tissue-autonomous process (Balsalobre *et al.*, 2000a).

Genome-wide expression analysis has identified many liver genes that show circadian expression dependent on the presence of glucocorticoids, including genes involved in key metabolic pathways.

Of 169 genes found to be differentially rhythmically expressed in the livers of sham-operated mice, 100 lost circadian rhythmicity in mice that had been adrenalectomised (Oishi *et al.*, 2005a). However, the circadian expression of a number of clock and clock-related genes was not lost, indicating that other mechanisms of governing circadian expression exist. From this experiment it is not possible to identify whether glucocorticoids were mediating circadian expression via signalling to the liver clock or by the direct action of rhythmic glucocorticoid secretion and synchronisation could have been restored by other SCN-dependent signals, such as feeding rhythms (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). In order to investigate the synchronising action of glucocorticoids, Reddy and colleagues (2007) examined the effect of DEX administration on the hepatic transcriptome in mice that had been subjected to surgical ablation of the SCN (SCNX), thus removing all other rhythmic influences. SCN-ablation resulted in the loss of cycling of 78% of the validated circadian transcripts and a single DEX treatment was able to re-synchronise 20% of the transcripts, while 57% showed other temporal changes, including core clock genes. Key metabolic genes were again among those that responded to DEX, indicating the potential importance of this synchroniser in circadian regulation of metabolism.

In this chapter the hearts of SCN-ablated mice were examined in order to further investigate the circadian transcriptome of the heart and identify clock-regulated genes that were dependent on signals from the master central clock mechanism in the SCN. SCN-ablated mice treated with DEX were also examined in order to investigate the synchronising effect of glucocorticoids on the cardiac clock and to assess the importance of different regulatory mechanisms of candidate cardiac CCGs - by the central SCN clock (via the direct action of glucocorticoids) or cardiac clock (via the synchronisation of clock genes).

4.2 Methods

4.2.1 SCN ablation

SCN ablation and DEX injection was carried out by Reddy and colleagues (2007) and mouse heart samples were generously donated by Dr. A. Reddy, Division of Neurobiology, MRC Laboratory of Molecular Biology, Cambridge. SCN ablation was performed according to method used by Akhtar and colleagues (2002). Briefly, adult male CD-1 mice (Harlan-Olac) were caged in groups of eight, in sound-proofed, ventilated environmental chambers under a lighting schedule of 12 hr bright white light and 12 hr dim red light (12L:12DR) and entrainment on release into continuous dim red light (DR:DR) was confirmed by monitoring spontaneous activity rhythms. Bilateral electrolytic lesion of the SCN was performed under general anaesthesia using an insulated gauge 0 insect pin directed stereotaxically at the SCN and the loss of activity rhythms was confirmed. Hearts were harvested from mice on the second cycle after transfer to DR:DR and immediately frozen prior to RNA extraction and RT-PCR. Primer and probes used are listed in Chapter 3.

4.2.2 Dexamethasone injection

Glucocorticoid receptor analogue Dexamethasone 21-phosphate (D-1159; Sigma) (DEX), 300μg/ml dissolved in phosphate-buffered saline (PBS), was delivered intraperioneally (2mg/kg) into SCNX mice at predicted CT6 under dim red light. PBS vehicle was used as a control with 0.15% ethanol. Hearts were harvested on the second cycle after transfer from 12L:12DR to DR:DR and immediately frozen prior to RNA extraction and RT-PCR.

4.2.3 Bioinformatics

Conserved predicted glucocorticoid response elements (GREs) where identified using the Evolutionary Conserved Regions (ECR) Browser (Ovcharenko *et al.*, 2004) and Multiple Sequence Local Alignment and visualization tool (Mulan) + Multi TF tools (Ovcharenko *et al.*, 2005) available from NCBI DCODE.org Comparative Genomics Developments (http://www.dcode.org).

4.2.4 Statistical analysis

Statistical analysis between individual data points was performed by one-way ANOVA followed by Newman-Keuls *post-hoc* multiple comparison tests where ANOVA was significant ($P<0.05$). Analysis between groups of samples was performed by two-way ANOVA.

Performing numerous ANOVA (multiple comparison) increases the probability of observing a significant result by chance (a Type I error). This probability can be calculated using the formula:

$$\% \text{ chance} = 100[1-(1-\alpha)^{N}]$$

where α is the critical value (*P*-value) at which the null hypothesis is rejected and N is the number of null hypotheses (tests) (Toothaker, 1993).

For example, with 10 tests (10 null hypotheses) and α of 0.05 there is:

$100[1-(1-0.05)^{10}] = 100(1-0.95^{10}) = 40\%$ chance of observing a significant result by chance.

The probability of observing a significant result by chance can be reduced to 5% by using a new critical value (α_n) at which the null hypothesis is rejected. This is calculated using a modified Bonferroni correction:

$\alpha_n = 1-[(1-\alpha)^{1/N}]$ (Holland and Copenhaver, 1988; Shaffer, 1995).

For example, with 10 tests and α of 0.05:

$\alpha_n = 1-[(1-0.05)^{1/10}] = 1-(0.95^{0.1}) = 0.0051,$

and the null hypothesis can be rejected where $P<0.0051$.

4.3 Results

4.3.1 Effect of SCN-ablation on cardiac gene expression

Semi-quantitative and quantitative real-time RT-PCR with Taqman probes was carried out on hearts taken from control mice and mice that had undergone surgical ablation of the SCN (SCNX) sacrificed at CT11, CT23 and CT35 as described in the methods. The time-points CT11 and CT35 are 24 hours apart and represent a complete circadian cycle, allowing repeated rhythmic expression (i.e. circadian) to be seen. Ablation of the SCN abolished endogenous circadian rhythmicity, as demonstrated by the loss of rhythmic wheel-running behaviour (Akhtar *et al.*, 2002).

Rhythmic expression of the clock genes *Bmal1*, *Mop4*, *Cry1* and *E4bp4* was observed in hearts from Control mice, with peak expression at CT23 (*Bmal1* phase; Figure 4.1 and Figure 4.7). Rhythmic expression of *Per3* was in anti-phase with peaks at CT11 and CT35 (*Per3* phase; Figure 4.2). An example semi-quantitative gel picture is shown in Figure 4.4. These rhythms are agreement with the cycling seen in Chapter 3 and peaks were confirmed by *post-hoc* analysis. Further clock gene and cardiac genes appeared to be rhythmically expressed (Figures 4.2, 4.3 and 4.5) but due to the limited sample number (n=2) these results were not significant (see Table 4.1) and an increased number of samples is required to determine the significance of these 'rhythms'. As this limitation may be masking biologically relevant results (Type II error), these results have been displayed.

Based on statistical analysis the rhythmic expression of all these genes was abolished or attenuated in SCNX mice (Table 4.1), that is, no significant rhythmicity was observed following SCN-ablation. A higher degree of variability between samples was also observed. The effect of SCN-ablation on the rhythmic expression of *Bmal1* was confirmed by the significant interaction value obtained from a two-way ANOVA followed by *post-hoc* analysis (Table 4.2). The attenuation or abolition of Control rhythms observed for other genes was not statistically significant, again this is likely due to the limited sample number. For this reason patterns that are suggested as a result of SCN-ablation and require further investigation are presented below.

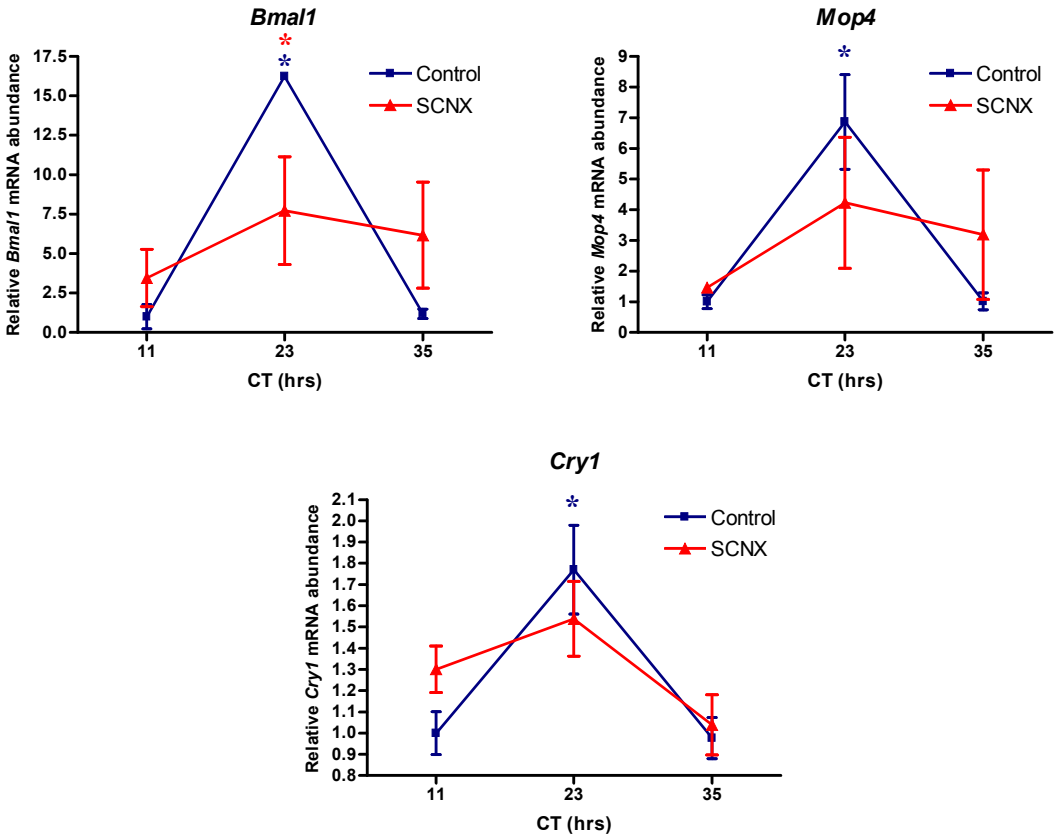


Figure 4.1. Circadian expression of genes in phase with *Bmal1* abolished or attenuated in the hearts of SCN-ablated mice. Taqman quantitative real-time RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *TATA box binding protein (mTbp)*. Values are the mean ±SD for two hearts per point (three observations) and are standardised to Control CT11 for each gene. Control = sham-operated mice, SCNX = SCN-ablated mice.

Statistical analysis was performed by ANOVA, see Tables 4.1 and 4.2. * (blue) Indicates significant difference between adjacent Control time-points following *post-hoc* analysis with Newman-Keuls multiple comparison test, $P<0.05$. * (red) Indicates significant difference between Control and SCNX following *post-hoc* analysis with Bonferroni test.

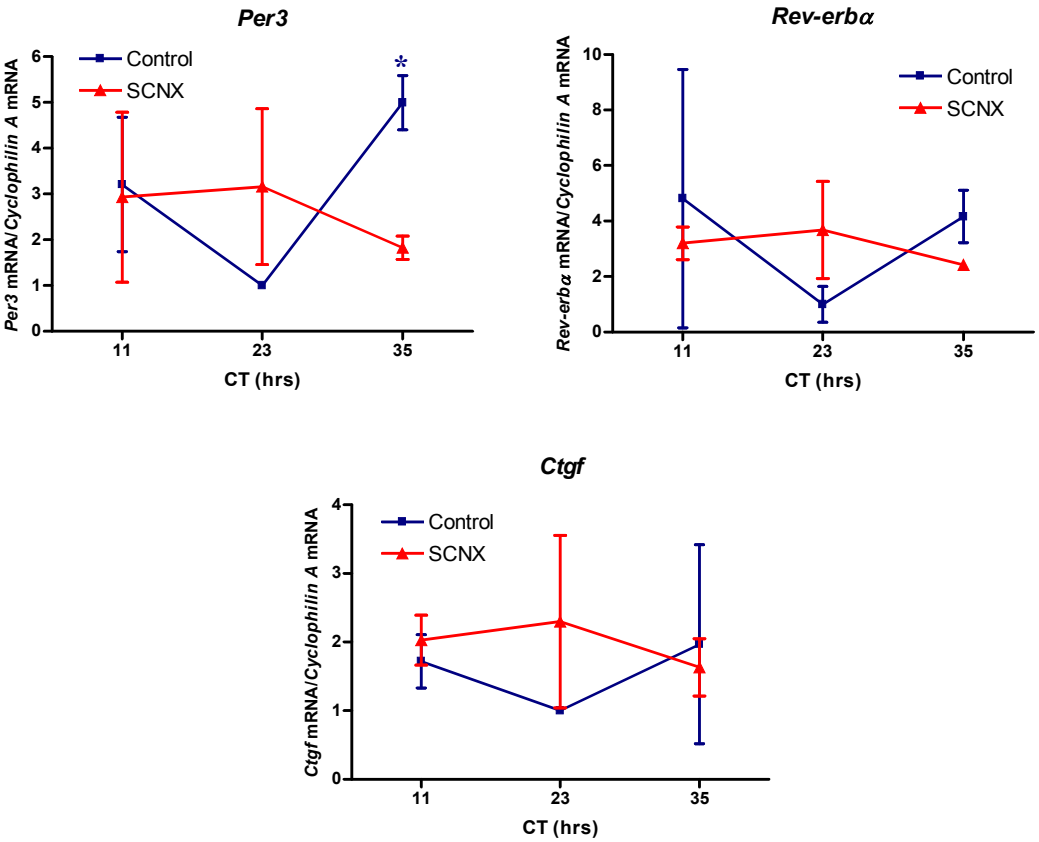


Figure 4.2. Circadian expression of genes in phase with *Per3* abolished or attenuated in the hearts of SCN-ablated mice. Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping genes mouse *Cyclophilin A*. Values are the mean ±SD for two hearts per point (two-three observations) and are standardised to Control CT23 for each gene. Control = sham-operated mice, SCNX = SCN-ablated mice.
Statistical analysis was performed by ANOVA, see Tables 4.1 and 4.2. * Indicates significant difference between adjacent time-points following *post-hoc* analysis with Newman-Keuls multiple comparison test, $P<0.05$.

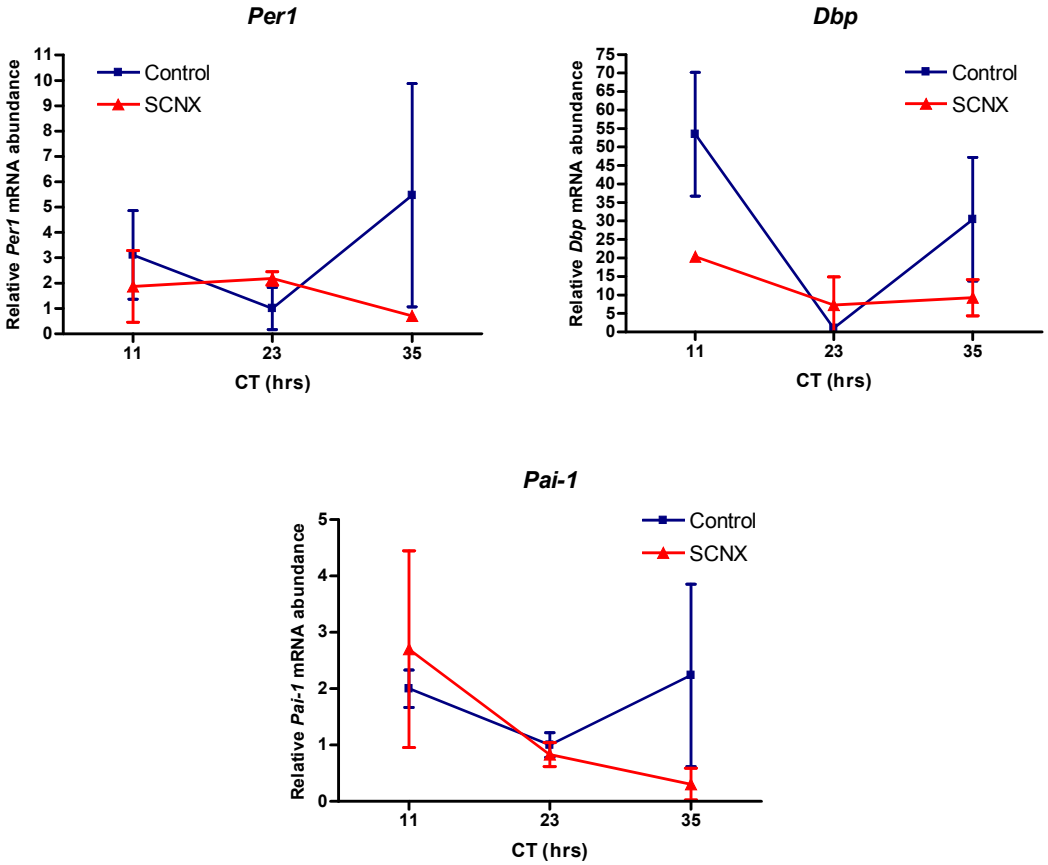


Figure 4.3. Circadian expression of genes in phase with *Per3* abolished or attenuated in the hearts of SCN-ablated mice. Quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping genes mouse *TATA box binding protein* (*mTbp*). Values are the mean ±SD for two hearts per point (two-three observations) and are standardised to Control CT23 for each gene. Control = sham-operated mice, SCNX = SCN-ablated mice. Statistical analysis was performed by ANOVA, see Tables 4.1 and 4.2.

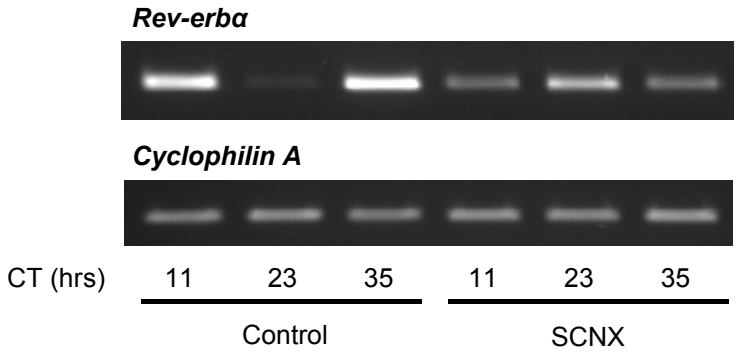


Figure 4.4. Circadian expression of genes in phase with *Per3* abolished or attenuated in the hearts of SCN-ablated mice. An example of an agarose gel image from semi-quantitative RT-PCR of mouse heart mRNA for *Rev-erbα* and *Cyclophilin A*. Control = sham-operated mice, SCNX = SCN-ablated mice.

In contrast to the observed loss of cycling seen for the majority of transcripts in SCNX mice, expression of four genes, *Per2*, *Dec1*, *Bnp* and *Pparα*, exhibited a phase shift in expression resulting in an inversion of the control rhythm in the case of *Per2*, *Dec1* and *Bnp* and the initiation of a rhythm for *Pparα* (Figure 4.5). *Pparγ* also exhibited a significant difference between the expression patterns in Control mice versus SCNX mice but neither of these patterns were rhythmic (i.e. expression was asymmetric) (Figure 4.5).

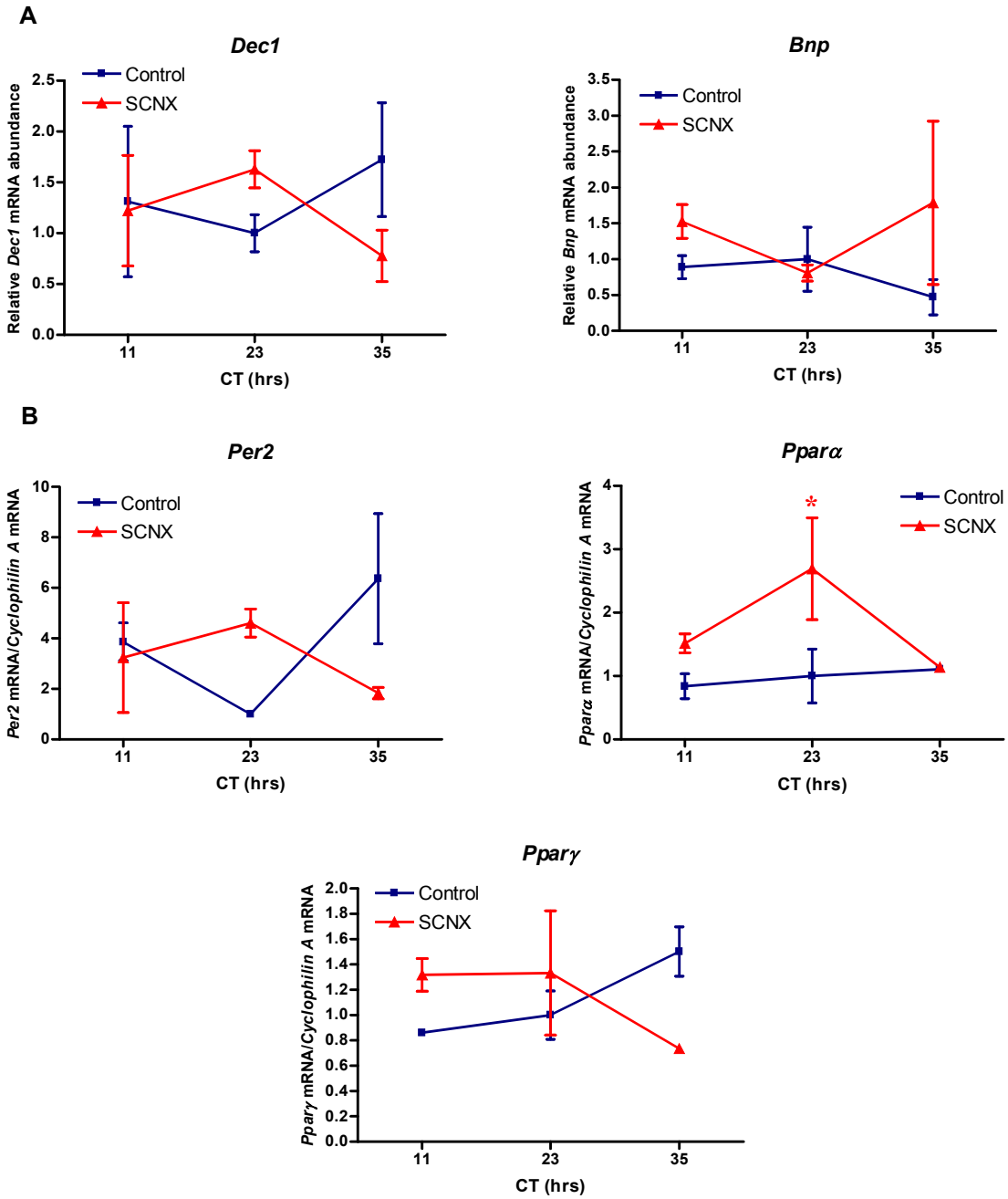


Figure 4.5. Circadian expression of genes showing a phase-shift in the hearts of SCN-ablated mice. Quantitative (A) and semi-quantitative (B) RT-PCR expression values were normalised against the corresponding expression values of the housekeeping genes mouse *TATA box binding protein* (*mTbp*) and mouse *Cyclophilin A* respectively. Values are the mean ±SD for two hearts per point (two-three observations) and are standardised to Control CT23 for each gene.
Statistical analysis was performed by ANOVA, see Table 4.1. * (red) Indicates significant difference between Control and SCNX following *post-hoc* analysis with Bonferroni test.

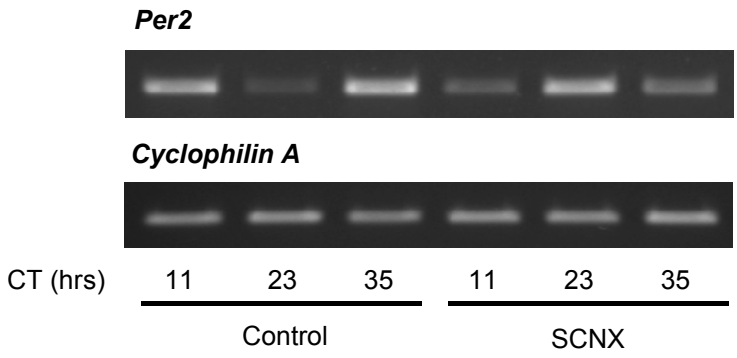


Figure 4.6. Circadian expression of genes showing a phase-shift in the hearts of SCN-ablated mice. An example of an agarose gel image from semi-quantitative RT-PCR of mouse heart mRNA for *Per2* and *Cyclophilin A*. Control = sham-operated mice, SCNX = SCN-ablated mice.

Two genes, *Clock* and *E4bp4*, exhibited rhythmic expression in phase with *Bmal1* in Control mice that appeared unaffected by SCN-ablation (Figure 4.7). The remaining genes showed no expression rhythm in Control or SCNX mice (*Anp*, *Klf5*, *Kv1.5*, *Kv4.2*, *Ucp2* and *Ucp3*) or the variation between samples was too great to determine an expression pattern (*Bmal2*, *Dec2*, *Mef2c* and *Glut4*) (see Figure 1, Appendix 1)

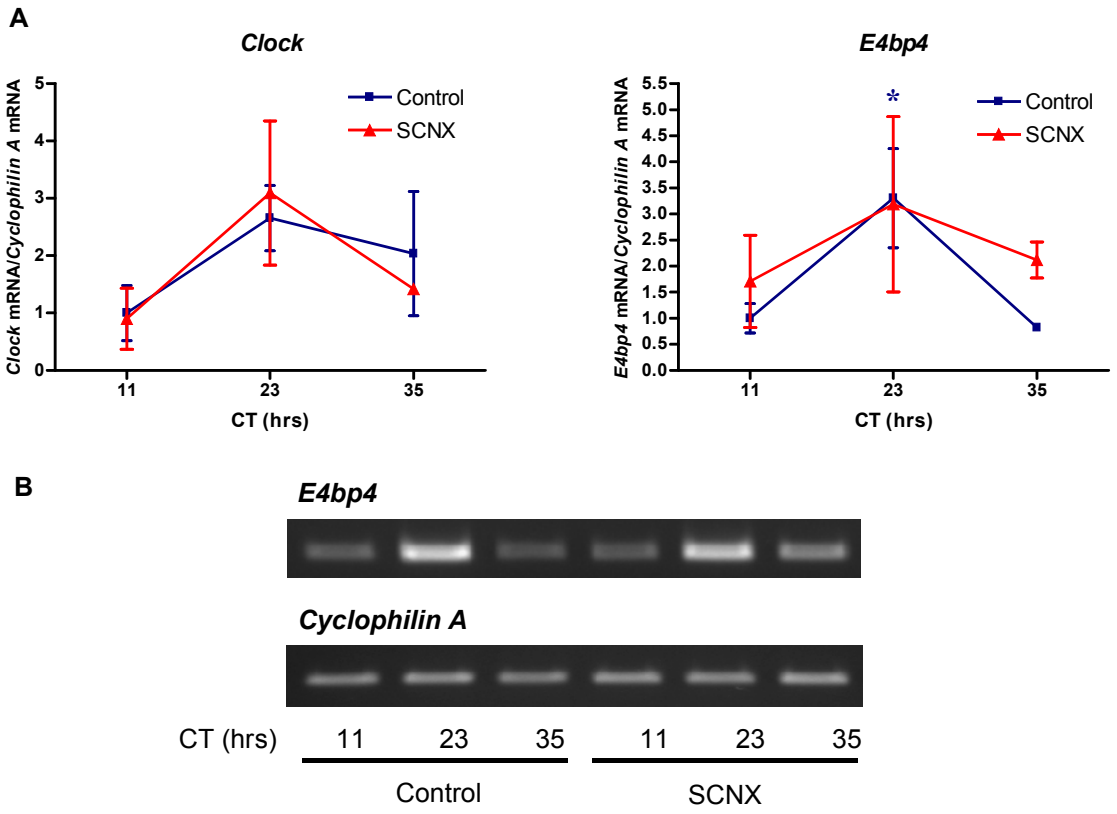


Figure 4.7. Circadian expression of genes that remained unchanged in the hearts of SCN-ablated mice. (A) Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SD for two hearts per point (two-three observations) and are standardised to Control CT11 for each gene. (B) An example of an agarose gel image from semi-quantitative RT-PCR of mouse heart mRNA for *E4bp4* and *Cyclophilin A*. Control = sham-operated mice, SCNX = SCN-ablated mice. Statistical analysis was performed by ANOVA, see Table 4.1. * Indicates significant difference between adjacent time-points following *post-hoc* analysis with Newman-Keuls multiple comparison test, $P<0.05$.

Table 4.1. Significance of temporal expression of genes in hearts taken from control (CON), SCN-ablated (SCNX) and dexamethasone-injected SCN-ablated mice (DEX). Significance of expression rhythms were determined by one-way ANOVA followed by *post-hoc* analysis with Newman-Keuls multiple comparison test. *P*-values in red = significant after correction for multiple testing ($P<0.00063$), blue = significant without correction for multiple testing ($P<0.05$) (see section 4.4.1 for explanation).

Gene	CON		SCNX		DEX	
	P	$F_{2,3}$	P	$F_{2,3}$	P	$F_{2,3}$
***Bmal1* phase**						
Bmal1	0.0001	635.3	0.446	1.069	0.009	35.37
Mop4	0.012	27.42	0.392	1.303	0.700	0.404
Cry1	0.019	19.34	0.091	5.927	0.023	17.30
E4bp4	0.038	11.69	0.481	0.943	0.254	2.238
Bnp	0.330	1.639	0.430	1.133	0.555	0.721
Clock	0.236	2.430	0.134	4.229	0.308	1.789
***Per3* phase**						
Per3	0.0498	9.588	0.662	0.476	0.618	0.567
Per1	0.393	1.298	0.311	1.768	0.014	24.12
Dbp	0.069	7.423	0.158	3.630	0.939	0.064
Per2	0.089	6.017	0.250	2.282	0.302	1.833
Dec1	0.4998	0.882	0.209	2.76	0.352	1.511
Rev-erbα	0.442	1.085	0.558	0.714	0.797	0.246
Pai-1	0.484	0.934	0.193	2.996	0.0024	83.08
Ctgf	0.573	0.673	0.726	0.357	0.184	3.136
Arrhythmic (Control)						
Pparγ	0.053	9.167	0.213	2.710	0.220	2.617
Ucp3	0.048	9.941	0.521	0.817	0.273	2.063
Pparα	0.649	0.502	0.092	5.888	0.095	5.711
Kv1.5	0.662	0.475	0.148	3.866	0.007	38.60
Ucp2	0.723	0.362	0.813	0.222	0.041	11.07
Kv4.2	0.845	0.179	0.224	2.561	0.940	0.063
Bmal2	0.932	0.072	0.422	1.167	0.418	1.185
Cry2	0.568	0.687	0.683	0.434	0.510	0.851
Anp	0.633	0.534	0.842	0.182	0.837	0.190
Klf5	0.579	0.659	0.699	0.404	0.790	0.255
Dec2	0.676	0.447	0.723	0.362	0.727	0.355
Glut4	0.987	0.014	0.313	1.754	0.412	1.210
Mef2c	0.725	0.358	0.565	0.694	0.779	0.272

Table 4.2. Effect of SCN-ablation (SCNX) and dexamethasone injection (DEX) on the temporal expression profiles from control mice or SCNX mice hearts respectively. Interaction between CON and SCNX or SCNX and DEX temporal expression profiles was determined by two-way ANOVA. P-values in blue = significant without correction for multiple testing (P<0.05).

Gene	CON v. SCNX		SCNX v. DEX	
	P	$F_{2,6}$	P	$F_{2,6}$
***Bmal1* phase**				
Bmal1	0.009	11.45	0.154	2.602
Mop4	0.119	3.092	0.274	1.617
Cry1	0.105	3.370	0.017	8.572
E4bp4	0.560	0.639	0.204	2.094
Bnp	0.206	2.080	0.262	1.687
Clock	0.648	0.467	0.062	4.583
***Per3* phase**				
Per3	0.057	4.790	0.727	0.337
Per1	0.200	2.132	0.045	5.411
Dbp	0.085	3.822	0.392	1.101
Per2	0.020	8.115	0.247	1.779
Dec1	0.132	2.889	0.712	0.359
Rev-erbα	0.309	1.440	0.842	0.178
Pai-1	0.242	1.813	0.704	0.372
Ctgf	0.427	0.983	0.373	1.166
Arrhythmic (Control)				
Pparγ	0.019	8.213	0.260	1.699
Ucp3	0.946	0.057	0.967	0.034
Pparα	0.058	4.733	0.034	6.265
Kv1.5	0.183	2.281	0.012	10.06
Ucp2	0.936	0.067	0.084	3.864
Kv4.2	0.758	0.291	0.334	1.325
Bmal2	0.415	1.022	0.818	0.208
Cry2	0.405	1.057	0.993	0.0075
Anp	0.752	0.299	0.835	0.186
Klf5	0.458	0.892	0.581	0.596
Dec2	0.489	0.808	0.807	0.222
Glut4	0.516	0.741	0.494	0.796
Mef2c	0.669	0.430	0.769	0.274

4.3.2 Effect of glucocorticoid injection on cardiac gene expression in SCN-ablated mice.

SCNX mice were injected with DEX or control vehicle (PBS) at CT6. Hearts were again collected from mice sacrificed at CT11, CT23 and CT35, representing 5, 17 and 29 hours post injection.

The expression patterns for two clock genes (*Cry1* and *Per1*) and two cardiac genes (*Pparα* and *Kv1.5*) were significantly affected by DEX injection, as shown by the significant interactions values obtained from two-way ANOVA (Table 4.2). Different responses to DEX injection were observed, with *Cry1* transcript levels decreasing over the time course and *Per1* (Figure 4.8), *Pparα* and *Kv1.5* (Figure 4.9) transcript levels acutely increasing. Further patterns were observed that did not reach statistical significance due to the limited sample number. Clock genes (*Clock*, *E4bp4*, *Per2* and *Bmal1*) and two cardiac genes (*Ucp2* and *Ctgf*) appeared to be acutely up-regulated by five hours post-injection (CT11) (Figure 4.8 and Figure 4.9). Down-regulation greater than 1.5-fold following DEX treatment was observed for three genes (*Dec2*, *Dbp* and *Bnp*). *Kv4.2* expression was also slightly down-regulated (Figure 4.10). The acute response to DEX treatment at CT11 is summarised in Figure 4.11.

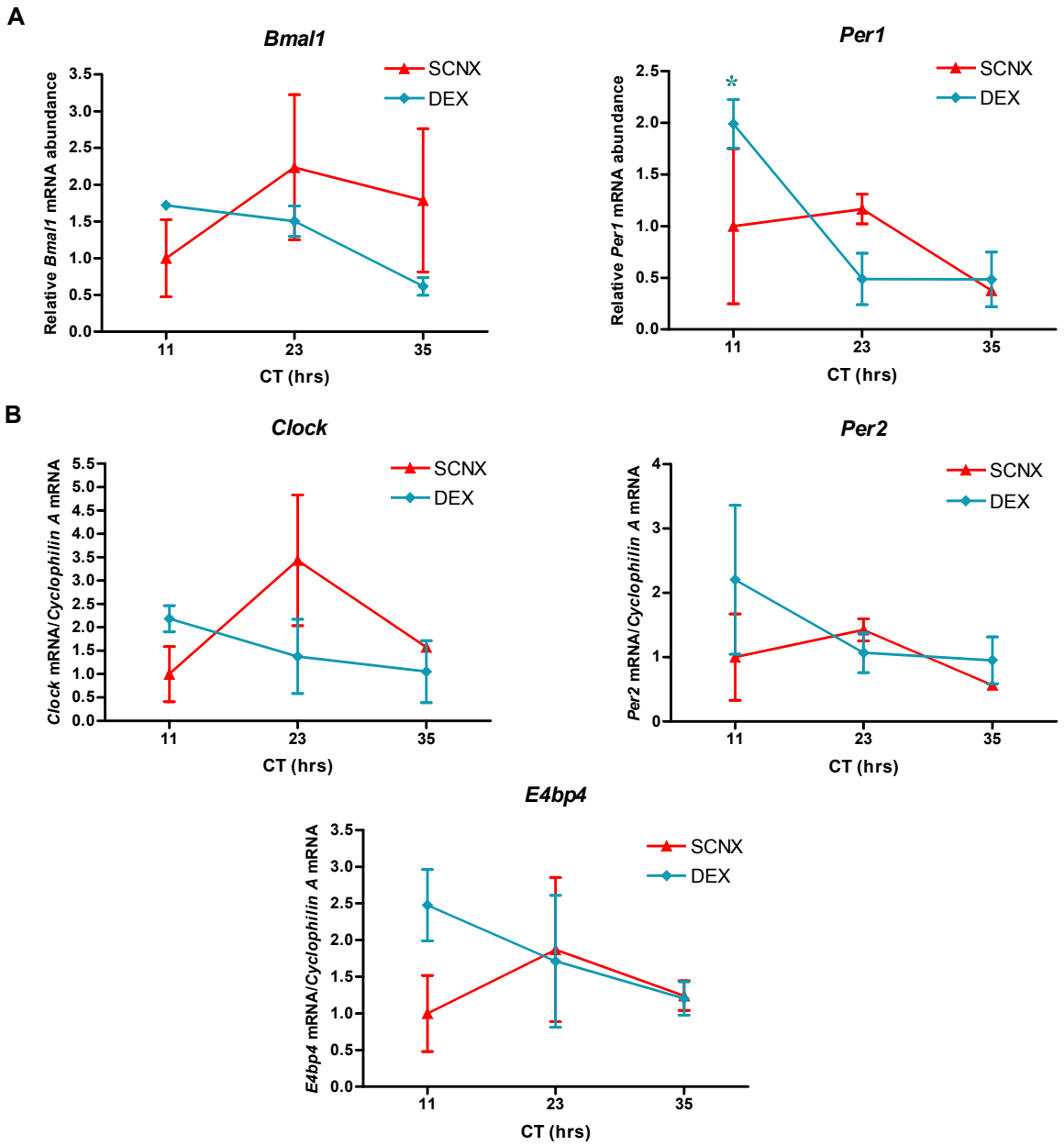


Figure 4.8. Up-regulation of clock gene expression by dexamethasone injection in the hearts of SCN-ablated mice. Quantitative (A) and semi-quantitative (B) RT-PCR expression values were normalised against the corresponding expression values of the housekeeping genes mouse *TATA box binding protein* (*mTbp*) and mouse *Cyclophilin A* respectively. Values are the mean ±SD for two hearts per point (two-three observations) and are standardised to SCNX CT11 for each gene. SCNX = SCN-ablated mice injected with a PBS control, DEX = dexamethasone-injected SCN-ablated mice. Statistical analysis was performed by ANOVA, see Table 4.1. * Indicates significant difference between adjacent time-points following *post-hoc* analysis with Newman-Keuls multiple comparison test, $P<0.05$.

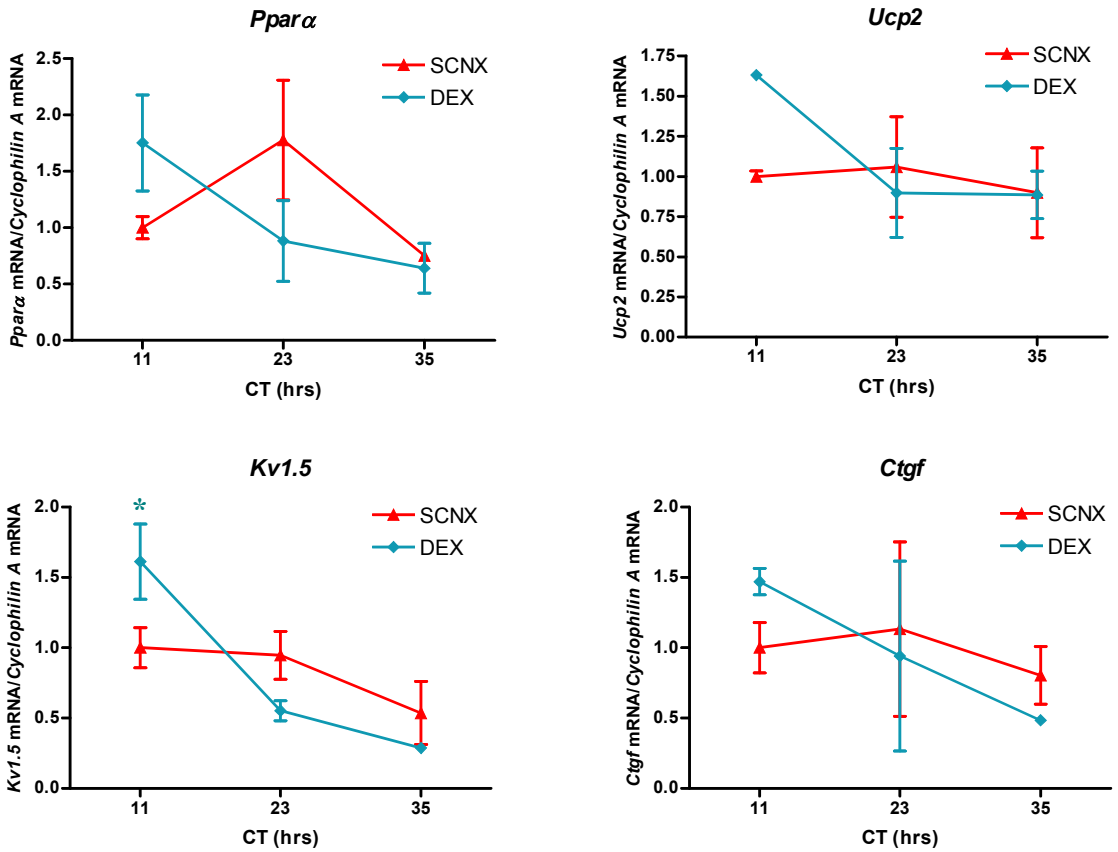


Figure 4.9. Up-regulation of gene expression by dexamethasone injection in the hearts of SCN-ablated mice. Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SD for two hearts per point (two-three observations) and are standardised to SCNX CT11 for each gene. SCNX = SCN-ablated mice injected with a PBS control, DEX = dexamethasone-injected SCN-ablated mice. Statistical analysis was performed by ANOVA, see Table 4.1. * Indicates significant difference between adjacent time-points following *post-hoc* analysis with Newman-Keuls multiple comparison test, $P<0.05$.

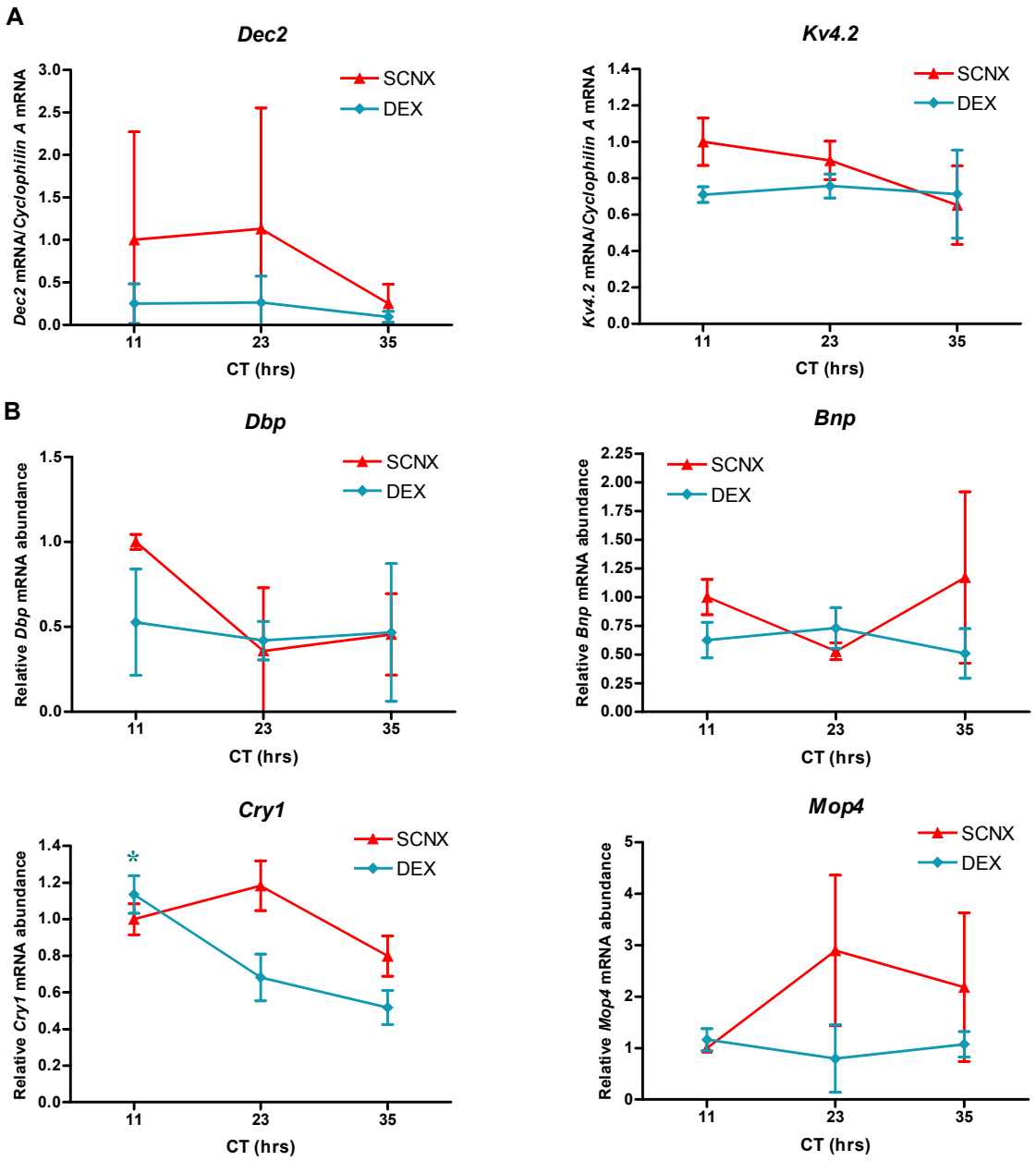


Figure 4.10. Down-regulation of gene expression by dexamethasone injection in the hearts of SCN-ablated mice. Semi-quantitative (A) and Quantitative (B) RT-PCR expression values were normalised against the corresponding expression values of the housekeeping genes mouse *Cyclophilin A* and mouse *TATA box binding protein* (*mTbp*) respectively. Values are the mean ±SD for two hearts per point (two-three observations) and are standardised to SCNX CT11 for each gene. SCNX = SCN-ablated mice injected with a PBS control, DEX = dexamethasone-injected SCN-ablated mice. Statistical analysis was performed by ANOVA, see Table 4.1. * Indicates significant difference between adjacent time-points following *post-hoc* analysis with Newman-Keuls multiple comparison test, $P<0.05$.

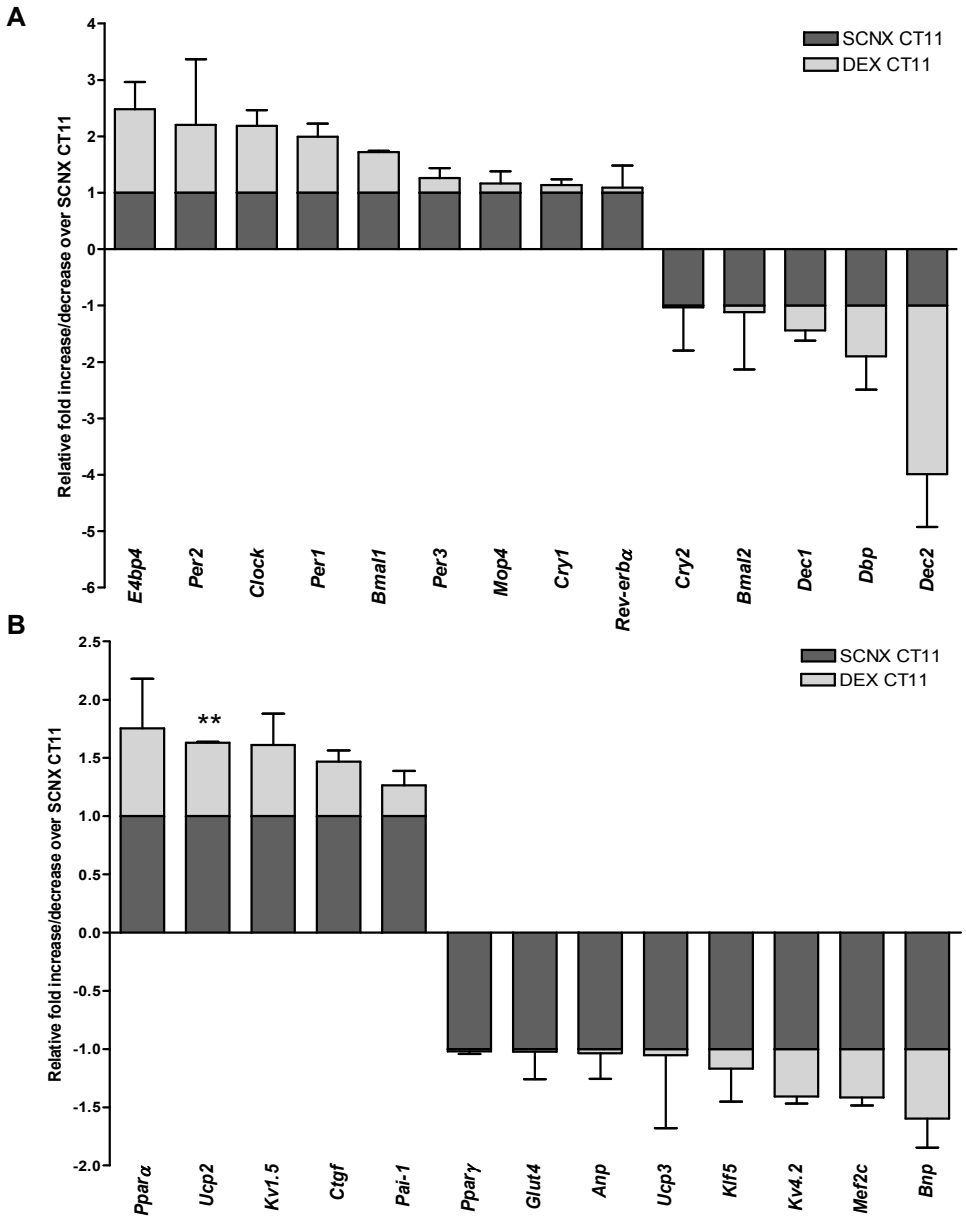


Figure 4.11. Relative induction or repression of gene expression by dexamethasone in the mouse heart at CT11. Fold changes in transcript level of clock genes (A) and cardiac genes (B) in the hearts of SCNX mice following dexamethasone injection (DEX) at CT11 were determined relative to transcript levels in the hearts of SCNX mice injected with a PBS control. Quantitative and semi-quantitative RT-PCR expression values at DEX CT11 were normalised against the corresponding expression values of the housekeeping genes mouse *TATA box binding protein* (*mTbp*) and mouse *Cyclophilin A* respectively, and standardised to SCNX CT11. Values are the mean ±SD for two hearts per point (two-three observations).

Statistical analysis was performed by ANOVA, ** $P<0.01$.

In addition to acutely affecting expression, injection of DEX also resulted in altered expression patterns over the three time-points compared to SCNX for some genes (Table 4.1). In the case of *Bnp* expression, as well as causing an initial down-regulation, treatment with DEX appeared to result in re-establishment of the control rhythm that was attenuated by SCN-ablation (Figure 4.12), although again these results did not reach significance. This was potentially also the case for *Per1* where transcript levels at CT11 were restored to those seen in the rhythmic control, although whether this is repeated at CT35 is difficult to ascertain due to the large error seen in the control rhythm (Figure 4.12). In other cases, an acute up-regulation in response to DEX was followed by a prolonged down-regulation of expression over the time-course, as seen for the clock genes *Cry1*, *Mop4*, *Dec2* (Figure 4.10) and *Clock* and *Bmal1* (Figure 4.8), or DEX injection did not affect expression compared to SCNX (see Figure 2, Appendix 1).

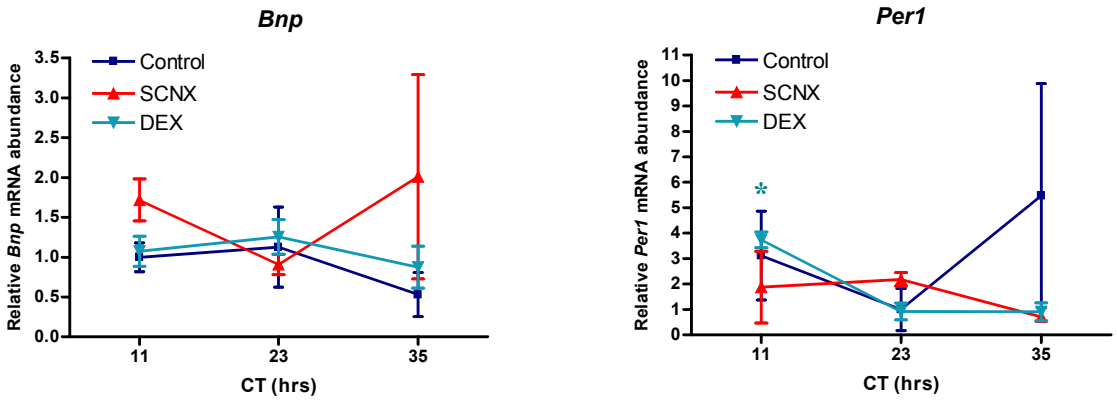


Figure 4.12. Re-establishment of the *Bnp* and *Per1* expression rhythms in SCN-ablated mice hearts following dexamethasone injection. Quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping genes mouse *TATA box binding protein* (*mTbp*). Values are the mean ±SD for two hearts per point (three observations) and are standardised to CON CT11 (*Bnp*) or CON CT23 (*Per1*). CON = sham-operated control mice, SCNX = SCN-ablated mice injected with a PBS control, DEX = dexamethasone-injected SCN-ablated mice.

* Indicates significant difference between adjacent time-points following *post-hoc* analysis with Newman-Keuls multiple comparison test, $P<0.05$.

4.3.3 Bioinformatics

The presence of conserved Glucocorticoid Response Elements (GRE) in the proximal promoter of a gene would potentially enable the Glucocorticoid Receptor (GR) to bind to and activate that promoter. Putative conserved GREs were identified in the first 10kb upstream of the putative transcription start site (where sequence was available) by *in silico* analysis (Table 4.3). It is important to note that

further sites may lie outside these regions, either further upstream or in downstream non-coding regions such as introns.

Table 4.3. Conserved potential GREs in clock and cardiac gene promoters. Putative conserved GREs were identified using the Evolutionary Conserved Regions (ECR) browser (Ovcharenko *et al.*, 2004) and rVISTA2.0 (Loots and Ovcharenko, 2004) tools from NCBI dCODE (www.dcode.org). The V$GR_Q6 and V$GRE_C TRANSFAC matrices were used for identification of conserved full GRE sites. UTR = un-translated regions.

Gene (distance analysed 5' of TSS)	GRE conserved with rat	GRE conserved with rat & human
Genes up-regulated by DEX treatment		
Per1 (10kb)	2	1
Per2 (10kb)	2	0
Bmal1 (10kb)	1	0
Clock (10kb)	1	0
Cry1 (10kb)	1	1
E4bp4 (10kb)	3	0
Pparα (10kb)	3	0
Ucp2 (6kb) + 5'UTR	5	2
Kv1.5 (10kb)	2	1
Ctgf (10kb)	5	2
Genes down-regulated by DEX treatment		
Dec1 (10kb)	0	0
Dec2 (10kb)	0	0
Kv4.2 (10kb)	2	0
Mop4 (10kb)	3	0
Bnp (10kb)	5	1
Genes unaffected by DEX treatment		
Dbp (10kb)	1	0
Bmal2 (10kb)	1	0
Per3 (10kb)	4	0
Cry2 (10kb)	2	0
Rev-erbα (10kb)	3	0
Pai-1 (10kb)	4	0
Anp (10kb)	1	0
Klf5 (10kb)	0	0
Ucp3 (5kb)	5	1
Pparγ (10kb)	4	0
Mef2c (6.5kb)	5	0
Glut4 (10kb)	0	1

4.4 Discussion

4.4.1 Findings and limitations

The results of this chapter indicate the importance of the SCN in the circadian regulation of cardiac gene expression *in vivo* and the varying effects of glucocorticoids on gene expression, contributing to the notion that glucocorticoids are key mediators of internal circadian synchrony. The findings of this chapter are however likely to be severely limited by the small number of samples used. Only two out of three hearts per data point were examined by RT-PCR in order to save time and one sample was missing, restricting Control CT11 to n=2. The results from RT-PCR on two samples displayed a high degree of disagreement and as such did not yield many statistically significant results. When correction for multiple comparisons is taken into account this becomes even more apparent as only one gene (*Bmal1)* demonstrated statistically significant rhythmicity in Control hearts. The trends observed for other genes may be real but RT-PCR of the third set and/or cross-validation with other techniques is required for confirmation. Cross-validation of cycling of a subset of genes from a microarray study was successfully achieved by *in-situ* hybridisation in a study of the mouse liver transcriptome (Akhtar *et al.*, 2002). This was done using three samples per time-point so cross-validation of the results presented in this chapter may require more than the one remaining set of samples and increasing the RT-PCR results to n=3 may be more fruitful.

Livers from the same mice as those used in the present study were examined in a similar experiment by Michael Hastings' group in Cambridge (Reddy *et al.*, 2007). Three hearts per data point were used for microarray analysis of transcript level, revealing significant cycling for 48% of putative circadian transcript by ANOVA. The cycling of four clock genes transcripts from this sub-set, and the loss of cycling in SCN-ablated mice hearts, was confirmed using quantitative (q) RT-PCR with Taqman probes (n=3), implying that qRT-PCR is a suitable technique. The effect of DEX administration of some transcripts was also confirmed using quantitative RT-PCR. Significant effects of DEX were observed despite some time-points displaying a high degree of error around the mean (SEM), implying that the use of three sets of samples (n=3) is sufficient. Therefore, using qRT-PCR (with Taqman probes) and three samples per time-point would have likely been sufficient to yield more meaningful results in the current study.

In addition to determining significance by ANOVA the importance of *post-hoc* testing (as used here) and visual interpretation of the result is evident when considering *Pparγ* and *Ucp2*. These genes both displayed statistically significant ($P<0.05$) expression patterns in Control samples but as the higher transcript levels at CT35 and CT11 respectively were not repeated at CT11/CT35 these patterns are not circadian (Figure 4.5 and Figure 1, Appendix 1). The significance of these patterns comes from

the levels of these two transcripts being less variable, therefore even with a reduction in variability that may come from an increased sample number, care should be taken when interpreting results.

Whilst acknowledging the limitations of the experimental design of this chapter, the significance of the findings are explored below.

4.4.2 Circadian gene expression in the heart is partially dependent on the SCN

The dependence of cardiac circadian gene expression on an intact SCN can be seen by the loss of cycling of all rhythmic genes in SCN-ablated mice. The majority of genes rhythmically expressed in control mice were clock or clock-related genes. This is likely to be a reflection of the strong amplitude of circadian oscillations seen for these transcripts (see Chapter 3). Expression rhythms were also observed for the cardiac genes *Pai-1*, *Ctgf* and *Bnp* (Figure 4.2, Figure 4.3 and Figure 4.5) which reflected their 20-hour expression profiles (see Chapter 3). Since the molecular clock hierarchy is organised into core clock genes, primary CCGs, secondary CCGs, etc., one would expect the regulation of core clock genes to be most dependent on signals from the SCN, as was observed.

As well as loss or attenuation of cycling, SCN-ablation may have resulted in alterations in phase and/or amplitude of expression. For some genes phase shifts of up to 12 hours were seen, resulting in inversion of the control expression rhythms (Figure 4.5). It is therefore possible that the apparent loss of cycling observed was actually the result of alterations in the phase of expression which was not fully apparent due to the limited number of time-points sampled. Expression of *Pparγ* was altered but neither the control nor SCNX temporal patterns were rhythmic, which may reflect unknown effects of SCN-ablation that are independent of the clock. Interestingly, SCN-ablation may have had little effect on the rhythmic expression of the core clock gene *Clock* and the clock output gene *E4bp4*, and the rhythmic expression of *E4bp4* was also not lost in the livers of adrenalectomised mice (Oishi *et al.*, 2005a). The effect of DEX on *E4bp4* expression was also short-lived, with rhythmic expression appearing to return within 18 hours of DEX administration (Figure 4.8). Therefore, robust expression of these genes, independent of signals from the SCN, may be an important characteristic of self-sustaining peripheral clocks, although this was not sufficient to maintain rhythmic expression of other core clock genes and outputs in the heart. In the liver and lungs, and presumably other peripheral tissues, CLOCK is required for rhythmicity (DeBruyne *et al.*, 2007b) (in contrast to the SCN where CLOCK is dispensable and can be substituted by MOP4 (DeBruyne *et al.*, 2007a), therefore the robust circadian expression of *Clock* may regulate the cycling of unknown target genes in the heart. Whether expression of *Clock* and *E4bp4* is unaffected by SCN-ablation in other tissues is unknown and the heart clock may be unique in retaining, in part, the capacity to generate daily rhythms in gene expression *in vivo*, independently of the SCN. It is interesting to speculate that this may be a reflection of the importance of the cardiac clock and the necessity for robust circadian expression rhythms in the heart.

SCN-ablation has previously been shown to alter rhythmic expression in peripheral tissues (Sakamoto *et al.*, 1998; Akhtar *et al.*, 2002; Terazono *et al.*, 2003) and all but two of the cycling genes in the present study exhibited changes in expression. Therefore, the SCN is clearly necessary for the maintenance of normal circadian expression in the heart. However, a study looking at peripheral tissues from SCN-ablated mice *ex vivo* found circadian expression of PER2 protein persisted but with phase-desynchrony between different tissues and animals (Yoo *et al.*, 2004). The SCN may therefore function as a phase synchroniser of self-sustained rhythms in peripheral tissues rather than an initiator of rhythms. The loss of rhythmicity observed in this chapter, and in particular the high degree of disagreement between samples, may then reflect differential phase-shifting of individual samples. For instance, the control expression rhythms of *Bmal1* and *Mop4* show little inter-sample variation but expression among SCNX samples is highly variable (Figure 4.1).

4.4.3 Changes in gene expression rhythms by glucocorticoids

SCN-ablation results in the loss of behavioural rhythms, such as rhythmic locomotor activity and feeding, as well as the rhythmic secretion of the glucocorticoid corticosterone from the adrenal gland (Moore and Eichler, 1972; Stephan and Zucker, 1972; Meyer-Bernstein *et al.*, 1999). The loss of these rhythms and the neuronal signals from the SCN are all likely to contribute to the disruption of the cardiac clock, although the relative importance of each signalling mechanism is unknown. The importance of glucocorticoids in clock synchronisation is suggested by the alteration in cortisol rhythms with age. In old age cycling of cortisol is maintained but the amplitude of the rhythm decreases and the peak is advanced, which may contribute to the altered sleep patterns of the elderly (Van Cauter *et al.*, 1996). The decline in cortisol rhythm amplitude may be a consequence of a decrease in the number of functioning SCN neurones seen with age and the ability of the SCN neurons to synchronise with the environment is diminished in aged mice (Weinert, 2000). Dampened activity and *Per2* expression rhythms in the SCN are also observed in older mice (Weinert *et al.*, 2001). It is tempting to speculate that declining SCN rhythmicity, and subsequent rhythmic glucocorticoid expression may lead to less robust expression rhythms in the heart, contributing to declining cardiac function with age and onset of disease.

Application of DEX against the arrhythmic background of SCN-ablated mice allows the relative importance of this SCN-derived circadian signal to be assessed. If DEX is an effective synchroniser, one would predict a common response to injection among groups of mice, leading to the re-establishment of common temporal expression patterns. However, if DEX elicits a response independently of the clock then common temporal expression may not be observed due to variations between mice. In this study a variety of common responses to DEX treatment were observed but re-establishment of the control rhythm was only indicated for *Bnp* and *Per1* (Figure 4.12), implying that DEX alone is not sufficient. Injection of DEX at CT6 may have led to peak DEX levels occurring

at a time different to the endogenous glucocorticoid peak since endogenous adrenal corticosterone peaks at ZT12 in rats (Moore and Eichler, 1972), which could account for differences in phase between control and DEX rhythms. The response of a gene to glucocorticoids and other stimuli can also depend on the time of stimulus relative to the expression phase of a gene. For example, administration of DEX to fibroblasts resulted in a different degree of phase advance or delay of *Dbp* and *Rev-erbα* expression depending on the time of day (Balsalobre *et al.*, 2000a) and in the SCN, *Per1* and *Per2* are only induced and phase-shifted by light during the subjective night (Yan and Silver, 2002). The response to a stimulus is also gene and tissue-specific – *Per1* and *Per2* in the SCN responded differently to phase-delaying and phase-advancing light pulses (Yan and Silver, 2002) as do different peripheral tissues (Yamazaki *et al.*, 2000).

Administration of DEX did temporally affect the expression of a number of clock and cardiac genes, resulting in a 'wave' of up- or down-regulation across the time-course. In the liver, 60% of the circadian transcriptome was synchronised by a wave of acute induction and repression followed by later induction (Reddy *et al.*, 2007). In the current study of the heart, acute up-regulation by CT11 was the most common response, being observed for one third of genes (5 of 14 clock or clock-related genes and 4 of 13 cardiac genes, Figure 4.8, Figure 4.9, and Figure 4.11). Acute and/or prolonged down-regulation of expression was also observed for clock-related and cardiac genes (Figure 4.10 and Figure 4.11). The increased power of the liver study due to the use of a microarray to investigate many more transcripts allowed further trends to be seen. 20% of the 366 target circadian genes were acutely resynchronised within 5 hours of DEX treatment, and 57% showed temporally specific induction or suppression in the following 29 hours. The results also showed that the inductive effect of DEX was anti-phasic to the suppressive effect. Chi(χ)-squared analysis of these differing patterns of response to DEX administration was highly significant, demonstrating that a single DEX stimulus can elicit different temporal responses and have an extensive effect on the liver circadian transcriptome, acting as principal synchroniser. The results in the heart presented here also show that DEX administration affects gene expression differently but due to the smaller number of genes investigated the significance of the different responses can not be tested in the same way as for the liver.

Many of the genes up-regulated by DEX possess Glucocorticoid Response Elements (GREs) in their promoters (Table 4.3), which are likely to govern these effects and directly mediate the re-synchronisation of circadian rhythms by acutely 'kick-starting' clock gene expression. In particular, *Per1* (which exhibited some re-establishment of the control rhythm) and *Per2* may be involved in this process, since they are immediate early genes, induced by a number of factors, including glucocorticoids (Schibler *et al.*, 2003) and are involved in entrainment of the SCN clock by light (Hastings and Herzog, 2004). The human and mouse *Per1* promoters contains a conserved GRE

(at -6393 bp relative to the TSS) and treatment of rat-1 fibroblasts with DEX rapidly activated *Per1* expression, resulting in cycling of clock and clock-related genes *in vitro* (Balsalobre *et al.*, 2000a). A GRE-like site in the *Per1* promoter is also involved in the up-regulation of *Per1* by acute stress (Yamamoto *et al.*, 2005), implying that *Per1* may integrate circadian and stress response. PER1 and PER2 have non-redundant roles in the mammalian circadian clock (Bae *et al.*, 2001; Zheng *et al.*, 2001) and PER2 may be particularly important in phase co-ordination of peripheral clocks since mutations in the *Per2* gene lead to abnormal circadian rhythms in mice and humans (Toh *et al.*, 2001). A recent study by Kornmann and colleagues (2007) temporally 'switched off' the liver clock by interfering with *Rev-erbα* expression and identified 31 genes that remained rhythmically expressed (10% of those cycling with a functioning clock). *Per2* was one of these genes being rhythmically controlled by systemic cues from the SCN, and since *Per2* expression rhythms also persist *ex vivo* (Yoo *et al.*, 2004), it is well placed to integrate synchronising cues and the core clock mechanism.

The mechanism of down-regulation of expression caused by DEX is likely to be less direct. The Glucocorticoid Receptor (GR) activates transcription via GREs on binding of glucocorticoids but is not commonly known to repress transcription (although some mechanisms have been described, adding to the complexity of GR signalling - see Torra *et al.*, (2000). Therefore, down-regulation in response to DEX administration is likely to be an indirect effect of glucocorticoids, mediated by a re-synchronised clock or a clock-independent, glucocorticoid-dependent mechanism. Prolonged reduction in expression levels may also be a consequence of a phase shift and the limited time-points sampled (see above). Expression of the transcriptional repressor *E4bp4* was acutely up-regulated by DEX in the heart (Figure 4.8) and liver (Reddy *et al.*, 2007, supplementary Figure S1) therefore it may mediate glucocorticoid-dependent down-regulation of target genes, potentially aided by decreased expression of its antagonistic activator *Dbp*. E4BP4-like binding sites have been identified in genes that were down-regulated by DEX in mouse fibroblasts (Wallace *et al.*, 1997), therefore E4BP4 may be an integrator of clock-dependent and clock-independent response to glucocorticoids. Following the DEX-mediated acute up-regulation *E4bp4* expression levels return to the pattern seen in SCNX and control mice, suggesting that circadian expression of *E4bp4* is quickly re-established and this factor may play a role in modulating the response of the clock to glucocorticoids. The clock gene *Rev-erbα* has also been shown to be transcriptionally down-regulated directly by DEX and the GR in the liver (Torra *et al.*, 2000) (although was not affected by DEX the heart in this experiment) so may also integrate the clock and glucocorticoid response.

The ability of glucocorticoids to up-regulate and down-regulate clock and cardiac gene expression would enable flexibility in response to synchronisation signals from the SCN and a distribution of circadian phases within or between tissues, suggesting that glucocorticoids may be sufficient as principal synchronisers of peripheral circadian gene expression. In addition to direct regulation by

glucocorticoids via GREs and indirect via synchronised clock gene expression, glucocorticoids may mediate different phases of response to SCN entrainment by the direct activation of genes that contain GREs and circadian regulatory elements (E-boxes/D-boxes/RREs), allowing convergence of systemic and intracellular synchronisation and providing further flexibility in the response of the system.

4.4.4 Tissue-specific effects of glucocorticoids

The analysis of gene expression in the heart (present study) and liver (Reddy *et al.*, 2007) from SCN-ablated mice allows comparison of the effects of DEX administration on these two contrasting organs. The responses of the clock and clock-related genes *Per1*, *Cry1*, *Bmal1* and *E4bp4* were similar in these two tissues but the delayed up-regulation of *Dbp* in the liver was not observed in the heart. Previous studies have suggested variation in the response of different tissues to synchronising stimuli and tissue-specific differences in phase have been observed *ex vivo* (Yoo *et al.*, 2004). Alternatively, the SCN may signal to different organs by different mechanisms, as suggested by the finding of Guo *et al*. (2005) that non-neural (behavioural or blood-borne) signals are adequate to maintain circadian rhythms of clock genes in the liver and kidney, but not in the heart, spleen or skeletal muscle. The presence of multisynaptic autonomic connections from the SCN to the heart (Scheer *et al.*, 2001) also suggests the need for neural signalling. The liver on the other hand is particularly responsive to a variety of metabolic signals, such as rhythmic feeding, which is believed to be an important synchroniser of gene expression (Damiola *et al.*, 2000; Hara *et al.*, 2001; Stokkan *et al.*, 2001). Therefore, the liver and heart may be programmed to respond differently to glucocorticoids and this may explain the difference in the response of *Dbp*, which is an important regulator of liver-specific genes (Lavery *et al.*, 1999).

Interestingly, the uncoupling of peripheral oscillators and the SCN by restricted feeding is inhibited by glucocorticoids (Le Minh *et al.*, 2001), therefore glucocorticoids may act as robust synchronisers controlling for the influence of external signals but allowing response to stronger stimuli as appropriate. In the case of the liver, feeding patterns controlled by the SCN may be the principal mediators of synchronisation, whereas the heart may need to be less sensitive to external changes (requiring neural and hormonal signalling; Guo *et al*., (2005), allowing tissue-specific adaptation of peripheral clocks to their environments.

The finding that a single glucocorticoid stimulus may be sufficient to restore the Control expression pattern of *Bnp* in the heart is significant given the cycling profile observed in Chapter 3 and its important role in cardiac pathophysiology, strengthening the hypothesis that it is a putative CCG. The effects of glucocorticoids may be direct or indirect (via the clock) due to identification of putative conserved GREs (Table 4.3, one conserved between mouse and human at -6037 bp relative to the TSS) and E-boxes (Table 3.8) in the *Bnp* promoter and the peak in expression observed in Chapter 3 may be governed by behaviour-induced glucocorticoid rhythms. Analysis of temporal transcript

abundance in adrenalectomised mice would shed light on this. The adrenal gland and the clock are believed to be involved in the circadian regulation of blood pressure (Sei *et al.*, 2008) and mice lacking a functional *Npr1* gene that encodes for the BNP and ANP receptor NPRA have elevated blood pressure (and exhibit marked hypertrophy) (Oliver *et al.*, 1997). It is therefore conceivable that rhythmic BNP expression may in part govern circadian blood pressure regulation.

The observation that conserved GREs are present in the promoters of many of the cardiac genes investigated (Table 4.3) suggests that glucocorticoids may regulate these genes and such regulation may result in circadian expression. This is also suggested by the loss of rhythmic *Ctgf* expression in livers of adrenalectomised mice (Oishi *et al.*, 2005a) suggesting circadian expression of *Ctgf* may be dependent on glucocorticoids. The circadian expression of *Pai-1* may also be affected by glucocorticoids since up-regulation has been seen in other studies and tissues (Andreasen *et al.*, 1990; Bruzdzinski *et al.*, 1993), although the induction in the heart was not significant. Glucocorticoids appear to be able to regulate related genes differently, for instance, *Kv1.5* expression was up-regulated at CT11 whereas *Kv4.2* expression was down-regulated. This may in part be a result of differences in transcript abundance at the time of DEX administration due to their opposing circadian expression profiles (see Chapter 3 and Yamashita *et al*. (2003) or the presence/absence of GREs. Previous studies have also shown the induction of *Kv1.5* mRNA by glucocorticoids in the rat heart ventricle and ventricular myocytes (Nishiyama *et al.*, 1995; Levitan *et al.*, 1996). Up-regulation of *Ucp2* and *Pparα* was also observed whereas levels of *Ucp3* and *Pparγ* did not change.

4.4.5 Conclusions

As discussed above the lack of statistical power has limited the finding of this chapter. However, in this first report studying the hearts of SCN-ablated mice the loss of rhythmic clock gene expression is apparent, suggesting that signals from the SCN are important in synchronising the cardiac clock. The heart may also possess the ability to autonomously regulate the clock since the results suggest that rhythmic expression of *Clock* and *E4bp4* was not affected by SCN-ablation. The variety of effects on temporal gene expression observed likely reflects the role of the SCN as a phase synchroniser rather than rhythm generator. The different responses to DEX administration point to the important role of glucocorticoids in mediating diverse internal synchrony. Glucocorticoids may play a role in a variety of cellular processes and thus provide a flexible mechanism for the integration of stress signalling with the synchronisation of peripheral clocks, allowing better adaptation to the environment. The restoration of the Control expression pattern of *Bnp* in hearts of SCN-ablated mice implicates glucocorticoids in the circadian regulation of this gene and adds to the suggestion that from Chapter 3 that *Bnp* is a CCG.

5. Circadian regulation in cardiac cells: *in vitro* model systems to examine the intrinsic cardiac clock

5.1 Introduction

Autonomous cellular clocks exist in most tissues, thus cell culture systems can be useful for examining the circadian regulation of genes in different cell types. Cell-based systems also allow the examination of regulation directly by molecular clock mechanisms since they are devoid of external influences, such as feeding rhythms and humoral factors. Rodent fibroblast cell lines are well established models of circadian regulation and exhibit self-sustained cell-autonomous circadian expression in culture with similar properties to isolated SCN cells (Nagoshi *et al.*, 2004). However, unlike SCN cells, fibroblasts do not communicate in culture and unless their individual cellular oscillators are synchronised circadian expression rhythms are distorted or undetectable. Circadian expression rhythms in cell lines were first demonstrated in Rat-1 fibroblasts by acutely treating them with a high concentration of serum (serum shock) (Balsalobre *et al.*, 1998). Subsequently, a number of other substances were shown to elicit circadian transcript oscillations, primarily in fibroblast cell lines (Rat-1 and NIH-3T3 cells; summarised in Nagoshi *et al*. (2005). One such substance is DEX and treatment of cells with this compound may mimic the synchronising effect of glucocorticoids on peripheral tissues (see Chapter 4). DEX has also been shown to trigger circadian expression rhythms in diverse cell types in culture, from Rat-1 fibroblasts (Balsalobre *et al.*, 2000a) to rat C6 glioma cell (Fujioka *et al.*, 2006) and cells derived from rat reproductive tissues (He *et al.*, 2007).

In this chapter cell culture systems were used to examine the molecular machinery of the cardiac clock *in vitro* and further investigate the direct regulation of putative CCGs, in the absence of external signals. The cardiac-derived rat cell line H9c2 was used and compared to the established NIH-3T3 fibroblast cell line, with the aim of investigating H9c2 cells as a model of the cardiac clock. The effect of different synchronising signals on different cells was also compared by DEX treatment and serum-shock.

5.2 Methods

5.2.1 Over-expression of hCLOCK and hBMAL1 in H9c2 cells

Expression plasmids for human (h) CLOCK and hBMAL1 were kindly donated by Dr John Hogenesch, The Genomics Institute of the Novartis Research Foundation, San Diego, California, USA.

Transfections were performed with jetPEI cationic polymer transfection reagent (Q-Biogen) as described in Chapter 2. Amounts of transfection reagent, DNA and media were optimised to give the least cell death and greatest over-expression (and therefore uptake by cells) of hCLOCK and hBMAL1. 1μg of each plasmid was used (and 2μg of empty pcDNA3.1(+) control plasmid) with 2μl jetPEI/μg DNA.

5.2.2 Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls *post-hoc* multiple comparison test for Figures 5.4 – 5.10.

5.3 Results

Two cellular model systems were used to test for direct targets of the clock: over-expression of core clock transcription factors in H9c2 cells, and synchronisation of circadian gene regulation by treatment with DEX and serum shock in H9c2 cells compared to NIH-3T3 cells.

5.3.1 Up-regulation of cardiac gene expression by over-expression of clock factors in H9c2 cells *in vitro*

To further identify *bona fide* targets of the molecular circadian clock in cardiac cells, the human clock transcription factors hCLOCK and hBMAL1 were ectopically over-expressed in the cardiac-derived rat cell line H9c2. Human factors were used because previous work in this lab has found them to activate target gene expression in rodent cell lines and they can be distinguished from endogenous rodent factors at the transcript level. To confirm over-expression and identify the time when over-expressed levels of hCLOCK and hBMAL1 transcripts were the highest, RNA was extracted from cells transfected with either hCLOCK and hBMAL1 or empty vector control (pcDNA3.1(+)) at 7, 12, 24 and 30 hours post-transfection and from untransfected cells. Extracted RNA was treated with DNase I and RT-PCR were performed on samples and un-transcribed controls with primers for hCLOCK and hBMAL1 (to confirm amplicons were from hCLOCK and hBMAL1 transcripts and not contaminating plasmid DNA). Transcript levels of hCLOCK and hBMAL1 were found to be highest at 24-hours post-transfection (Figure 5.1). Analysis of candidate gene transcripts at these time-points also demonstrated that up-regulation of expression was greatest after 24 hours for all genes (decreasing by 30 hours post-transfection), implying that hCLOCK and hBMAL1 levels may have peaked before 24 hours. Figure 5.2 shows the expression of *Dbp* peaking at 24 hours post-transfection as an example. Gene expression was subsequently analysed from RNA extracted from H9c2 cells 24 hours post-transfection.

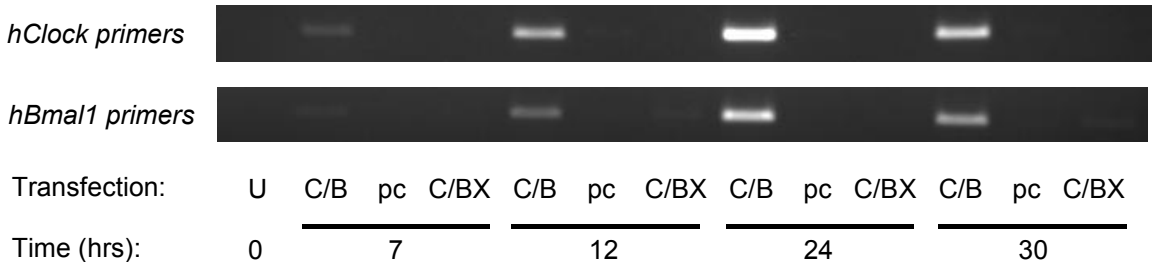


Figure 5.1. Expression of *hClock* and *hBmal1* transcripts from plasmids is highest at 24 hours post-transfection. An agarose gel image of *hClock* and *hBmal1* transcripts amplified by RT-PCR from DNase I-treated RNA extracted from H9c2 cells transfected with hCLOCK and hBMAL1 expression plasmids or empty vector pcDNA3.1(+). Cells were lysed and RNA extracted at 7, 12, 24 and 30 hours post-transfection. RNA was also extracted from untransfected cells. U = Untransfected, C/B = transfected with hCLOCK and hBMAL1 expression plasmids, pc = transfected with pcDNA3.1(+), C/BX = control RT reaction carried out without enzyme on DNase I-treated RNA from cells transfected with hCLOCK and hBMAL1 expression plasmids. Successful amplification of endogenous RNA was detected by amplification of the control gene transcript 36b4 in reverse-transcribed samples only.

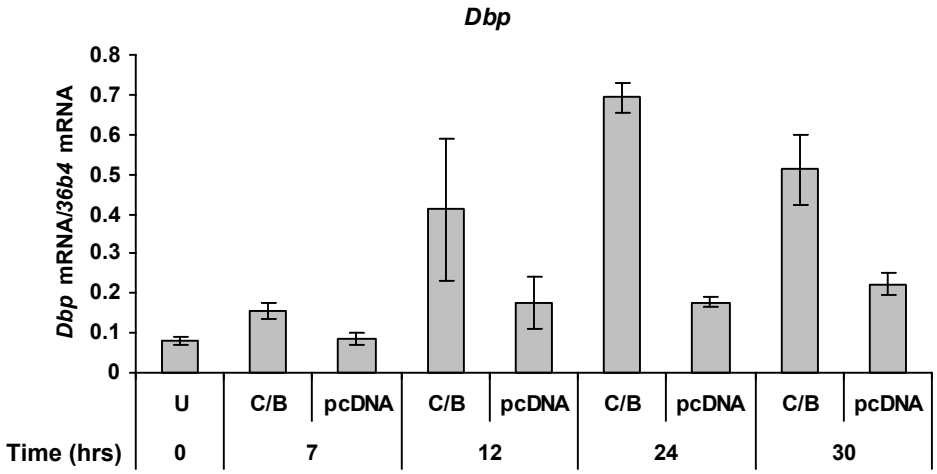


Figure 5.2. Expression of *Dbp* peaks at 24 hours post-transfection. Semi-quantitative RT-PCR expression values from triplicate wells of H9c2 cells transfected with hCLOCK + hBMAL1 (C/B), empty vector pcDNA3.1(+) (pcDNA) or untransfected (U) were normalised to the corresponding expression values of the housekeeping gene *36b4*. Values represent the means ±SEM from three triplicate wells.

Expression of candidate CCGs from Chapters 3 and 4, together with known CCGs, such as *Dbp* and *Rev-erbα*, was investigated. As *Dbp* transcript levels displayed the biggest peak to trough difference in Chapter 3, this gene was selected as a positive control over other clock genes such as *Per1*. Endogenous mRNA levels of *Anp*, *Pai-1* and *Dbp* were significantly higher (2.3-, 1.9-, 1.7-fold respectively) in cells over-expressing CLOCK and BMAL1 compared to cells transfected with empty vector plasmid pcDNA3.1(+) (Figure 5.3). *Rev-erbα*, *Bnp* and *Arc* appeared to be higher (1.4-, 1.4- and 1.3-fold respectively) but did not reach significance, which may be due to the lower number of samples tested for these three genes (n=2). Levels of two of the genes investigated, *Rorα* and *Usf2*, were modestly but significantly reduced following over-expression of CLOCK and BMAL1. *Rorα* levels were 22% lower whereas *Usf2* levels were only slightly reduced by 4.5% (Figure 5.3).

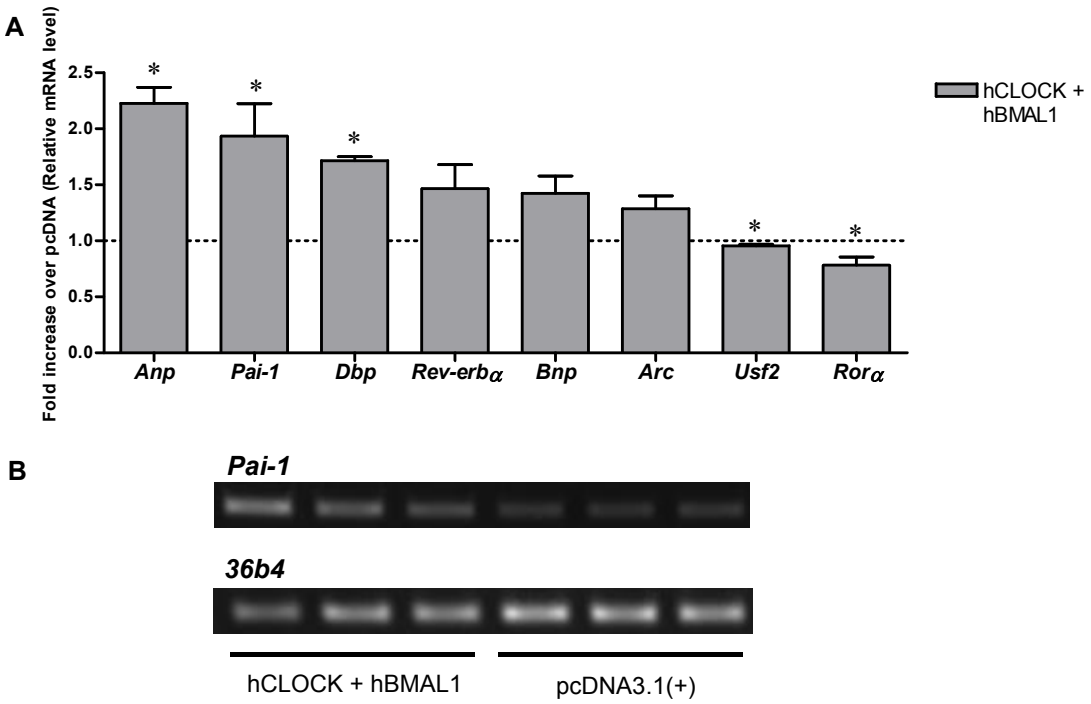


Figure 5.3. Alterations in transcript levels following over-expression of CLOCK and BMAL1 in H9c2 cells. (A) Semi-quantitative RT-PCR expression values from triplicate wells of H9c2 cells transfected with hCLOCK + hBMAL1 were normalised to the corresponding expression values of the housekeeping gene *36b4* and standardised to the expression values from triplicate wells transfected with empty vector pcDNA3.1(+). Values represent the means ±SEM from three independent experiments performed in triplicate, except for *Bnp*, *Rev-erbα* and *Arc* where means ±standard deviation from two independent experiments are shown. Dotted line represents levels of expression in cells transfected with pcDNA3.1(+). * $P<0.05$. (B) An example of an agarose gel image from semi-quantitative RT-PCR of H9c2 mRNA for *Pai-1* and *36b4*. CLOCK + BMAL1 = triplicate transfected with CLOCK and BMAL1, pcDNA3.1(+) = triplicate transfected with empty vector pcDNA3.1(+).

5.3.2 Synchronisation of rhythmic gene expression by DEX treatment of H9c2 cells *in vitro*

To investigate the rhythmic regulation of putative CCGs at the transcript level H9c2 cells in culture were exposed to an acute treatment (2 hours) of 100nM DEX to synchronise circadian gene expression. RNA was extracted from three wells of one 24-well plate per time-point every four hours for 52 hours (>2 days) and treated with DNase I and gene expression analysed by RT-PCR. Of the known clock and CCGs investigated only synchronised *Dbp* circadian expression was detected, shown by the peaks at 20-24 and 44-48 hours and troughs at 12 and 36 hours post treatment (Figure 5.4). Expression of *Bmal1*, *Per1* and *Rev-erbα* was not constant but did not demonstrate any notable rhythmicity. Likewise, expression of the putative clock output genes *Pai-1*, *Bnp* and *Anp* is variable but not rhythmic (Figure 5.5). *Bnp* and *Anp* expression patterns do show a similar trend, with possible clock-governed peaks with 24 hour periodicity at 8 and 32 hours post-treatment, however, the peaks are not statistically significant. Expression of *Rorα* and *Usf2* (Figure 5.6) was also not rhythmic but increased after 32-36 hours, as seen for *Rev-erbα*.

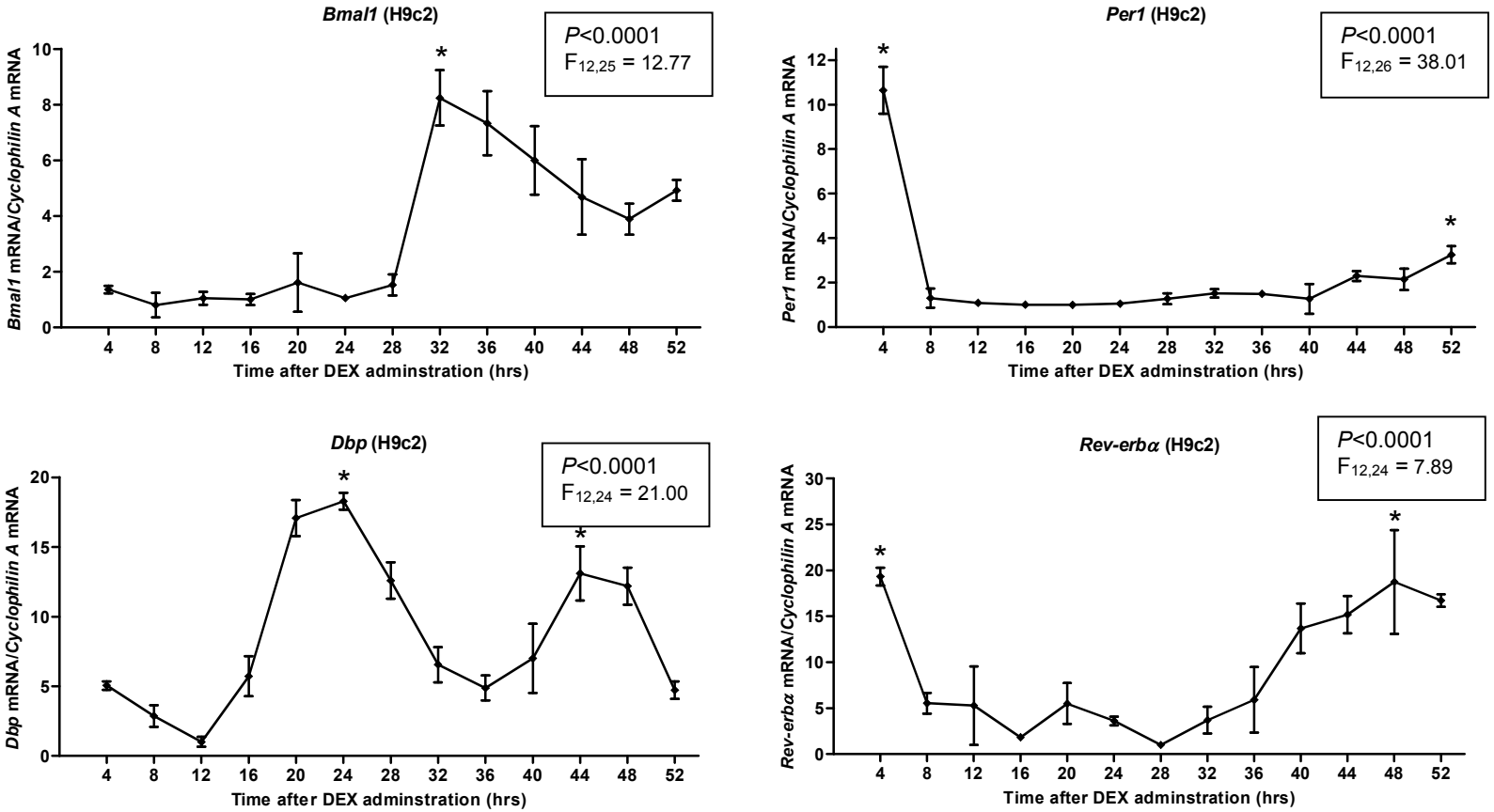


Figure 5.4. Circadian/temporal expression of putative clock-controlled genes in H9c2 cells following DEX treatment. H9c2 cells were treated with 100nM DEX (0 hours) for two hours. Triplicate cells were lysed every four hours and total RNA extracted followed by DNase I treatment and semi-quantitative RT-PCR. Expression values were normalised against the corresponding expression values of the housekeeping gene *Cyclophilin A*. Values are the mean ± SEM of three samples (one-two observations), standardised to the lowest expression time-point.

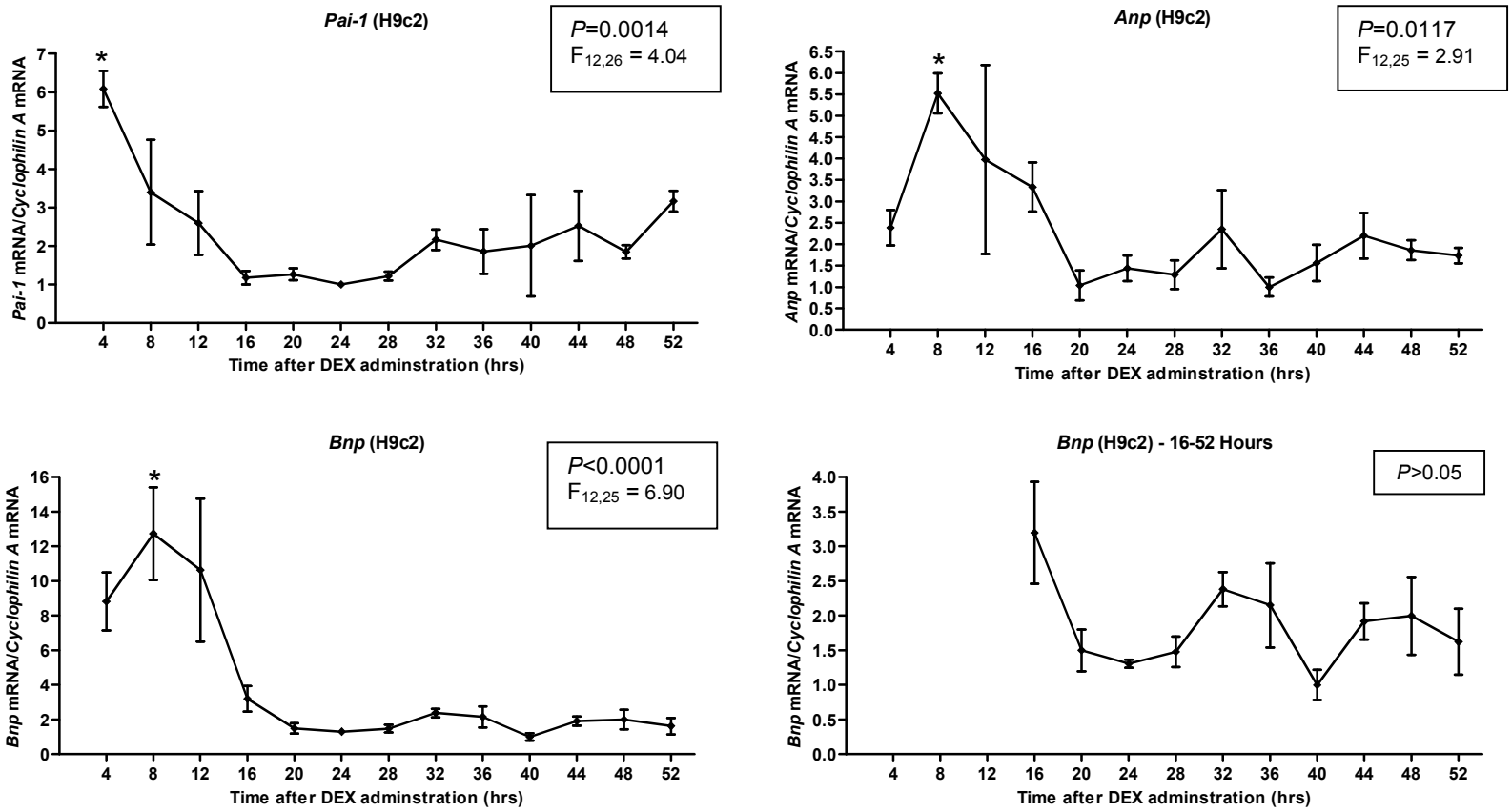


Figure 5.5. Temporal expression of putative clock-controlled genes in H9c2 cells following DEX treatment. H9c2 cells were treated with 100nM DEX (0 hours) for two hours. Triplicate cells were lysed every four hours and total RNA extracted followed by DNase I treatment and semi-quantitative RT-PCR. Expression values were normalised against the corresponding expression values of the housekeeping gene *Cyclophilin A*. Values are the mean ± SEM of three samples (one-two observations), standardised to the lowest expression time-point.

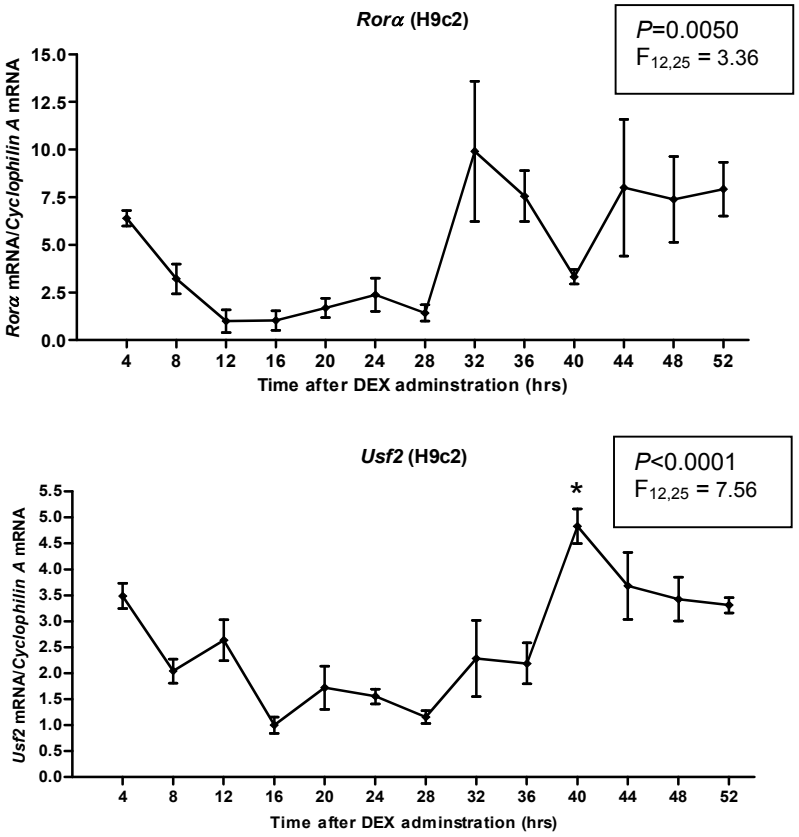


Figure 5.6. Temporal expression of putative clock-related genes in H9c2 cells following DEX treatment. H9c2 cells were treated with 100nM DEX (0 hours) for two hours. Triplicate cells were lysed every four hours and total RNA extracted followed by DNase I treatment and semi-quantitative RT-PCR. Expression values were normalised against the corresponding expression values of the housekeeping gene *Cyclophilin A*. Values are the mean ± SEM of three samples, standardised to the lowest expression time-point.

5.3.3 Synchronisation of rhythmic gene expression by DEX treatment in NIH-3T3 cells *in vitro*

To compare the circadian regulation of genes in cardiac cells with that of another cell type, mouse fibroblast NIH-3T3 cells were also treated with DEX under the same conditions. RNA was extracted from three wells of a 24-well plate per time-point and the experiment was repeated. Expression of *Dbp* was again strongly rhythmic, as was *Bmal1* and *Per1* expression (unlike in H9c2 cells; Figure 5.7). Expression of *Bmal1* was in anti-phase to *Dbp* and *Per1* expression, as would be expected from the phases of clock gene expression seen in the whole mouse heart (Chapter 3). *Pai-1* expression was again not rhythmic but was acutely up-regulated by DEX (Figure 5.8). *Bnp* expression was also acutely up-regulated by DEX and significantly variable from 16-52 hours post-treatment but with no significant peaks (Figure 5.8). Acute up-regulation of *Anp* expression was delayed by 4 hours compared to *Bnp* (Figure 5.9). *Anp* expression was significantly up-regulated again at 24 hours post-treatment, but this peak was not repeated after 24 hours. In contrast to H9c2 cells, *Rorα* appeared to be rhythmically expressed in 3T3 cells in response to DEX treatment but the peaks at 20-24 hours and 48 hours post-treatment were not statistically significant following *post-hoc* testing and were only observed in one of the two experiments (Figure 5.7).

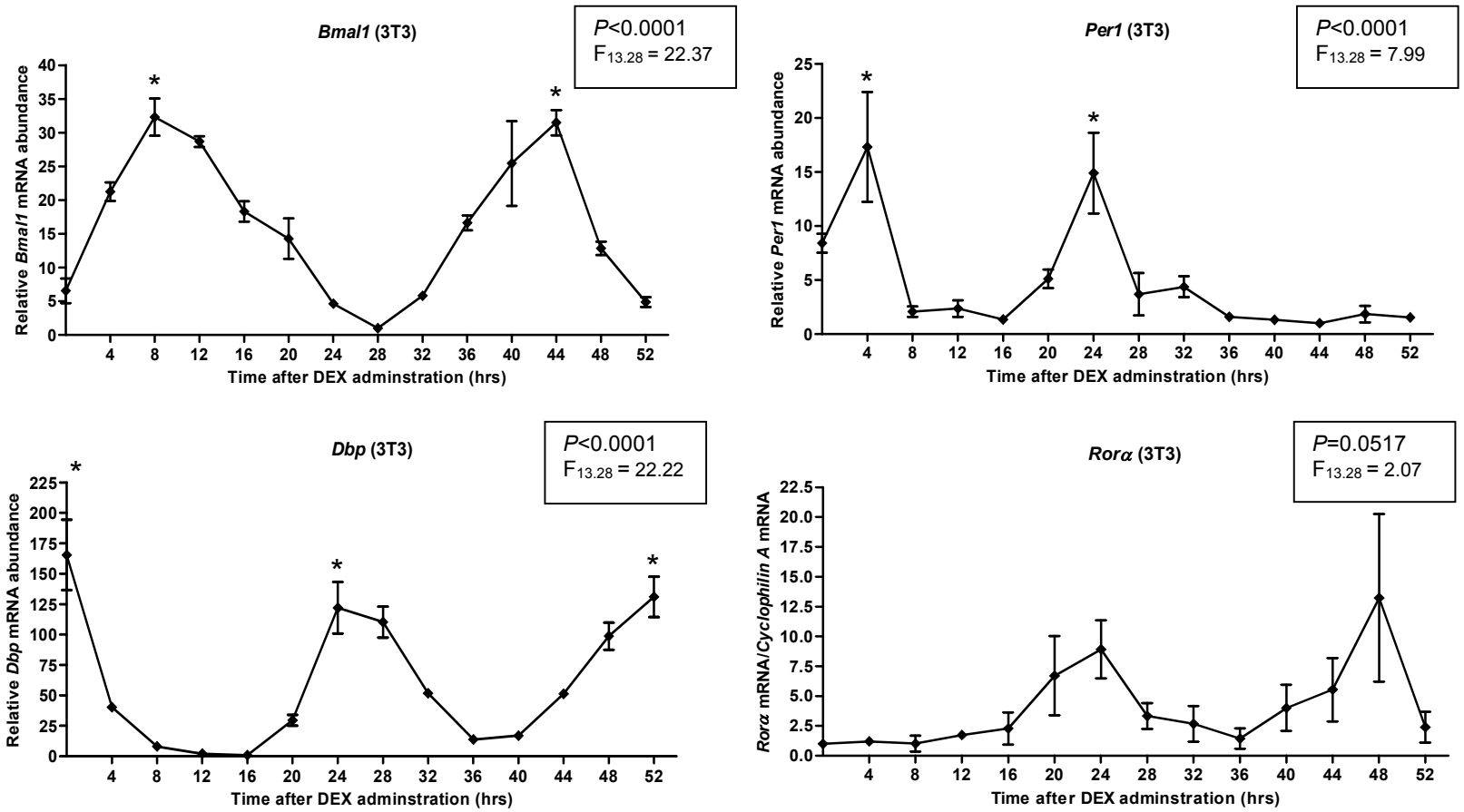


Figure 5.7. Circadian expression of clock and clock output genes in NIH-3T3 cells following DEX treatment. NIH-3T3 cells were treated with 100nM DEX (0 hours) for two hours. Triplicate cells were lysed every four hours and total RNA extracted followed by DNase I treatment and quantitative (*Bmal1*, *Per1* and *Dbp*) and semi-quantitative (*Rorα*) RT-PCR. Expression values were normalised against the corresponding expression values of the housekeeping gene TATA box-binding protein (*Tbp*) (quantitative) and *Cyclophilin A* (semi-quantitative). Values are the mean ± SEM six samples from two independent experiments, standardised to the lowest expression time-point.

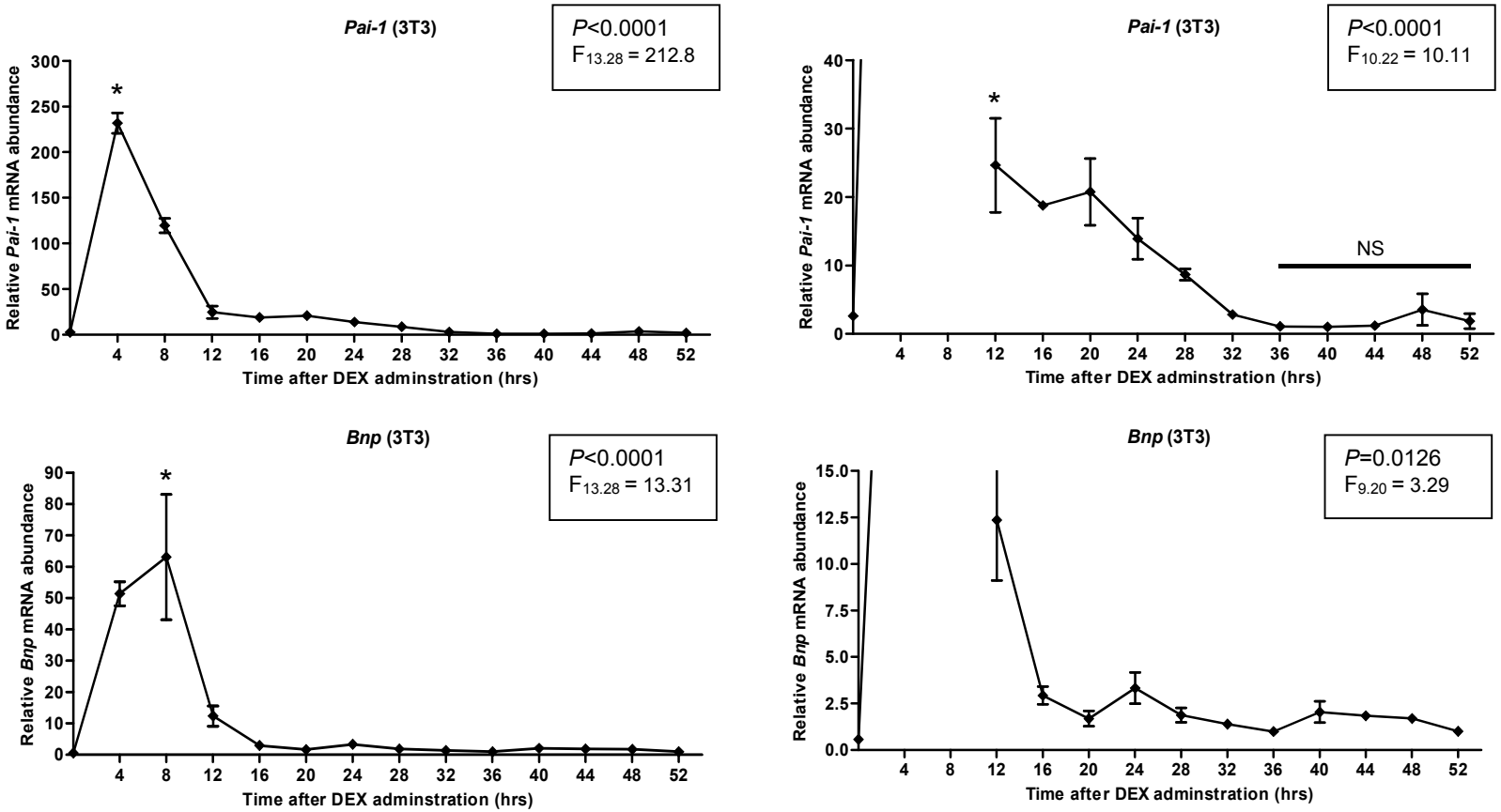


Figure 5.8. Temporal expression of putative clock-controlled genes in NIH-3T3 cells following DEX treatment. NIH-3T3 cells were treated with 100nM DEX (0 hours) for two hours. Triplicate cells were lysed every four hours and total RNA extracted followed by DNase I treatment and quantitative RT-PCR. Expression values were normalised against the corresponding expression values of the housekeeping gene TATA box-binding protein (*Tbp*). Values are the mean ± SEM six samples from two independent experiments, standardised to the lowest expression time-point.

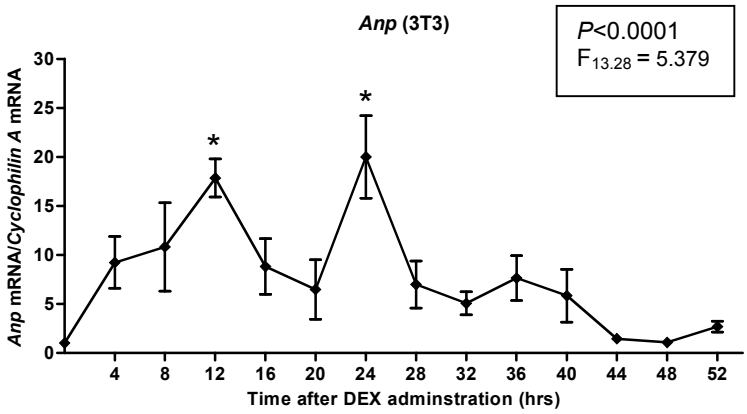


Figure 5.9. Temporal expression of putative clock-controlled gene *Anp* in NIH-3T3 cells following DEX treatment. NIH-3T3 cells were treated with 100nM DEX (0 hours) for two hours. Triplicate cells were lysed every four hours and total RNA extracted followed by DNase I treatment and semi-quantitative RT-PCR. Expression values were normalised against the corresponding expression values of the housekeeping gene *Cyclophilin A* (semi-quantitative). Values are the mean ± SEM six samples from two independent experiments, standardised to the lowest expression time-point.

5.3.4 Synchronisation of rhythmic gene expression by serum shock *in vitro*

To compare the response of cells to DEX with that of another stimulus, serum-starved H9c2 cells were treated with 50% horse serum for 30 minutes (serum shock) and transcript levels determined by RT-PCR as before. RNA was extracted from three wells of one 24-well plate per time-point (except for *Per1* = one well). Serum shock resulted in highly variable gene expression with no significant patterns (Figure 5.10). *Per1* and *Pai-1* expression appeared to be acutely up-regulated by serum shock.

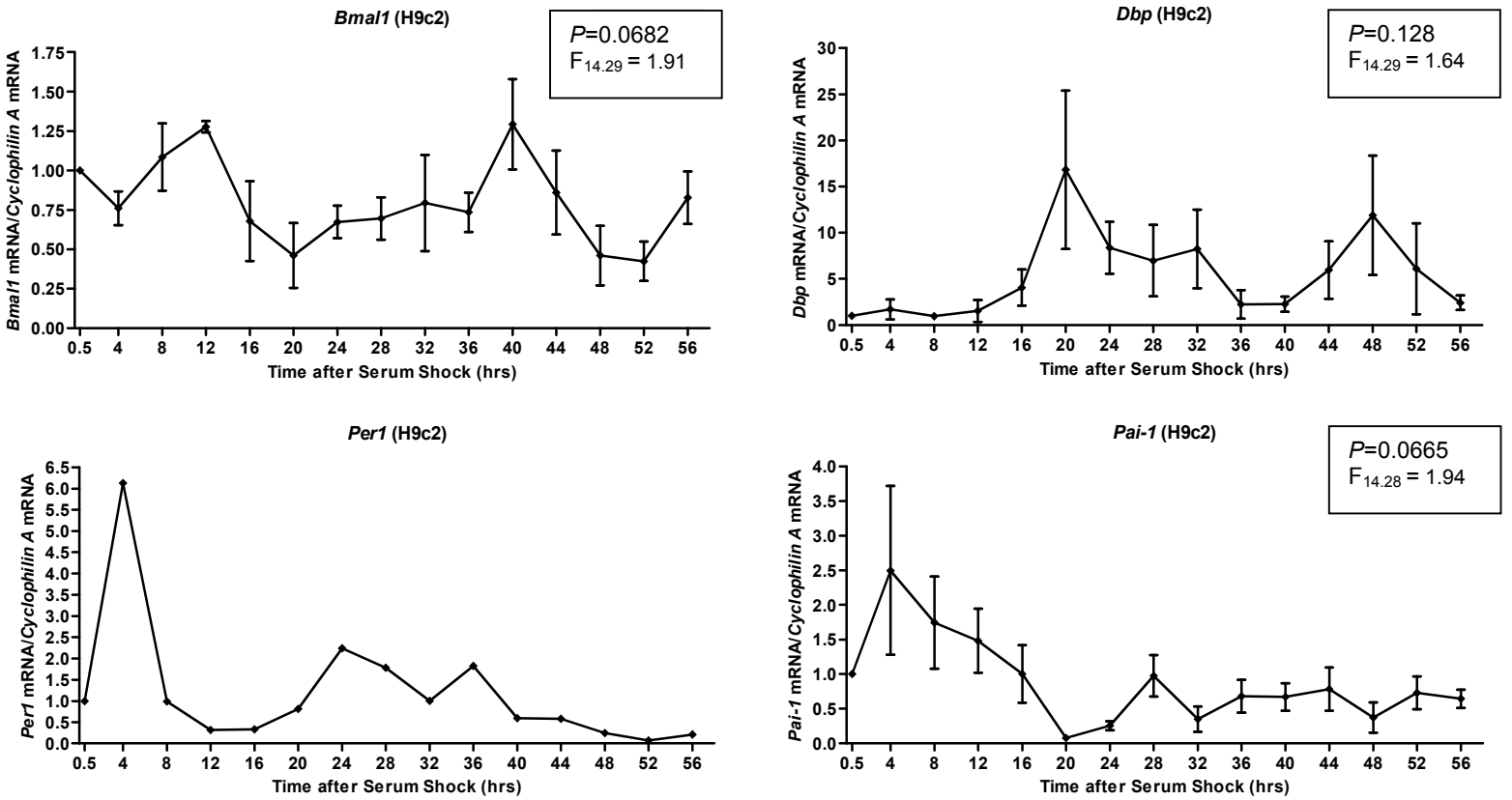


Figure 5.10. Cycling of transcripts in H9c2 cells following serum shock. H9c2 cells were serum starved (0.5% FBS) for 24 hours and then treated with 50% horse serum for two hours (serum shock), followed by supplementation with 0.5% FBS culture medium. Triplicate cells were lysed every four hours and total RNA extracted followed by semi-quantitative RT-PCR. Expression values were normalised against the corresponding expression values of the housekeeping gene *Cyclophilin A*. Values are the mean ± SEM of three samples (one-two observations), except for *Per1* where n=1. Values were standardised to the first time-point, 0.5 hours post serum shock.

5.4 Discussion

5.4.1 Model systems to identify targets of the intracellular circadian clock

The observed up-regulation of candidate CCGs following the over-expression of clock factors *in vitro* strengthens the hypothesis that these genes are regulated by the intracellular molecular clock mechanism in cardiac cells, rather than through circadian rhythms generated systemically by the SCN or the external environment. As expression of the primary CCG *Dbp* is highly rhythmic (see Chapter 3) and directly regulated by CLOCK:BMAL1 (Ripperger *et al.*, 2000) it was used here as a positive control to confirm the use of these techniques in identifying clock targets. *Dbp* expression was significantly up-regulated by over-expression of hCLOCK and hBMAL1 (Figure 5.3). Interestingly both *Anp* and *Pai-1* were more strongly up-regulated than *Dbp*. All these genes have conserved canonical E-boxes in their promoters (see Chapter 3), therefore *Anp* and *Pai-1* may be regulated directly by CLOCK and BMAL1 through the E-box as is the case for *Dbp* (see Chapter 6 for E-box-dependent *Pai-1* regulation). Since *Anp* and *Pai-1* were up-regulated to a greater extent than *Dbp* it is possible that multiple clock-dependent mechanisms are working together in activating these genes. For example, both *Anp* and *Pai-1* promoters contain conserved E-box-like sites and DBP-binding sites (see Chapter 3, Table 3.8) so the increased expression of *Dbp* by CLOCK and BMAL1 may increase the amount of DBP protein available to augment *Anp* and *Pai-1* activation.

Bnp, a gene closely related to *Anp* that was rhythmically expressed in the mouse heart (see Chapter 3), also contains DBP-binding sites in its promoter and appeared to be up-regulated (although this was not significant, possibly due to the use of two samples instead of three). *Bnp* activation was at a similar level to that of *Rev-erbα* but lower than *Anp* and *Pai-1*. One possible explanation is the higher number of putative conserved RREs (RORα/REV-ERBα-binding elements) in the *Bnp* promoter. Since *Rev-erbα* was also up-regulated by CLOCK and BMAL1 (whereas *Rorα* expression was repressed), REV-ERBα may be acting as a negative regulator of *Bnp*, in competition with CLOCK:BMAL1, DBP and other positive regulators. Alternatively, more of the 'circadian' regulatory sites may be functional in the promoter of *Anp*. The relative levels of up-regulation of CCGs may also be dependent on how well they are activated by different clock factors. CLOCK and BMAL1 may not be the best combination of activators for all the genes examined and other heterodimer, such as MOP4:BMAL2, may be preferred.

All transcripts that were measured in three independent experiments showed significant changes in level in cells transfected with CLOCK and BMAL1. The three transcripts that were only measured in two independent experiments did not significantly increase due to the modest magnitude and higher variance. Three repetitions are therefore necessary to yield meaningful results from this technique and further candidate CCGs should be investigated.

Synchronisation of circadian gene expression in H9c2 cells was less successful in demonstrating the clock regulation of cardiac genes, although treatment with DEX did mediate the circadian expression of *Dbp*, confirming the novel finding that DEX can synchronise the molecular clocks within H9c2 cells in culture (Figure 5.4). However, expression of the core clock genes *Bmal1* and *Per1*, and clock output gene *Rev-erbα* was not synchronised, despite expression of these genes being highly rhythmic in the whole rodent heart (see Chapter 3). This is somewhat surprising since one would expect circadian *Dbp* expression to be governed by the synchronisation of the core circadian feedback loop and the rhythmic expression of CLOCK and BMAL1. Treatment of NIH-3T3 cells with the same concentration of DEX also synchronised the cells in two independent experiments, as shown by the circadian expression of *Bmal1*, *Per1* and *Dbp* (Figure 5.7). It would seem unlikely that *Dbp* expression is uncoupled from the circadian expression of *Bmal1* and *Per1*. The lack of core clock gene cycling in H9c2 cells may therefore be a result of cell-type differences (see section 5.4.2) or of experimental conditions, compounded by the lack of repetition of H9c2 treatment with DEX (whereas the experiment was repeated in NIH-3T3 cells). *Dbp* peak transcript levels were 7-fold higher in NIH-3T3 cells compared to H9c2 cells, therefore the techniques used in this chapter may not have been sensitive enough to detect lower levels of cycling in H9c2 cells, particularly as all transcripts had a lower peak-trough ratio than *Dbp* in the whole heart (Chapter 3).

Since the activity of clock transcription factors is also dependent on post-translational modifications (Reppert and Weaver, 2001; Harms *et al.*, 2004), it is possible that the core clock mechanism was synchronised at the post-transcriptional level and that rhythmic CLOCK:BMAL1 activity is sufficient to generate robust *Dbp* cycling. Alternatively, *Dbp* may be a target of clock factors other than CLOCK and BMAL1, be regulated by rhythmic histone acetylation/deacetylation of its promoter (as shown in Nakahata *et al*., (2008a) or an uncharacterised circadian regulatory mechanism, such as rhythmic mRNA degradation (as has been proposed to operate in mice (Garbarino-Pico and Green, 2007), the plant *Arabidopsis thaliana* (Mas, 2008) and the green alga *Chlamydomonas reinhardtii* (Hwang *et al.*, 1996). The observation that *Dbp* was the only gene to display rhythmicity in H9c2 cells further points to its importance as a mediator of circadian regulation in cardiac cells, despite its targets in the heart being unknown.

Rorα expression may have been synchronised in NIH-3T3 cells, although the observed peaks are not significant due to disagreement between samples (the pattern of peaks was only observed in one of the two experiments). Since temporal variation in *Rorα* expression in the whole heart were not significant (Chapter 3) and circadian expression of *Rorα* has been observed in the SCN but not in the liver (Preitner *et al.*, 2002; Ueda *et al.*, 2002), cycling of the *Rorα* transcript may be tissue- and cell type-specific. Despite *Pai-1* expression being strongly circadian in the whole heart (see Chapter 3) cycling was not observed in H9c2 or NIH-3T3 cells (with DEX). Treatment with serum shock has

previously been shown to initiate circadian *Pai-1* expression in NIH-3T3 cells (Wang *et al.*, 2006), whereas treatment of H9c2 cells with aldosterone resulted in circadian expression of clock genes but not *Pai-1* (Tanaka *et al.*, 2007). Therefore, the initiation of *Pai-1* circadian expression depends on the stimulus used and cell type. In this experiment, *Pai-1* expression was acutely up-regulated by DEX, and to a lesser extent by serum. In the experiment by Tanaka and colleagues (2007) aldosterone also acutely increased *Pai-1* expression in H9c2. Therefore, the acute response to DEX and aldosterone is likely to be independent of the clock and may have over-ridden clock regulation (as discussed in Chapter 4). Synchronisation of *Pai-1* expression by serum shock also resulted in an initial large induction prior to cycling (Wang *et al.*, 2006), therefore the response of *Pai-1* to serum may be clock dependent.

Treatment of cells with DEX also acutely induced expression of *Bnp*, although this was delayed by 4 hours compared to *Pai-1* (Figure 5.7). If this acute induction is clock-dependent the delay may reflect the different temporal expression profiles of *Pai-1* and *Bnp in vivo*. The levels of *Pai-1* and *Bnp* induction were very high (approximately 230-fold and 63-fold respectively), and if this acute induction is clock-independent the disruption to the normal transcript levels may mask any subsequent cycling at a lower level, especially since transcript levels do not return to baseline until approximately 16 hours post-treatment. After this time *Bnp* expression exhibits some possible, but non-significant, cycling with peaks at 24 and 40-48 hours. *Anp* expression is highly variable following DEX treatment but also exhibits a peak in expression at 24 hours, possibly reflecting a common circadian regulatory mechanism for *Bnp* and *Anp*. As the synchronisation of H9c2 and NIH-3T3 cells with DEX or serum shock did not demonstrate cycling of the *Pai-1* transcript, and we know that this gene is rhythmically expressed in the heart (Chapter 3), it is unlikely that this technique would demonstrate cycling of other candidate CCGs and was not pursued. Improvement of the sampling and detection methods (e.g. by using real-time quantitative RT-PCR) may enable the detection of more subtle rhythms in transcript abundance.

5.4.2 Circadian gene expression in H9c2 cells compared to NIH-3T3 cells.

Since DEX was able to synchronise the circadian expression of *Bmal1* and *Per1* in NIH-3T3 but not H9c2 cells, the NIH-3T3 cell line appear to be the better model for examining the effects of DEX as a synchroniser of circadian expression. Fibroblast cell lines, such as NIH-3T3 cells, have traditionally been favoured as models of circadian expression as the fibroblast molecular clock is robust (Nagoshi *et al.*, 2004) and has very similar properties to SCN neurons (Yagita *et al.*, 2001). However, the transcriptomes and complement of regulatory factors of NIH-3T3 and H9c2 cells are different; for example, *Pai-1* expression is eight times greater in NIH-3T3 cells than in H9c2 cells (see Figure 1, Appendix 2). Therefore, a cardiac cell model is most appropriate to investigate the circadian regulation of cardiac enriched genes. Adult rat cardiomyocytes have been shown to be synchronised

with serum and norepinephrine (Durgan *et al.*, 2005) but the isolation and maintenance of primary cells can be difficult, labour intensive and costly compared to the ease of use of a cell line. H9c2 cells are morphologically similar to embryonic cardiomyocytes and also possess several elements of the electrical and hormonal signalling pathways found in adult cardiac cells and are believed to be a suitable model for the study of cardiomycocyte biology (Hescheler *et al.*, 1991). Unfortunately the results presented here demonstrate that the H9c2 cell line may not be a good model for the study of circadian biology dynamics. As H9c2 cells are a myoblasts cell line derived from embryonic rat heart (Kimes and Brandt, 1976) they can be differentiated into either cardiac or skeletal muscle depending on the culture conditions (Pagano *et al.*, 2004). In particular, culturing H9c2 in low-serum containing medium causes them to differentiate into myocyte/myotubes. Therefore, serum-starving these cells prior to serum shock may have started the differentiation process and altered the transcriptome. Muscle-specific factors, such as MyoD and Myogenin can bind to E-boxes and if H9c2 cells contain a particularly high concentration of these factors the ability of clock factors to activate the circadian expression of cardiac genes via E-box elements may be compromised.

The difference in the ability to synchronise fibroblast and cardiac cell lines in the present experiment may imply that cardiac cells are less able to maintain circadian expression rhythms in culture and are more dependent on synchronising cues from the SCN, as suggested by SCN-ablation in Chapter 4. However, if this is the case it may only be true of cardiac myoblasts or immortalised cell lines since circadian oscillations in gene expression have recently been observed in cultured isolated adult rat cardiomyocytes following synchronisation (Durgan *et al.*, 2005). The primary cells used by Durgan and colleagues (2005) were synchronised with serum and norepinephrine, therefore the synchronisation of cardiac cells may depend on the stimulus used, although serum shock was only able to initiate weak circadian rhythms in H9c2 cells. Norepinephrine is a major sympathetic neurotransmitter and may relay signals from diurnal variations in sympathetic activity since its levels in the plasma are rhythmic (Linsell *et al.*, 1985). Acute exposure to norepinephrine was able to induce oscillations in clock gene expression in primary cardiomyocytes but these were short-lived in the absence of serum (Durgan *et al.*, 2005). Therefore, a combination of stimuli may be needed to initiate and maintain circadian gene expression, with different stimuli contributing to different signalling pathways.

5.4.3 DEX and serum as synchronisers of circadian gene expression

As previously shown (Balsalobre *et al.*, 2000a), DEX was able to synchronise the clocks of fibroblast cells, as demonstrated by the strong and anti-phase circadian expression rhythms of *Bmal1*, *Per1* and *Dbp* (Figure 5.7). Serum shock has also been commonly used to synchronise circadian expression in fibroblast cell lines but neither serum shock nor DEX have been used to synchronise H9c2 cells previously. DEX was able to synchronise the clocks of cardiac H9c2 cells in this experiment, as

shown by the oscillations in *Dbp* expression (Figure 5.4), but the other clock genes investigated did not become rhythmically expressed, as discussed above. Serum shock appeared to be moderately successful in synchronising clock gene expression in H9c2 cells but resulted in a high level of variability between samples (Figure 5.10). Serum contains many components, such as growth factors and hormones, and is well known to induce the transient expression of several immediate early genes (Nagoshi *et al.*, 2005). As a non-specific stimulus, multiple cellular pathways (and gene networks) may be activated by serum, potentially interfering with regulation by the clock. Glucocorticoids may be one of the components of serum that elicits circadian synchronisation. Therefore, the use of DEX as a specific stimulus may elicit more robust circadian expression and not require prior treatments such as serum starvation, which may interfere with gene expression (see above). However, like serum, DEX can elicit the acute up-regulation of a number of genes and in the whole heart DEX induced the expression of one third of the genes examined (Chapter 4). This may potentially interfere with clock regulation of target genes, but DEX can also induce the circadian expression of many genes that do not possess GREs in their promoters (Reddy *et al.*, 2007). In the present study, DEX did appear to elicit stronger transcript cycling of *Dbp* in H9c2 cells than did serum and a screen for zeitgebers for the cardiomyocyte clock revealed that only DEX was able to induce *Bmal1* and *Per2* expression (Young and Bray, 2007). Therefore cortisol may be a strong synchroniser of the cardiomyocyte clock, as observed in other cell types (Balsalobre *et al.*, 2000a).

DEX and serum are only two of an array of substances that have been shown to trigger circadian expression in cultured cells (Akashi and Nishida, 2000; Balsalobre *et al.*, 2000a; Balsalobre *et al.*, 2000b; Yagita and Okamura, 2000; Hirota *et al.*, 2002; Durgan *et al.*, 2005). The recent demonstration that aldosterone can synchronise the clocks of H9c2 cells (Tanaka *et al.*, 2007) but not *Pai*-1 expression, coupled with the fact that serum shock can elicit rhythms of clock genes and *Pai-1* expression in NIH-3T3 cells (Wang *et al.*, 2006), but not elicit rhythmic clock gene expression in cultured endothelial cells (Westgate *et al.*, 2008), highlights the importance of different cell types and stimuli in circadian cell culture models. Also, the clock mechanisms of NIH-3T3 cells and a cell line derived from the SCN (SCN2.2) are very similar in terms of core clock gene expression but only four of the 323 rhythmic transcripts identified in NIH-3T3 cells were also rhythmic in SCN2.2 cells (Menger *et al.*, 2007). Therefore, to fully understand the circadian regulation of a particular gene it may be necessary to test different cell types and stimuli to find the most appropriate model.

5.4.4 Conclusions

The primary aim of this chapter was to further investigate the regulation of candidate CCGs by the clock using two *in vitro* cellular approaches. The first approach, over-expression of the clock factors CLOCK and BMAL1, resulted in the up-regulation of some candidate genes, adding weight to the evidence gathered in previous chapters that *Anp*, *Bnp* and *Pai-1* are CCGs. Further repetition of this

experiment and the examination of more candidate CCG transcripts is now required. The second approach, synchronisation of circadian gene expression, proved to be less useful in identifying CCGs due to the difficulty in generating robust cycling of clock gene expression in H9c2 cells. However, the regulation of these three candidate CCGs directly by the intracellular clock can not be discounted and further work in the next chapter will attempt to elucidate the mechanisms of circadian regulation of these genes.

6. Transcriptional regulatory mechanisms of putative clock-controlled genes

6.1 Introduction

As discussed in Chapter 1, PAI-1 plays an important role in many processes throughout the cardiovascular system and is tightly regulated at the transcriptional level by a variety of stimuli. Of these stimuli hypoxia is one of the main contributors to CVD and the regulation of *Pai-1* expression by hypoxia responsive factors is likely to be important in CVD aetiology.

In humans and rodents the regulation of *Pai-1* expression in response to hypoxia is believed to be an E-box-mediated mechanism. In the human promoter a non-canonical E-box (CACGTA) is sufficient for hypoxia-induced up-regulation of *Pai-1* via HIF-1α binding (Fink *et al.*, 2002) whereas the main HIF-1α target in the rat promoter is believed to be a canonical E-box located at -174 to -179bp relative to the transcriptional start site (TSS), although a CACGTA E-box located four bases down-stream is also likely to be involved (Kietzmann *et al.*, 1999). The CACGTA site in the human promoter can also be bound by the closely related hypoxia-responsive factor EPAS1 (HIF-2α) (Sato *et al.*, 2004b), although regulation of *Pai-1* expression by EPAS1 appears to be cell-type specific (Carroll and Ashcroft, 2006).

E-box dependent regulation of the human *Pai-1* promoter by circadian factors has also been recently demonstrated. In particular CLOCK:BMAL1 and CLOCK:BMAL2 heterodimers can activate the human *Pai-1* promoter in endothelial cells *in vitro* via two canonical E-box elements which are both necessary and sufficient for activity (Maemura *et al.*, 2000; Minami *et al.*, 2002; Schoenhard *et al.*, 2003; Chong *et al.*, 2006). These two E-boxes are not directly conserved with the rodent promoters but the circadian expression of *Pai-1* appears to be conserved between species and the cycling of *Pai-1* mRNA and protein is lost in the hearts and plasma of *Clock* mutant mice (Minami *et al.*, 2002; Oishi *et al.*, 2005b; Ohkura *et al.*, 2006). During the course of the current study Oishi and colleagues (2007) demonstrated activation of the mouse *Pai-1* promoter by CLOCK and BMAL1 at a canonical E-box. However, the precise mechanism of *Pai-1* transcriptional regulation by E-box-binding factors is still unclear as cross-talk between circadian and hypoxic pathways and interplay between E-boxes and HRE is likely. Ubiquitously expressed E-box-binding factors such as USF may also be involved. In addition to regulation via E-boxes, the clock can influence gene expression through the binding of clock-output factors REV-ERBα/RORα and DBP/E4BP4 to their associated elements, adding to the complexity of circadian regulation.

Bnp expression is also regulated by a number of mechanisms in the heart (LaPointe, 2005; Ma *et al.*, 2005) and is stimulated by acute myocardial hypoxia (Goetze *et al.*, 2004). As *Bnp* appears to be

rhythmically expressed and contains conserved E-boxes in its promoter (see Chapter 3) it may also be regulated by a balance of circadian and hypoxic factors.

The aim of this chapter was to investigate the transcriptional regulatory mechanisms of putative CCGs identified in this PhD project. One pivotal candidate is *Pai-1* because of its strong circadian expression rhythm in the mouse heart and other peripheral tissues yet the regulatory mechanism has not been fully characterised. Functional E-boxes have been identified in the rodent *Pai-1* promoter that may govern *Pai-1* regulation via a number of pathways. *Bnp* is another important candidate due to it role in cardiac pathology and regulation by multiple stimuli but transcriptional regulation by clock factors has not yet been documented. *In silico* analysis and *in vitro* experiments were performed to characterise the regulation of the promoter elements of these genes in order to gain understanding of the cross-talk between circadian and other pathways, such as hypoxia, and how this might affect circadian expression under different physiological conditions. Transcript cycling may also involve unidentified circadian regulatory elements so the identification and contribution of further putative regulatory motifs to transcriptional circadian regulation was investigated.

6.2 Methods

6.2.1 Cloning of the mouse *Pai-1* proximal promoter and mouse *Pai-1* distal module

The mouse *Pai-1* 5' proximal promoter and mouse *Pai-1* distal module were amplified from 50ng mouse genomic DNA by high fidelity PCR and cloned into the pGEM-T Easy vector (Promega) (see Chapter 2, section 2.2). The addition of restriction sites for *Mlu* I and *Bgl* II into the amplification primers (Table 6.1) allowed excision of the amplicons and cloning into the pGL3-Basic (proximal promoter) or SV40 minimal promoter-containing pGL3-Promoter (distal module) luciferase reporter vectors (Promega). Two proximal fragments were cloned, the -707 construct from -707bp to +108bp relative to the Transcription Start Site (TSS) and the -314 construct from -314bp to +108bp relative to the TSS (Figure 6.1). The distal module was located at -2997bp to -3405bp relative to the TSS (Figure 6.2). Putative transcription factor binding sites were identified *in silco* by orthologous sequence alignment (see below) and are shown diagrammatically in Figure 6.1 and Figure 6.2. Mutants were generated by site-directed mutagenesis using primers designed using the Stratagene QuikChange Primer Design programme and listed in Table 6.1. Authenticity of all constructs was checked by sequencing.

6.2.2 Cloning of the mouse *Bnp* proximal promoter

The mouse *Bnp* 5' proximal promoter from -883bp to +39bp was also amplified and cloned into the pGL3-Basic vector using primers listed in Table 6.1.

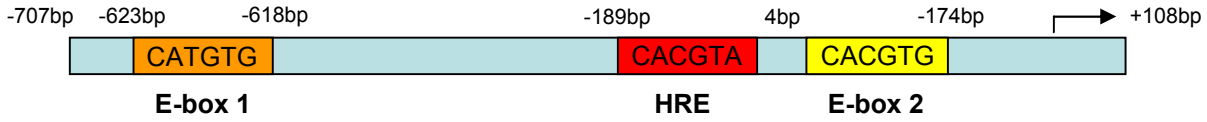
Table 6.1. Proximal promoter and distal module amplification primers. Restriction sites are underlined. Primers sequences are 5' to 3'.

Primer	Sequence
mPai-1 proximal promoter *Mlu I* F	CTACGCGTCCCACCCAGTACACCTCAAAA
mPai-1 proximal promoter *Bgl II* R	CTAGATCTGATTGGCTCTTGTTGGCTGTC
mPai-1 promoter module *Mlu I* F	TACACGCGTAAGACACTCTCCACCTCCTCCAG
mPai-1 promoter module *Bgl II* R	CTTAGATCTCAGGCAATGGGCCTACATTATGG
mBnp proximal promoter *Bgl II* F	CAGATCTCATGGTGGGGGAGGAGACTTTG
mBnp proximal promoter *Hind III* R	AAGCTTGCAATGATGCCGTGTTCTCCCTT

Table 6.2. *Pai-1* proximal promoter and distal module putative transcription factor binding site mutation primers. Mutated putative sites are underlined. Primers sequences are 5' to 3'.

Primer	Sequence
mPai-1 proximal promoter E-box 1 mutant F	CAGGAGAGTCTGGCCCGTCGACGGGAGTC AGACATGCTTC
mPai-1 proximal promoter E-box 1 mutant R	GAAGCATGTCTGACTCCCGTCGACGGGCC AGACTCTCCTG
mPai-1 proximal promoter E-box 2 mutant F	CCCCCATGCCCTTTCAGTCGACCACACACG TGTCCCAG
mPai-1 proximal promoter E-box 2 mutant R	CTGGGACACGTGTGTGGTCGACTGAAAGG GCATGGGGG
mPai-1 proximal promoter E-box 3 mutant F	CCATGCCCTTTCACACGTACACAGTCGACT CCCAGCAAGTC
mPai-1 proximal promoter E-box 3 mutant R	GACTTGCTGGGAGTCGACTGTGTACGTGT GAAAGGGCATGG
mPai-1 proximal promoter E-box 2+3 mutant F	CCATGCCCTTTCAGTCGACCACAGTCGACT CCCAGCAAGTC
mPai-1 proximal promoter E-box 2+3 mutant R	GACTTGCTGGGAGTCGACTGTGGTCGACT GAAAGGGCATGG
mPai-1 distal module E-box 1 mutant F	GTGTATTCAGGTATAGTCGACTGATACAG AGTATG
mPai-1 distal module E-box 1 mutant R	CATACTCTGTATCAGTCGACTATACCTGAA TACAC
mPai-1 distal module RRE mutant F	GTCCCCCATGTCCAACGAAAGACAAAGCT TTGGGGGTGGGGAG
mPai-1 distal module RRE mutant R	CTCCCCACCCCCAAAGCTTTGTCTTTCGTT GGACATGGGGGAC

***mPai-1* promoter -707:**



***mPai-1* promoter -314:**

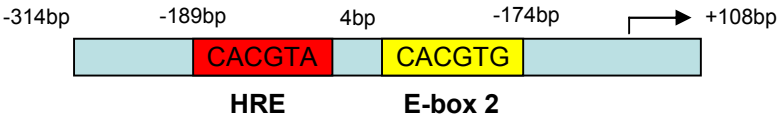


Figure 6.1. Mouse *Pai-1* proximal promoter constructs. Graphical representation of the mouse *Pai-1* promoter -707 and -314 (-707/-314bp to +108bp relative to the TSS) fragments cloned into the multiple cloning site of the pGL3-Basic reporter vector.

***mPai-1* distal module:**

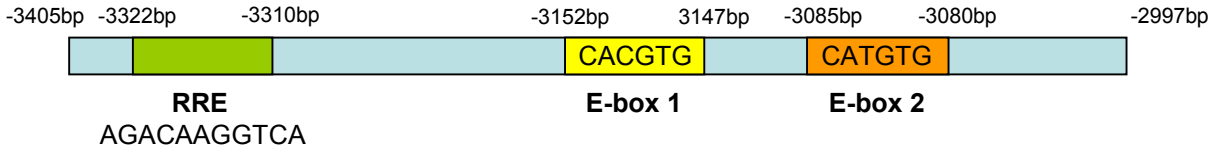


Figure 6.2. Cloning of the mouse *Pai-1* promoter module. Graphical representation of the mouse *Pai-1* upstream promoter element (termed 'module') (-3405bp to -2997bp relative to transcription start site) fragments cloned into the SV40 promoter-containing pGL3-Promoter reporter vector (bottom).

6.2.3 Cloning of the mouse *Pai-1* distal module-destabilised luciferase construct

The destabilised pGL3-Promoter report vector containing the 3xE-box insert (3xE-box-dLuc) was kindly provided by Dr Hiroki Ueda, Molecular Medicine Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., Miyukigaoka, Japan. The destabilised *Luciferase* gene was made by deleting the *Luciferase* gene stop codon to make a continuous sequence with the proteolytic "PEST" sequence from mouse ornithine decarboxylase (Ueda *et al.*, 2005). The WT and E-box-mutated mouse *Pai-1* distal module constructs were generated by substituting the 3xE-box insert with the WT and mutant fragment excised from the pGL3-Promoter vector following *Mlu* I and *Bgl* II digestion. The destabilised empty vector control (SV40-dLuc) was generated by excising the PEST sequence from the dLuc vector and ligating it into the WT pGL3-Promoter vector following *Xba* I digestion.

6.2.4 Bioinformatics.

Comparative sequence analysis was carried out using the Evolutionary Conserved Regions (ECR) Browser (Ovcharenko *et al.*, 2004) from NCBI dCODE.org Comparative Genomics Developments (http://www.dcode.org). Conserved predicted transcription factor binding sites where identified from ECRs by rVISTA 2.0 (Loots and Ovcharenko, 2004), also available from dCODE, using the TRANSFAC professional library V10.2. Conserved predicted transcription factor binding sites from extra-ECR regions were identified using CONSITE (Sandelin *et al.*, 2004) (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite) and sequences were aligned using Clustal W2 (Larkin *et al.*, 2007) (www.ebi.ac.uk/Tools/clustalw2).

6.2.5 Over-expression co-transfection assays.

Expression plasmids were kindly donated by the following people:

- Human CLOCK, BMAL1, BMAL2 and MOP4 – Dr John Hogenesch, The Genomics Institute of the Novartis Research Foundation, San Diego, California, USA.
- Human EPAS1 expression plasmid – Dr Koji Maemura, Cardiovascular and the Pulmonary and Critical Care Division, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA.
- Mouse USF2 and A-USF expression plasmids – Dr Nanyue Chen, Department of Molecular Patholgy, The University of Texas, M.D, Anderson Cancer Centre, Houston, Texas, USA.
- Mouse RORα expression plasmid – Dr Hiroki Ueda, Molecular Medicine Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., Miyukigaoka, Japan

Transfections were performed with jetPEI cationic polymer transfection reagent (Q-Biogen) as described in Chapter 2. Amounts of transfection reagent, DNA and media were optimised (see Chapter 5) and 2µl jetPEI was used per µg DNA.

6.2.6 Statistical analysis.

Statistical analysis of three or more groups was performed by one-way ANOVA followed by Newman-Keuls *post-hoc* multiple comparison tests where ANOVA was significant ($P<0.05$). Interaction between treatments (Figures 6.14 and 6.19) was performed by two-way ANOVA. Analysis of two groups was performed using Students t-test (Figure 6.18).

6.3 Results

6.3.1 Circadian regulatory elements in the mouse *Pai-1* promoter.

To further elucidate the mechanisms governing circadian regulation of the *Pai-1* gene *in silico* analysis of the 5' upstream regulatory region was performed. Conserved regulatory regions were first identified by a cross-species alignment of syntenic regions from the mouse, rat, dog, rhesus monkey and human genomes using the Evolutionary Conserved Region (ECR) browser (Ovcharenko *et al.*, 2004) (Figure 6.3). By comparing sequences from species that share a common ancestry conserved functional cis-elements can be identified.

Two E-boxes and a HRE were identified within the evolutionary conserved proximal region (Figure 6.4A). The most distal E-box in the proximal region (E-box 1, CATGTG) located at -618bp/-623bp relative to the TSS is a non-canonical E-box and conserved between mouse and rat. It aligns with a canonical E-box in the human promoter that has been identified as a target of CLOCK:BMAL heterodimers (Maemura *et al.*, 2000; Chong *et al.*, 2006). The second E-box (E-box 2) located at -174bp/-179bp relative to the TSS has the canonical CLOCK:BMAL-binding sequence CACGTG and is conserved between mouse and rat. It also aligns with a non-canonical E-box in the dog, human and monkey sequences. A conserved consensus HRE (CACGTA) that is similar in sequence to the consensus E-box was identified 4 bases upstream of E-box 2.

Regulation of the human *Pai-1* proximal promoter by CLOCK:BMAL heterodimers via two canonical E-boxes has been documented (Maemura *et al.*, 2000; Chong *et al.*, 2006). However, the potential for clock and hypoxic factors to regulate these E-boxes and HRE in the mouse proximal promoter has not been extensively investigated. Therefore, wild-type and mutant reporter constructs of the mouse *Pai-1* proximal promoter were generated (see Section 6.2) and used in co-transfection assays to determine the activity of these sites. Regulation via mouse E-box 1 (non-canonical) has been characterised the least so a truncated (-314) construct without this E-box was made in addition to the full length proximal promoter (-707).

Through comparative sequence analysis an E-box containing region approximately 3-3.4kb upstream of the TSS was also identified (Figure 6.3). This 408bp region contains two E-boxes conserved between mouse and rat, one of which has the canonical CLOCK:BMAL-binding consensus sequence (Figure 6.4B). The region also contains a consensus RRE (the binding site for RORα and REV-ERBα) and is referred to as the distal module. Whether the regulatory elements in this newly-identified module are functional and involved in the circadian regulation of the mouse *Pai-1* gene was unknown so the module was also cloned into a reporter vector (see Section 6.2) for use in co-transfection assays.

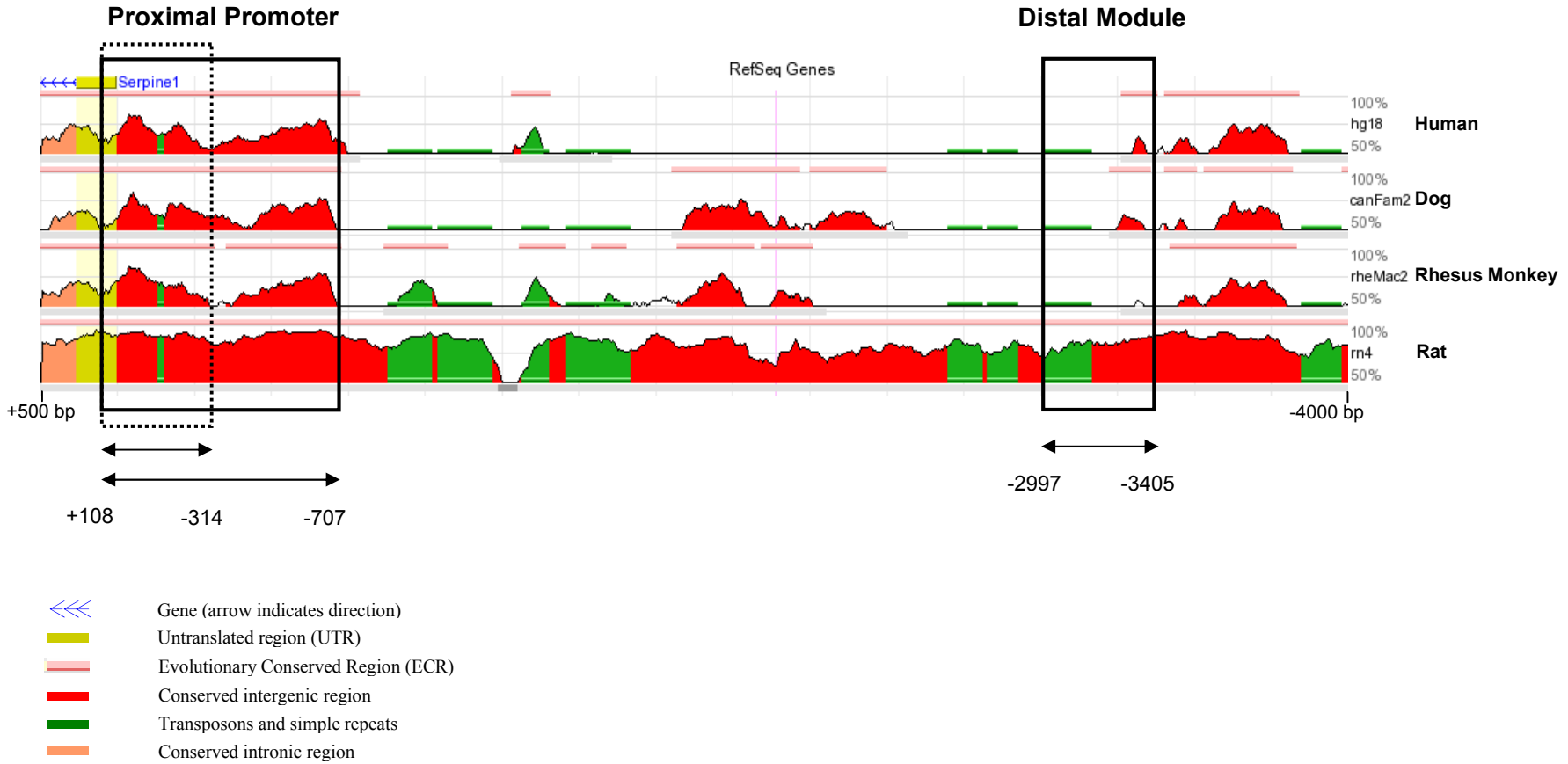


Figure 6.3. *In silico* analysis of the *Pai-1* 5'-flanking region. Phylogenetic comparative analysis of the *Pai-1* (aka *Serpine1*) 5' upstream regulatory sequences from human (*Homo sapiens*), dog (*Canis familaris*), rhesus monkey (*Rhesus macaque*) and rat (*Rattus norvegicus*) compared to the reference sequence (baseline) from mouse (*Mus musculus*). Sequences were obtained from and aligned by the ECR browser from NCBI dCODE (www.dcode.org).

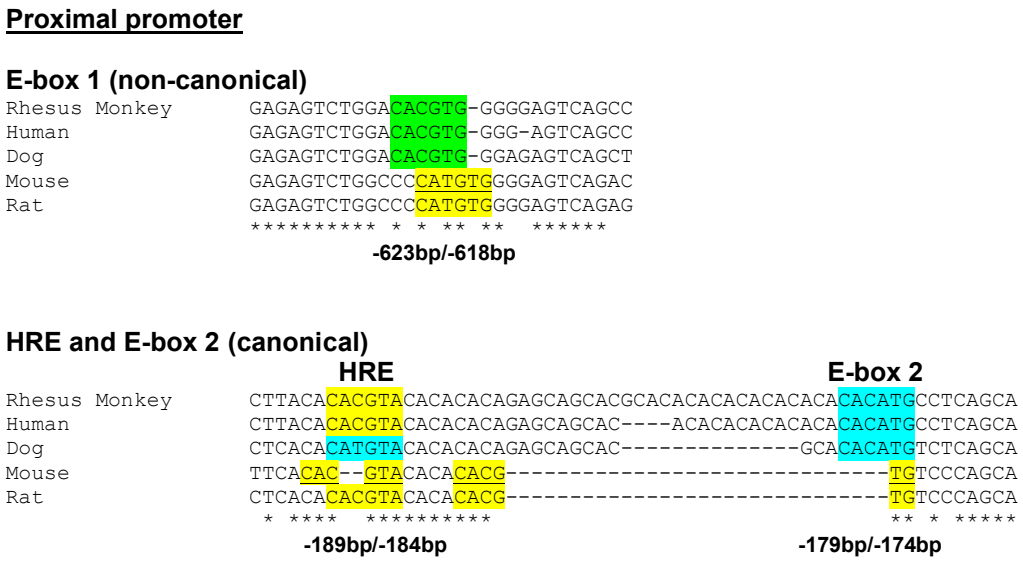


Figure 6.4. Clustal alignment of the *Pai-1* proximal promoter showing conserved transcription factor binding sites. Sequences were obtained from the ECR browser (Ovcharenko *et al.*, 2004) from NCBI dCODE (www.dcode.org) and aligned using Clustal W2 (Larkin *et al.*, 2007) (www.ebi.ac.uk/Tools/clustalw2). Transcription factor binding sites were identified using the rVISTA2.0 tool (Loots and Ovcharenko, 2004) and CONSITE (Sandelin *et al.*, 2004). Conserved circadian sites are shown in yellow and aligned alternative E-boxes in green (canonical E-box) or blue (non-canonical E-box).

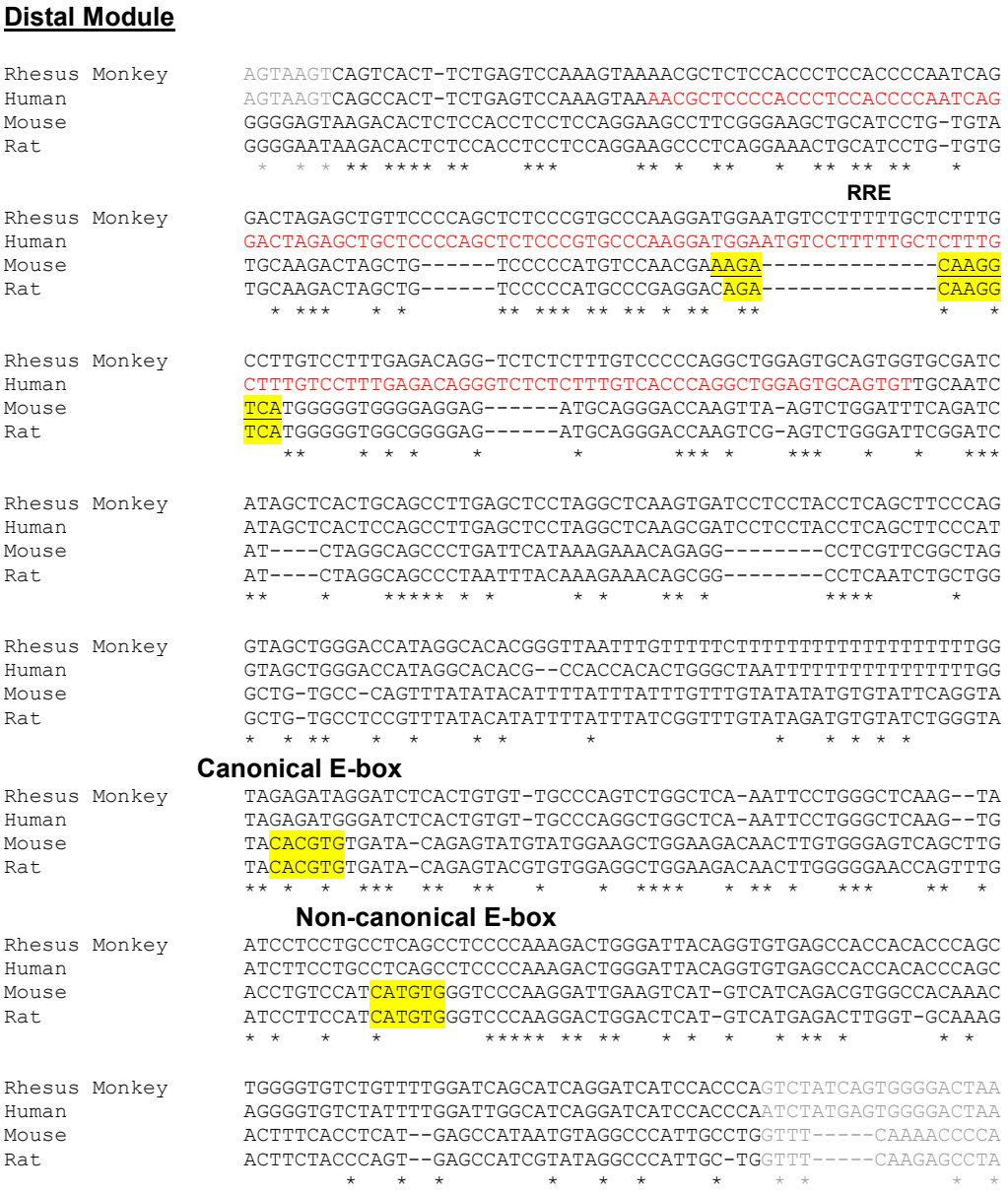


Figure 6.5. Clustal alignment of the *Pai-1* distal module showing conserved transcription factor binding sites. Sequences were obtained from the ECR browser (Ovcharenko *et al.*, 2004) from NCBI dCODE (www.dcode.org) and aligned using Clustal W2 (Larkin *et al.*, 2007) (www.ebi.ac.uk/Tools/clustalw2). Transcription factor binding sites were identified using the rVISTA2.0 tool (Loots and Ovcharenko, 2004) and CONSITE (Sandelin *et al.*, 2004). Distal module is shown in black with human ECR peak from Figure 6.3 shown in red for positioning. Conserved circadian sites are shown in yellow.

6.3.2 Basal activity of the mouse *Pai-1* proximal promoter *in vitro*.

To assess the basal activity of the two proximal constructs 500ng of each was transfected into NIH-3T3 and H9c2 cells for 24 hours and bioluminescence was measured as described. 500ng of each reporter (a relatively large amount) was used in order to allow subtle changes in response to be detected. The levels of activity were also within the optimal range of the assay (according to the manufacture's protocol) so 500ng of reporter plasmid per well was used for all subsequent experiments. Basal activity of both constructs was greater than 100-fold that of the empty pGL3-Basic vector and similar in both cell types (Figure 6.6). Activity of the two constructs was the same in NIH-3T3 cells but differed in H9c2 cells where the longer -707 construct had higher activity, indicating that the region between -314 and -707, which includes E-box 1 (non-canonical), may contain a binding site for a cardiac-specific factor.

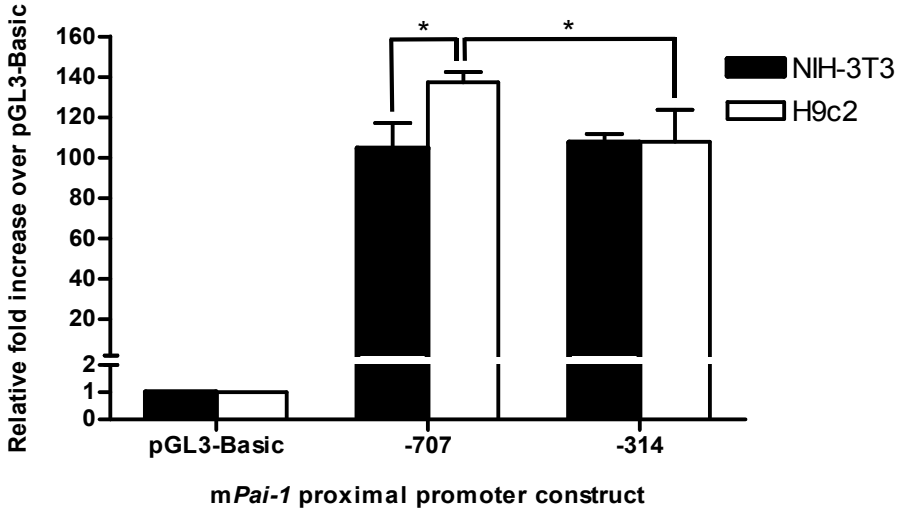


Figure 6.6. Mouse *Pai-1* proximal promoter basal activity. Basal activity of wild-type (WT) -707 and -314 mouse *Pai-1* proximal promoter constructs. H9c2 (white bars) and NIH-3T3 (black bars) cells were transfected with 500ng of a pGL3-Basic reporter plasmid containing the wild-type -707 or -314 m*Pai-1* proximal promoter. Values represent the mean ± standard deviation of two independent experiments performed in triplicate and were standardised to the activity of empty pGL3-Basic. ANOVA P=0.033, $F_{3,4}$=8.49, * P<0.05.

6.3.3 Regulation of the mouse *Pai-1* proximal promoter by circadian and E-box-binding factors.

To investigate the effects of circadian transcription factors on the mouse *Pai-1* promoter 500ng of the -707 *mPai-1* promoter-pGL3-Basic construct was co-transfected into NIH-3T3 cells with plasmids over-expressing circadian and E-box-binding factors. 500ng of each expression plasmid was used to

allow combinations of plasmids to be co-transfected without surpassing the total amount of transfection DNA that begins to cause increased cell death (see Chapter 5). NIH-3T3 cells were used rather than H9c2 cells as they express higher levels of *Pai-1* (approximately 8-fold higher; Figure 1 Appendix 2) and were recently used to demonstrate circadian rhythms of *Pai-1* transcript expression following synchronisation by serum-shock (Wang *et al.*, 2006). Also, as H9c2 cells are a myoblast-derived cell line (as mentioned in Chapter 5) they may have an increased abundance of muscle-specific bHLH factors that could interfere with the binding of clock factors to the *Pai-1* promoter in this *in vitro* model.

Over-expression of CLOCK and BMAL1 significantly activated the mouse *Pai-1* proximal promoter in NIH-3T3 cells by 1.8-fold (Figure 6.7). Mutation of the HRE had no effect on this activation whereas mutation of the canonical E-box 2 completely abolished activation. Replacing BMAL1 with BMAL2 increased activation of the WT promoter to 2.5-fold (Figure 6.7), as seen for the human *Pai-1* promoter (Chong *et al.*, 2006).

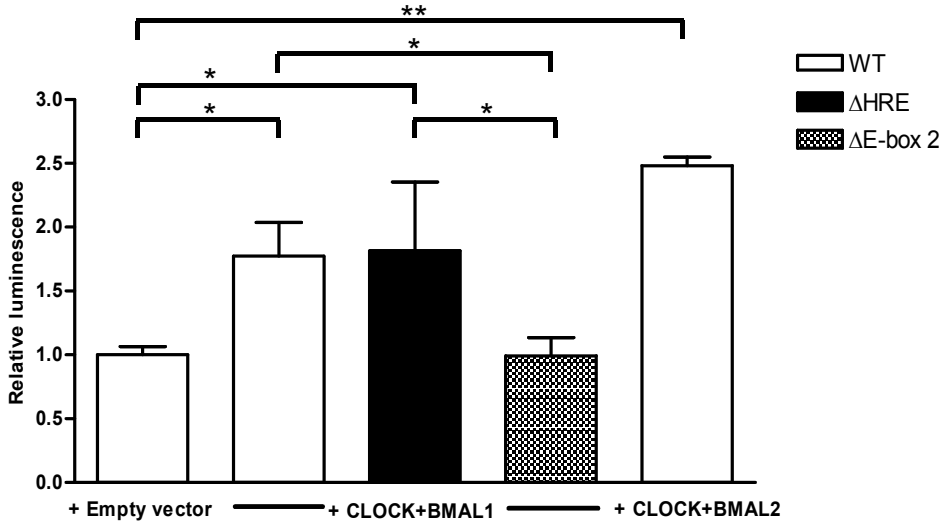


Figure 6.7. Regulation of mouse Pai-1 proximal promoter by clock factors in NIH-3T3 cells.
NIH-3T3 cells were transfected with 500ng of a pGL3-Basic reporter plasmid containing the wild-type (WT, white bars), HRE mutated (ΔHRE, black bars) or E-box 2 (canonical) mutated (ΔE-box 2, hatched bars) mouse *Pai-1* proximal promoter (-707 to +108bp relative to transcription start site). Luciferase activity was induced by co-transfection with plasmids expressing hCLOCK, hBMAL1 and hBMAL2 proteins (500ng each). Values represent the mean ± standard deviation of two independent experiments (three for ΔE-box 2) performed in triplicate and were standardised to the basal activity of the WT promoter as shown by co-transfection of the construct with empty vector control (pcDNA3.1(+)).
ANOVA P=0.004, $F_{4,6}$=13.15, ** P<0.01, * P<0.05**.**

Transfection with the hypoxia-responsive bHLH factor EPAS1 strongly activated the WT proximal promoter 13-fold (Figure 6.8A). Activation was reduced approximately 70% when the HRE was mutated, indicating that the HRE is important in EPAS1-mediated activation of the proximal promoter. However, activation of this mutant was still 3.8-fold higher than the empty vector control so another site, such as E-box 2 (canonical) is likely to be involved in EPAS1 transactivation. Interestingly mutation of E-box 2 augmented activation by EPAS1 compared to WT rather than reducing it. Co-transfection of plasmids expressing BMAL1 or BMAL2 did not augment the activation by EPAS1 on the WT or mutated promoter constructs (Figure 6.8B & C) in contrast to the augmentation seen for the human *Pai-1* proximal promoter (4G and 5G haplotypes) where EPAS1+BMAL2 activated more strongly than EPAS1+BMAL1 and EPAS1 alone (Chong *et al.*, 2006). Mutation of both the HRE and E-box 2 completely abolished activation by EPAS1 and BMAL1 (Figure 6.8B), further demonstrating that these two site are co-operatively involved in the activation of the *mPai-1* promoter by EPAS1.

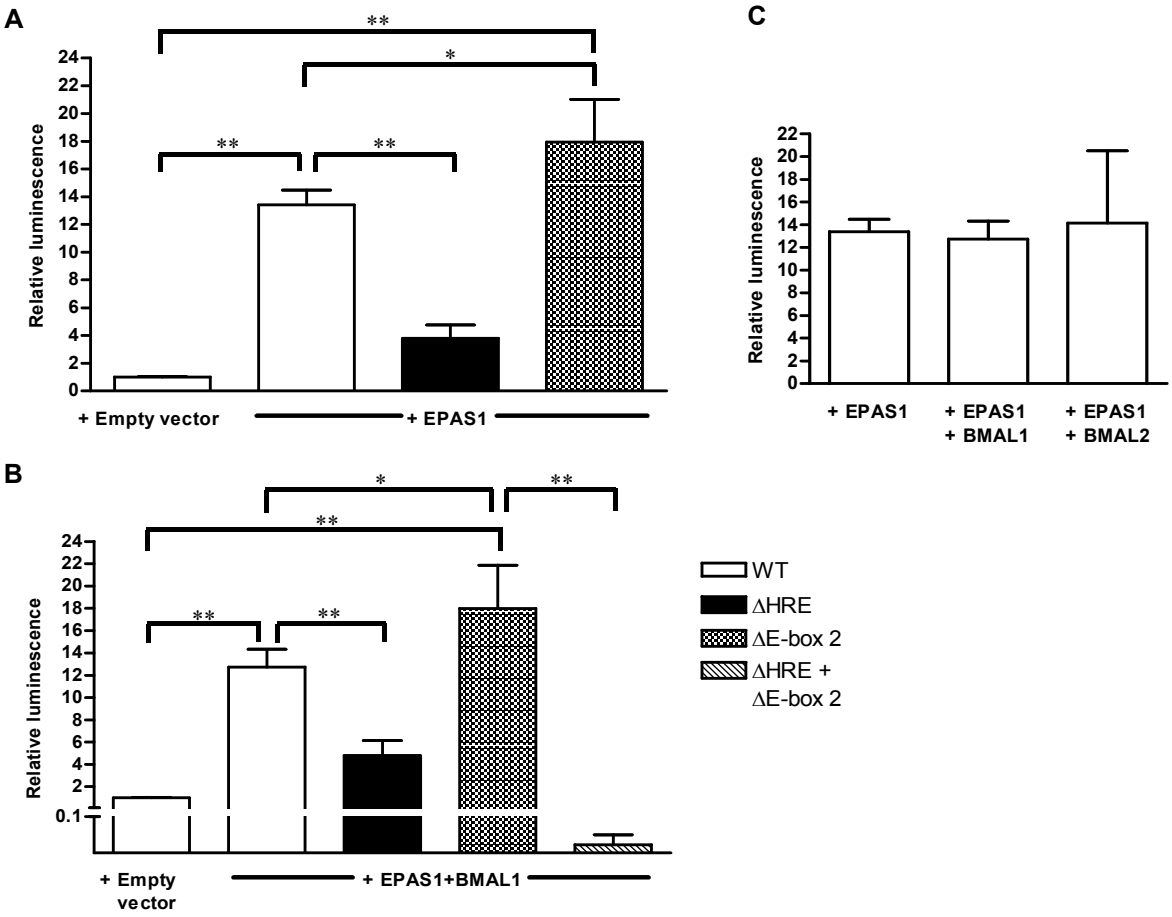


Figure 6.8. Regulation of mouse *Pai-1* proximal promoter by EPAS1 and clock factors in NIH-3T3 cells. NIH-3T3 (black bars) cells were transfected with 500ng of a pGL3-Basic reporter plasmid containing the wild-type (WT, white bars), HRE mutated (ΔHRE, black bars), E-box 2 (canonical) mutated (ΔE-box 2, hatched bars) or HRE + E-box 2-mutated (ΔHRE + ΔE-box 2, striped bars) mouse *Pai-1* proximal promoter (-707 to +108bp relative to transcription start site). Luciferase activity was induced by co-transfection with plasmids expressing hEPAS1 (A), or hEPAS1, hBMAL1 and hBMAL2 (B + C) proteins (500ng each). Values represent the mean ± SEM of three-five independent experiments performed in triplicate (except for ΔE-box 2, ΔE-box 2 + ΔHRE, and WT + EPAS1+BMAL2, where values represent the mean ± standard deviation of two independent experiments performed in triplicate). Values were standardised to the basal activity of the WT promoter as shown by co-transfection of the construct with empty vector control (pcDNA3.1(+)). (A) ANOVA $P<0.0001$, $F_{3,11}=62.74$, (B) ANOVA $P<0.0001$, $F_{4,11}=31.96$, ** $P<0.01$, * $P<0.05$. $P<0.01$ significance bars between ΔHRE and ΔE-box 2 (A and B) and WT and ΔHRE+ΔE-box 2 (B) are omitted for clarity.

To examine whether the canonical E-box (E-box 2) and HRE containing region is similarly regulated by clock and hypoxic factors in cardiac cells transfections were repeated in H9c2 cells. The -314

fragment was used since present data indicated possible cell-specific activation of the region between -314 and -707bp upstream (see Figure 6.6), which could potentially have interfered with the observed results. In contrast to NIH-3T3 cells, over-expression of CLOCK and BMAL1 did not activate the mouse *Pai-1* proximal promoter in H9c2 cells (Figure 6.9). BMAL2 was also unable to activate the construct and may cause some repression in combination with CLOCK.

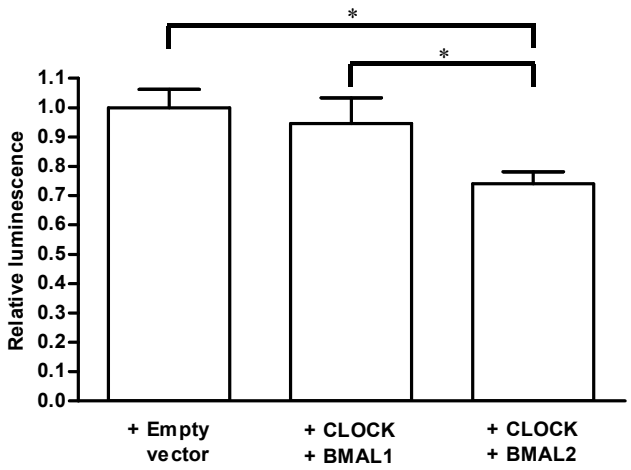


Figure 6.9. Regulation of mouse *Pai-1* proximal promoter by clock factors in H9c2 cells. H9c2 cells were transfected with 500ng of a pGL3-Basic reporter plasmid containing the wild-type mouse *Pai-1* proximal promoter (-314 to +108bp relative to transcription start site). Luciferase activity was induced by co-transfection with plasmids expressing hCLOCK, hBMAL1 and hBMAL2 proteins (500ng each). Values represent the mean ± standard deviation of two independent experiments performed in triplicate and were standardised to the basal activity of the WT promoter as shown by co-transfection of the construct with empty vector control (pcDNA3.1(+)).
ANOVA P=0.037, $F_{2,3}$=12.07, *P<0.05.

Although CLOCK + BMAL1 did not activate the mouse *Pai-1* promoter in H9c2 cells transfection with plasmids expressing EPAS1 strongly activated the promoter approximately 12-fold compared to transfection with BMAL1 (Figure 6.10). Co-transfection with BMAL1-expressing plasmid further augmented this activation to approximately 16-fold (and increase of approximately 30%), as seen for the human *Pai-1* promoter (Chong *et al.*, 2006) but not for the mouse promoter in NIH-3T3 cells (Figure 6.8C). Over-expression of CLOCK significantly reduced activation by EPAS1+BMAL1.

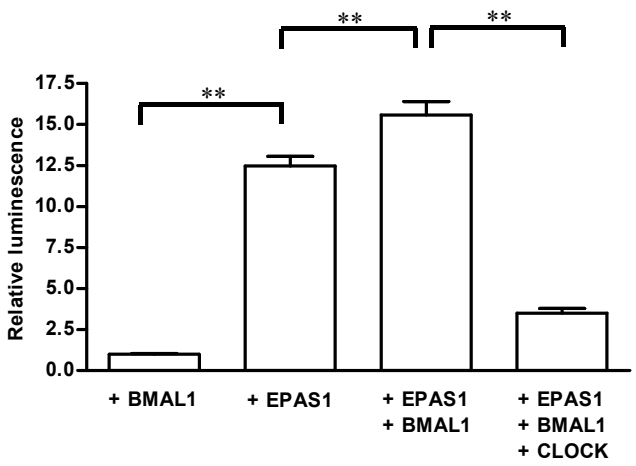


Figure 6.10. Regulation of mouse *Pai-1* proximal promoter by EPAS1 and clock factors in H9c2 cells. H9c2 cells were transfected with 500ng of a pGL3-Basic reporter plasmid containing the wild-type mouse *Pai-1* proximal promoter (-314 to +108bp relative to transcription start site). Luciferase activity was induced by co-transfection with plasmids expressing hBMAL1, hEPAS1 and hCLOCK proteins (500ng each, except where three factors were co-transfected when 330ng of each was used). Values represent the mean ± standard deviation of two independent experiments performed in triplicate and were standardised to WT promoter + BMAL1.
ANOVA $P<0.0001$, $F_{3,4}=356.5$, $^{**}P<0.01$.

As CLOCK + BMAL1/2 did not activate the mouse *Pai-1* promoter in H9c2 cells an alternative bHLH factor, USF2, was tested. USF2, a factor that has previously been demonstrated to regulate the rat and human *Pai-1* promoters via E-boxes (Samoylenko *et al.*, 2001; Dimova and Kietzmann, 2006). Over-expression of USF2 modestly, but significantly, activated the -707 and -314 constructs by 1.8- and 1.3-fold respectively (Figure 6.11). H9c2 cells were also transfected with plasmids expressing the dominant-negative USF factor A-USF. Substitution of the WT basic DNA-binding domain with acidic residues renders A-USF unable to bind DNA but able to form strong heterodimers with USF1 and USF2 proteins, resulting in attenuation of DNA-biding and activation by WT USF factors (Allen *et al.*, 2005). Over-expression of A-USF had no effect on the basal activity of the m*Pai-1* promoter therefore the activation by USF observed here may be artificial and USF factors are unlikely to have a significant role in regulating basal m*Pai-1* promoter activity in rat H9c2 cells. As the two different length reporter constructs were activated by different amounts it would have been wise to examine the response of both to CLOCK, BMAL and EPAS1 in both cell types.

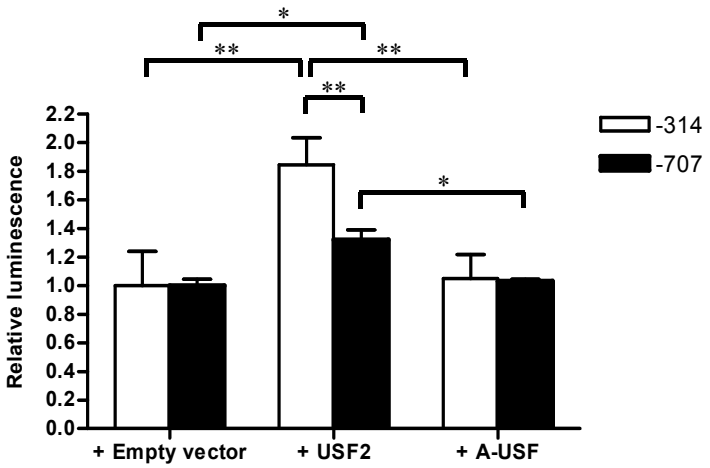


Figure 6.11. Regulation of mouse *Pai-1* proximal promoter by E-box binding factor USF2 in H9c2 cells. H9c2 cells were transfected with 500ng of a pGL3-Basic reporter plasmid containing the wild-type -314 (white bars) or -707 (black bars) mouse *Pai-1* proximal promoter. Luciferase activity was induced by co-transfection with plasmids expressing mUSF2 or dominant negative A-USF (500ng each). Values represent the mean ± standard deviation of two independent experiments performed in triplicate and were standardised to the basal activity of the WT promoters as shown by co-transfection of constructs with empty vector control (pcDNA3.1(+)).
ANOVA P=0.0003, $F_{5,6}$=31.16, *P<0.05, **P<0.01.

6.3.4 Basal activity of the mouse *Pai-1* distal module *in vitro*.

To determine whether the distal module is required for *Pai-1* activity and whether the canonical E-box is functionally active, the WT- and canonical E-box-mutated constructs were transfected into NIH-3T3 and H9c2 cells and bioluminescence was measured after 48 hours as described. Basal activity of the WT module was approximately 1.5-fold greater than the pGL3-SV40 promoter control and activity was abolished by canonical E-box mutation in both cell types (reduction of 69% in NIH-3T3 and 68% in H9c2; Figure 6.12). Mutation of the RRE modestly increased basal WT module activity in NIH-3T3 cells. All other trends were similar between the two cell types.

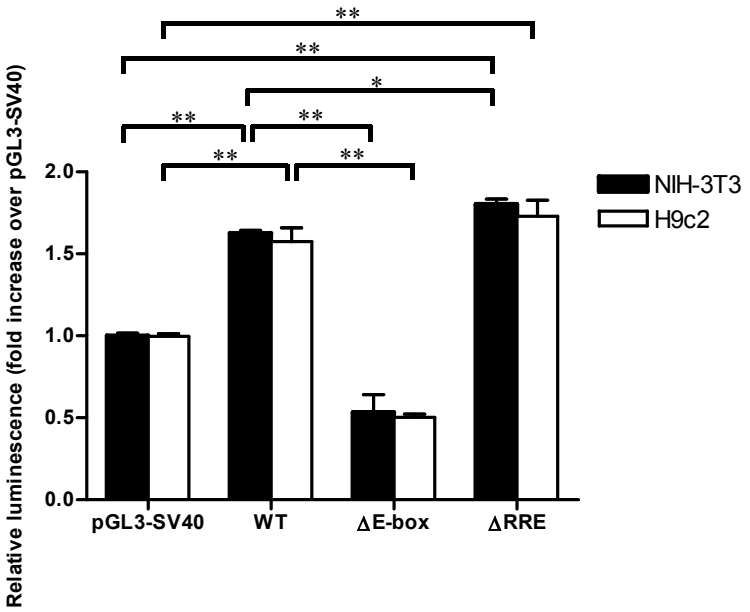


Figure 6.12. Mouse *Pai-1* distal module basal activity. H9c2 (white bars) and NIH-3T3 (black bars) cells were transfected with 500ng of a pGL3-SV40 reporter plasmid containing the wild-type (WT), canonical E-box-mutated (ΔE-box) or RRE-mutated (ΔRRE) mouse *Pai-1* distal module and bioluminescence measured after 48 hours. Values represent the mean ± standard deviation of two independent experiments performed in triplicate for all transfections, except NIH-3T3 WT and ΔE-box which represent mean ± SEM of five independent experiments performed in triplicate. Values were standardised to the activity of the empty vector pGL3-SV40 in each cell type.
ANOVA $P<0.0001$, $F_{7,14}=45.9$, $*P<0.05$, $**P<0.01$.

6.3.5 Regulation of the mouse *Pai-1* distal module by circadian and E-box-binding factors

To investigate whether circadian transcription factors activate the mouse *Pai-1* distal module and whether the canonical E-box and RRE are involved the WT and mutant constructs were co-transfected into NIH-3T3 cells with plasmids over-expressing circadian and E-box-binding factors. Over-expression of CLOCK and BMAL1 failed to activate the module but significantly reduced the basal activity by 49% (Figure 6.13). Mutation of the canonical E-box did not affect this loss in activity, resulting in a significant reduction of 52%.

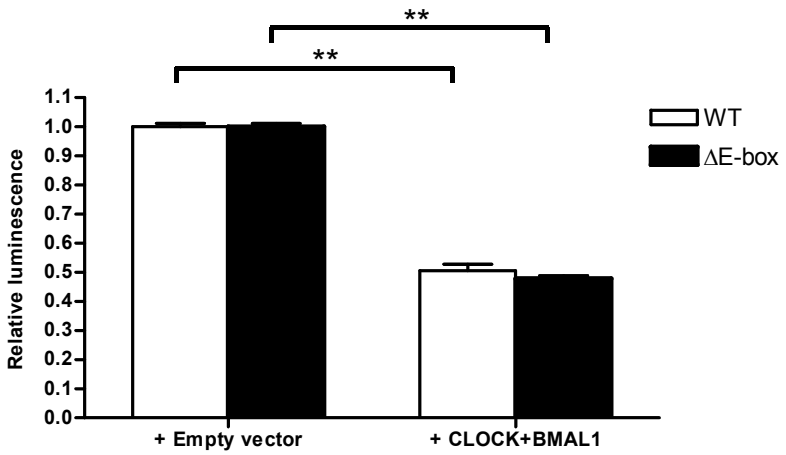


Figure 6.13. Regulation of mouse *Pai-1* distal module by clock factors in NIH-3T3 cells. NIH-3T3 cells were transfected with 500ng of a pGL3-SV40 reporter plasmid containing the wild-type (WT) or canonical E-box-mutated (ΔE-box) mouse *Pai-1* distal module. Luciferase activity was measured following co-transfection with plasmids expressing hCLOCK and hBMAL1 proteins (500ng each). Values represent the mean ± SEM of three independent experiments performed in triplicate, except for ΔE-box + CLOCK+BMAL1 where value represents mean ± standard deviation of two independent experiments. Values were standardised to the basal activity of the WT or ΔE-box module as shown by co-transfection of the constructs with empty vector control (pcDNA3.1(+)). ANOVA $P<0.0001$, $F3_{,7}=525.3$, $^{**}P<0.01$.

In contrast to CLOCK and BMAL1, EPAS1 over-expression did significantly activate the mouse *Pai-1* distal module by 1.8-fold, which was augmented by over-expression of BMAL2 (3.4-fold) (Figure 6.14). Augmentation by BMAL1 (2.7-fold) did not reach significance. Canonical E-box mutation did not abolish the activation by EPAS1 but increased it to 2.7-fold. This increase between wild-type and E-box mutant was not significant. Mutation of the E-box did however abolish the augmentation of EPAS1 activity by BMAL2. Whilst the activities of the wild-type and E-box mutant reporters in response to over-expression were not significant, analysis by two-way ANOVA demonstrated that the overall trends for the two reporters were significantly different.

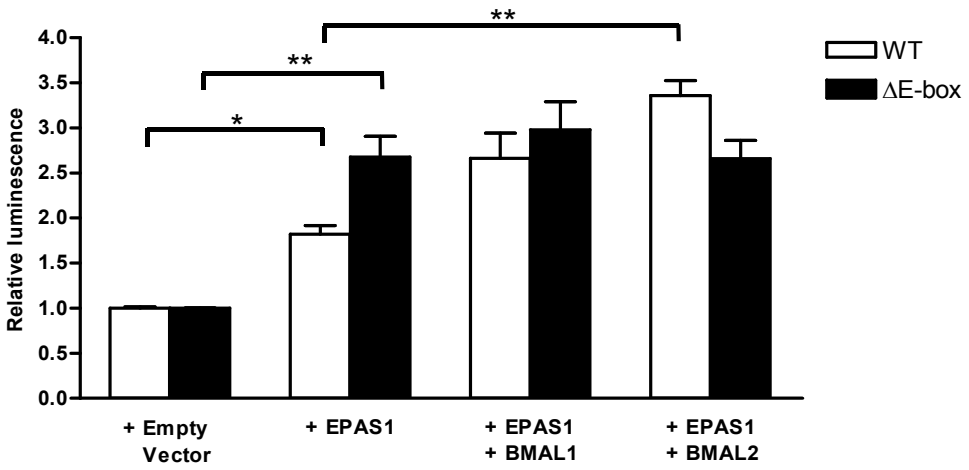


Figure 6.14. Regulation of mouse Pai-1 distal module by EPAS1 and clock factors in NIH-3T3 cells. NIH-3T3 cells were transfected with 500ng of a pGL3-SV40 reporter plasmid containing the wild-type (WT) or canonical E-box-mutated (ΔE-box) mouse *Pai-1* distal module. Luciferase activity was induced by co-transfection with plasmids expressing hEPAS1, hBMAL1 and hBMAL2 proteins (500ng each). Values represent the mean ± SEM of three-five independent experiments performed in triplicate except for WT and ΔE-box + EPAS1+BMAL2, where value represents mean ± standard deviation of two independent experiments. Values were standardised to the basal activity of the WT or ΔE-box module as shown by co-transfection of the constructs with empty vector control (pcDNA3.1(+)).
One-way ANOVA $P<0.0001$, $F_{7,14}=19.56$, ** $P<0.01$, * $P<0.05$. Two-way ANOVA interaction $P=0.028$, $F_{3,13}=4.20$. $P<0.01$ significance bars between empty vector and EPAS1+BMAL1 and EPAS1+BMAL2 for both WT and ΔE-box are omitted for clarity.

As CLOCK and BMAL1 were unable to activate the distal module the effect of USF2 over-expression was investigated. Over-expression of USF2 significantly lowered the basal activity of the WT- and canonical E-box-mutated distal module construct by 45% and 25% respectively.

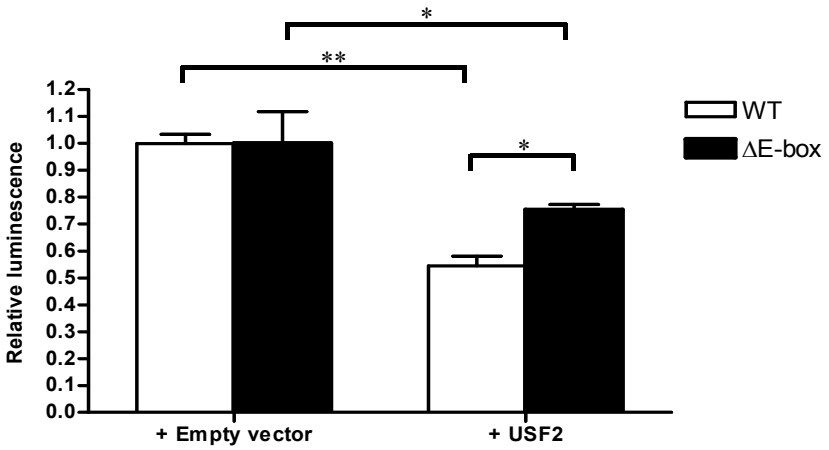


Figure 6.15. Regulation of mouse *Pai-1* distal module by E-box binding factor USF2 in NIH-3T3 cells. NIH-3T3 cells were transfected with 500ng of a pGL3-SV40 reporter plasmid containing the wild-type (WT) or canonical E-box-mutated (ΔE-box) mouse *Pai-1* distal module. Luciferase activity was measured following co-transfection with plasmids expressing mUSF2 protein (1000ng). Values represent the mean ± standard deviation of two independent experiments performed in triplicate and were standardised to the basal activity of the WT or ΔE-box module as shown by co-transfection of the constructs with empty vector control (pcDNA3.1(+)).
ANOVA P=0.0057, $F_{3,4}$=22.71, ** P<0.01, * P<0.05.

In addition to a canonical E-box the distal module also contains a RRE site. Mutation of the RRE had little effect on basal activity of the module (Figure 6.12), indicating that it may not be a physiologically relevant site. This hypothesis was strengthened by over-expression of the RRE-binding activator RORα, which was unable to activate the wild-type module and mutation of the RRE had no effect (Figure 6.16).

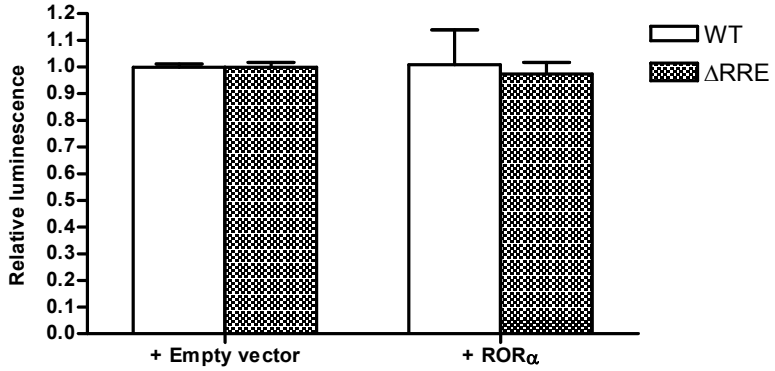


Figure 6.16. Lack of regulation of mouse *Pai-1* distal module by RRE-binding factor RORα in NIH-3T3 cells. NIH-3T3 cells were transfected with 500ng of a pGL3-SV40 reporter plasmid containing the wild-type (WT) or RRE-mutated (ΔRRE) mouse *Pai-1* distal module. Luciferase activity was measured following co-transfection with plasmids expressing mRORα protein (1000ng). Values represent the mean ± SEM of three independent experiments performed in triplicated for WT module and mean ± standard deviation of two independent experiments performed in triplicate for ΔRRE module. Values were standardised to the basal activity of the WT or ΔRRE module as shown by co-transfection of the constructs with empty vector control (pcDNA3.1(+)).
ANOVA P=0.994, $F_{3,6}$=0.027.

Since mutation of the canonical E-box demonstrated that this E-box is crucial for activity of the distal module in NIH-3T3 and H9c2 cells (Figure 6.12), and CLOCK, BMAL1 (Figure 6.13) and USF2 (Figure 6.15) failed to activate the module in NIH-3T3 cells, the response of the construct to E-box binding factors was investigated in H9c2 cells.

Over-expression of CLOCK and BMAL1 again lowered the basal activity of the wild-type- and canonical E-box-mutated modules, by 67% and 66% respectively (Figure 6.17), as observed in NIH-3T3 cells. Over-expression of USF2 increased the activity of the WT module by 1.9-fold (Figure 6.18), similar to the activation of the -314 proximal promoter construct in H9c2 cells but in contrast to the reduction in activity observed in NIH-3T3 cells.

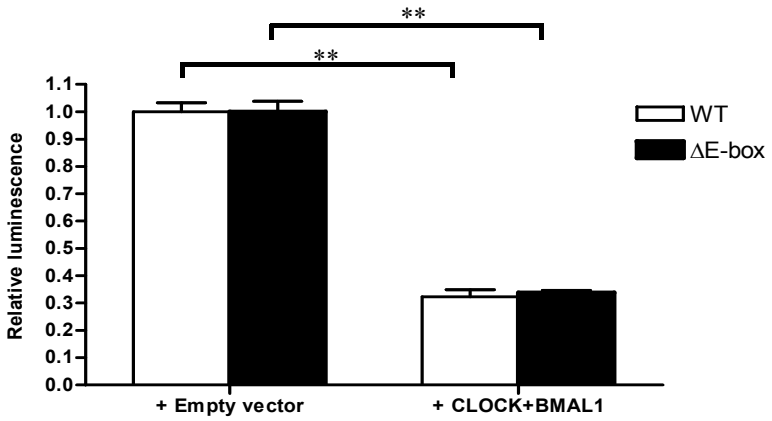


Figure 6.17. Regulation of mouse *Pai-1* distal module by clock factors in H9c2 cells. H9c2 cells were transfected with 500ng of a pGL3-SV40 reporter plasmid containing the wild-type (WT) or E-box-mutated (ΔE-box) mouse *Pai-1* distal module. Luciferase activity was measured following co-transfection with plasmids expressing hCLOCK and hBMAL1 proteins (1000ng each). Values represent the mean ± SEM of three independent experiments performed in triplicate for WT module and mean ± standard deviation of two independent experiments performed in triplicate for ΔE-box module. Values were standardised to the basal activity of the WT or ΔE-box module as shown by co-transfection of the constructs with empty vector control (pcDNA3.1(+)).
ANOVA $P<0.0001$, $F_{3,6}=404.1$, ** $P<0.01$.

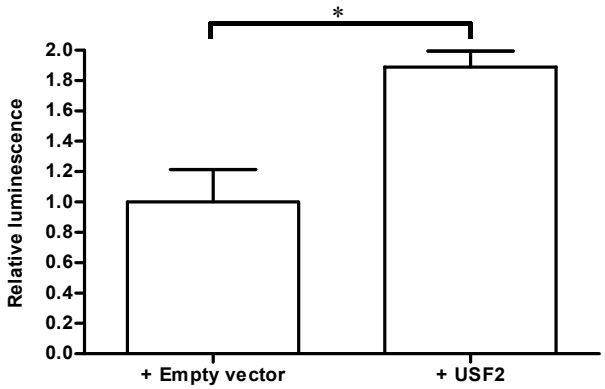


Figure 6.18. Regulation of mouse *Pai-1* distal module by E-box binding factor USF2 H9c2 cells. H9c2 cells were transfected with 500ng of a pGL3-SV40 reporter plasmid containing the wild-type (WT) mouse *Pai-1* distal module. Luciferase activity was measured following co-transfection with plasmid expressing mUSF2 (1000ng). Values represent the mean ± standard deviation of two independent experiments performed in triplicate and were standardised to the basal activity of the WT module as shown by co-transfection of the constructs with empty vector control (pcDNA3.1(+)).
T-test $P=0.034$, $t_2=5.26$, ** $P<0.05$.

6.3.6 The canonical E-box may be sufficient to generate cycling of the mouse *Pai-1* distal module in synchronised NIH-3T3 cells.

As shown in Chapter 5, treatment with DEX can synchronise NIH-3T3 cells and generate rhythmic gene expression. Rhythmic mouse *Pai-1* transcript expression has also been demonstrated in NIH-3T3 following synchronisation by serum-shock (Wang *et al.*, 2006). To determine whether the distal module, and the canonical E-box in particular, can confer rhythmicity the WT and canonical E-box-mutated modules were cloned into a destabilised luciferase promoter-reporter vector and transfected into cells that were then synchronised by DEX. Turnover of luciferase produced from the reporter is therefore high and changes in luminescence can be measured in real-time, allowing the detection of circadian rhythms. This technique has been used to demonstrates the rhythmicity of short circadian (Ueda *et al.*, 2005) and larger promoter elements (Ohno *et al.*, 2007).

Treatment with DEX resulted in an acute activation of the WT distal module that persisted for the initial 15 hours and then fell to near-baseline level at 25 hours post-stimulus (Figure 6.19). What appeared to be rhythmic expression of the module reporter was detected over the subsequent 50 hours, as shown by the peaks in reporter activity at 35 and 63 hours post-stimulus. However, these peaks were not statistically significant by *post-hoc* analysis. Activation of the E-box-mutated module reporter by DEX was less dramatic as shown by the relatively constant reporter levels for the first 25 hours of the time-course (Figure 6.19). Reporter levels then declined steadily with no peaks in expression. The WT module pattern was clearly different to that of the E-box-mutated module, as shown by the significant two-way ANOVA interaction value. The E-box-mutated reporter profile was also very similar to the profile of the empty vector negative control, further implying the necessity of the canonical E-box to confer rhythmicity of the module.

Whether this distal module can confer rhythmicity in isolation must be further investigated as this experiment was performed three times and only generated meaningful results once (Figure 6.19), where the peaks in activity were not significant. The inability to repeat these results may be largely due to the inadequacy of the equipment (see section 2.5.2) and sampling method used and the experiment should be repeated using optimised specialised equipment (i.e. real-time monitoring in an incubator).

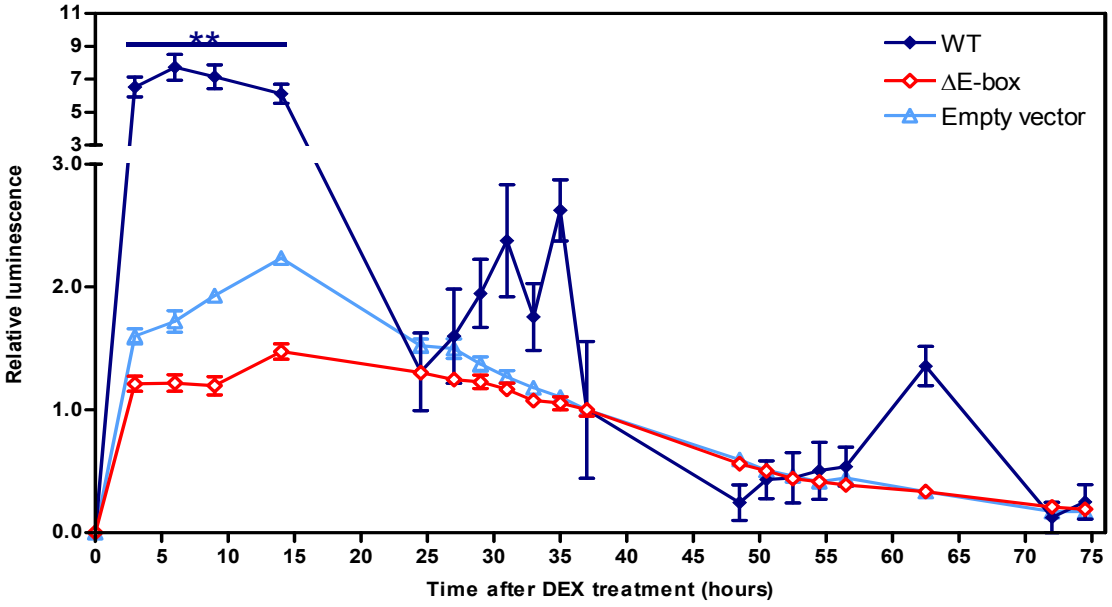


Figure 6.19. Canonical E-box may mediate rhythmic mouse *Pai-1* distal module activity in synchronised cells *in vitro*. NIH-3T3 cells were transfected with a SV40-driven destabilised luciferase reporter plasmid containing the wild-type (WT, blue, closed diamonds) or canonical E-box mutated (ΔE-box, red, open diamonds) mouse *Pai-1* distal module (-3405 to -2998bp relative to transcription start site) or empty SV40-driven vector as a negative control (empty vector, aqua, open triangles) (C). Cells were synchronised by treatment with 100nM DEX at 0 hours prior to addition of assay medium containing luciferin. Bioluminescence was measured at regular intervals in an assay plate reader at 37ºC. Values represent the mean ± SEM of six observations from one experiment and were standardised to the mid-point of the time-course, 37 hours post-treatment.
One-way ANOVA: WT $P<0.0001$, $F_{19,100}=42.03$, ΔE-box $P<0.0001$, $F_{19,100}=114.6$, Empty vector $P<0.0001$, $F_{19,100}=198.0$. Two-way ANOVA interaction between WT and ΔE-box $P<0.0001$, $F_{19,190}=41.45$. ** $P<0.01$.

6.3.7 Regulation of the mouse *Bnp* proximal promoter by circadian and E-box-binding factors.

Previous chapters have identified *Bnp* as a putative novel CCG. The 5' regulatory region of this gene has a canonical E-box conserved between mouse and rat at -528 to -534bp relative to the TSS. Activation of the *Bnp* proximal promoter by clock factors has not previously been demonstrated and the rodent promoter was investigated here. The first 1kb 5' upstream of the mouse TSS was cloned into a luciferase reporter vector and transfected into NIH-3T3 cells together with plasmids over-expressing hCLOCK and hBMAL1. Over-expression of CLOCK and BMAL1 activated the mouse *Bnp* promoter construct approximately 5-fold (Figure 6.20).

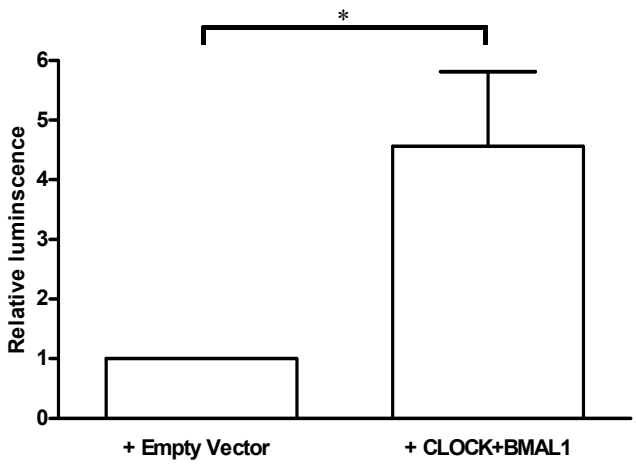


Figure 6.20. Regulation of mouse *Bnp* proximal promoter by clock factors in NIH-3T3 cells.
NIH-3T3 cells were transfected with 200ng of a pGL3-Basic reporter plasmid containing the wild-type mouse *Bnp* proximal promoter (-890 to +57bp relative to transcription start site). Luciferase activity was measured following co-transfection with plasmids expressing hCLOCK and hBMAL1 proteins (200ng each). Values represent the mean ± standard deviation of two independent experiments performed in triplicate and were standardised to the basal activity of the promoter as shown by co-transfection of the construct with empty vector control (pcDNA3.1(+)).
T-test P=0.046, t_2=4.03, * P<0.05.

Repetition of CLOCK and BMAL1-mediated activation of the mouse *Bnp* proximal promoter was not possible in further experiments (not shown) therefore further investigation into the circadian regulation of mouse *Bnp* gene is required. The variation in results may reflect the high basal activity of the mouse *Bnp* reporter construct in NIH-3T3 cells, which was 120-fold greater than that of the empty reporter vector (not shown).

6.4 Discussion

6.4.1 Regulation of the mouse *Pai-1* promoter by circadian and hypoxic factors

Regulation of the rodent *Pai-1* gene by the circadian clock and hypoxia has been described but many aspects of the regulatory mechanisms remain unclear and the combined effects of circadian and hypoxic factors on the *Pai-1* promoter has not previously been investigated. In this chapter the ability of circadian and hypoxic factors to regulate the mouse *Pai-1* promoter via known and novel transcription factor binding sites was examined.

Although the number and location of circadian regulatory elements in the human and rodent *Pai-1* promoters differ the results here demonstrate that the mouse *Pai-1* proximal promoter can be activated by CLOCK and BMAL1/BMAL2 via the canonical E-box (E-box 2) in NIH-3T3 cells. These same results were also demonstrated by Oishi and colleagues (2007) during the course of the current study. Therefore, the regulation of *Pai-1* by CLOCK and BMAL1 (or BMAL2) in mice and humans suggests *Pai-1* is a direct target of the circadian clock. Mutation of E-box 2 alone completely abolished CLOCK+BMAL1-mediated activation of the proximal promoter construct, implying that the other two identified sites (E-box 1 and HRE) are not necessary for this regulatory mechanism.

EPAS1 over-expression also activated the mouse *Pai-1* promoter, and this was mainly but not totally dependent on the HRE. Mutation of the HRE and canonical E-box (E-box 2) completely abolished activity therefore these two sites in combination are sufficient for activation of the mouse *Pai-1* proximal promoter by EPAS1. In fact, the double mutation completely abolished basal activity of the construct (Figure 2A, Appendix 2), demonstrating the functional importance of these two sites. Interestingly mutation of the canonical E-box augmented EPAS1-induced activation of the construct, suggesting that it is not bound by EPAS1 and may contribute to activity of the region via the occupancy of other factors such as CLOCK and BMAL1. In support of this, co-expression of CLOCK and BMAL1 dramatically reduced EPAS1 activation of the proximal promoter in H9c2 cells. Both the HRE and canonical E-box match the consensus HIF-1 binding site at 6 out of 8 bases (Kietzmann *et al.*, 1999) but only the HRE sequence (CACGTA) was found to be necessary for hypoxia-dependent activation of the human *Pai-1* promoter, via HIF-1α (Fink *et al.*, 2002) and EPAS1 (Sato *et al.*, 2004b). Studies in the rat however have demonstrated that the canonical E-box is the hypoxia-responsive element, with the HRE having a supporting role in mediating HIF-1α-DNA complex formation by forming a complex with factors other than HIF-1α (Kietzmann *et al.*, 1999).

Together the results of these transfection assays suggest that the balance between regulation of the mouse *Pai-1* promoter by CLOCK:BMAL1 and EPAS1 is complex. EPAS1, like HIF-1α, binds DNA as a heterodimer and has been shown to form functional heterodimers with BMAL1 (Hogenesch *et al.*, 1998; Takahata *et al.*, 1998), adding a further degree of complexity. EPAS1 together with BMAL1

can activate the human *Pai-1* promoter more strongly than EPAS1 alone (Chong *et al.*, 2006) but the addition of BMAL1 had no effect on the mouse proximal promoter in NIH-3T3 cells.

6.4.2 A novel distal regulatory module may integrate regulation of mouse *Pai-1* by circadian and hypoxic factors

In addition to investigating circadian and hypoxic regulation of the mouse *Pai-1* proximal promoter a putative novel distal E-box-containing module was identified and characterised. The importance of this canonical E-box can be seen by 70% reduction in basal activity following mutation. The module was also activated by EPAS1 *in vitro*, although mutation of the canonical E-box did not abolish activation, as observed with the proximal promoter. Addition of BMAL2 augmented activation by EPAS1, in agreement with the augmentation seen for the human proximal promoter (Chong *et al.*, 2006). Augmentation by BMAL2 was lost following E-box mutation, further demonstrating that the E-box is a target of combined regulation by EPAS1 and BMAL. Therefore the distal module canonical E-box may be a target for EPAS1:BMAL heterodimers and represent a conserved mechanism with humans. The mechanism mediating action by EPAS1 alone however remains unclear and may involve the second non-canonical E-box (CATGTG). Interestingly over-expression of CLOCK and BMAL1 decreased activity of the region, independently of the canonical E-box and RRE. CLOCK and BMAL1 are traditionally known as activators so may indirectly prevent activation by blocking the binding of endogenous activators to either of the two E-boxes. The canonical E-box (CACGTG) in the rodent *Pai-1* promoter can be bound by the ubiquitously expressed USF factors (Viollet *et al.*, 1996; Coulson *et al.*, 2003) and the non-canonical E-box sequence (CATCTG) may be a target for HAND2 (Dai and Cserjesi, 2002), which is primarily expressed during development but also found in the failing heart (Natarajan *et al.*, 2001). Alternatively, CLOCK might sequester BMAL1 and prevent EPAS1:BMAL1 heterodimer formation. Recently it has also been shown that CLOCK and BMAL1 may act as a repressor when bound to CRY in a complex and interfere with the activation of promoters by other factors (Kondratov *et al.*, 2006). Whatever the mechanism involved, this distal canonical E-box rather than the proximal canonical E-box may therefore be a target of interplay between the circadian and hypoxic pathways and act as a site that modifies the circadian response of the proximal promoter.

Despite the distal module not being activated by CLOCK and BMAL1 it may be sufficient to generate circadian expression rhythms following treatment with DEX *in vitro*, via a mechanism dependent on the canonical E-box. Therefore, the non-canonical E-box and the RRE are unlikely to be involved in circadian regulation of the region directly. As discussed in Chapters 4 and 5, glucocorticoids play an important role in communicating temporal information between the central clock and peripheral tissues and DEX can synchronise rhythmic expression in different cell types. The distal module does not contain a consensus glucocorticoid response element (GRE) but preliminary results suggest that it

may be rhythmically expressed following DEX treatment and that DEX can mediate circadian expression independently of the GRE, via a clock/E-box-dependent mechanism, as suggested by Reddy and colleagues (2007).

6.4.3 *Pai-1* promoter modules can mediate co-ordinated and specific transcriptional regulation

In this chapter two regions (modules) have been identified that may mediate co-operative regulation of the *Pai-1* promoter, the closely spaced HRE and canonical E-box in the proximal promoter, and the canonical and non-canonical E-boxes and consensus RRE in the distal module. A module typically consists of two or more transcription factor binding sites within an approximately 300bp region of DNA (Klingenhoff *et al.*, 2002). Transcription factors that bind these sites can work synergistically or antagonistically depending on the spacing of sites and binding factor present in a particular cell type, enabling the integration of signals in a cell-specific manner. The presence of a module can identify candidate genes involved in a particular pathway and conserved modules can be found in promoter regions with little overall sequence homology (Klingenhoff *et al.*, 1999). Only half of the distal module identified here overlaps with an evolutionary conserved region (ECR) but the combination of an E-box and RRE is present in the mouse and human *Pai-1* up-stream regulatory regions (Wang *et al.*, 2006) and may represent a circadian module present in other clock-regulated genes. The functionality of the novel distal module is implied here through its suggested ability to generate rhythmic reporter expression in synchronised cells, an approach that has been used before to identify the circadian regulatory elements of the *Per2* promoter (Akashi *et al.*, 2006) and investigate the rhythm-generating properties of isolated circadian elements *in vitro* (Ueda *et al.*, 2005). This approach enables the identification of regions regulated by the clock within a cell type due to the exclusion of internal temporal cues. It also provides temporal information and avoids the potential non-physiological effects of ectopic over-expression in transient reporter assays and knock-out mice models.

The study by Ueda and colleagues (2005) demonstrated that conserved E-boxes, RREs and DBP/E4BP4-binding sites present in clock and clock-controlled genes are able to generate the variety of circadian phases manifested in the clock transcriptional network, with some elements altering amplitude and others phase. In particular, the combination of canonical and non-canonical E-boxes and RREs could delay transcriptional activity, as seen in the regulation of *Cry1* where an E-box-like element gave rise to cyclic expression in phase with the *Per2* promoter and a RRE mediated expression in phase with the *Bmal1* promoter (Ueda *et al.*, 2005). *In vivo* this is reflected by the peak of *Cry1* transcript expression in the SCN and peripheral tissues occurring between the anti-phase peaks of *Per2* and *Bmal1* [see Chapter 3 and Yamamoto *et al.* (2004)] and the circadian expression of *Cry1* is therefore likely to be dependent on the combined regulation via both elements. The *Pai-1*

transcript cycles robustly in the mouse heart (Chapter 3), peaking at CT12-16, slightly later than core clock and clock-output genes such as *Per1* and *Dbp*, suggesting that *Pai-1* circadian expression may involve elements other than canonical E-boxes, such as the identified conserved RRE. Two RREs were also recently identified in the human *Pai-1* promoter (at -418 and -265 bp relative to the TSS, proximal to the two canonical E-boxes) that mediate *Pai-1* repression by REV-ERBα (Wang *et al.*, 2006).

Variations in expression phase may also be generated by the presence of non-canonical E-boxes. *Per1* is regulated via five well-conserved canonical E-boxes (Hida *et al.*, 2000), giving rise to peak expression around CT12 in mouse peripheral tissues [see Chapter 3 and Yamamoto *et al.* (2004)]. *Per2* expression on the other hand peaks slightly after *Per1* in many peripheral tissues and the mouse *Per2* promoter does not contain a canonical E-box. Instead, robust *Per2* expression is generated by a non-canonical E-box (CACGTT) and E4BP4-binding site in combination, suggesting that the combination of different E-boxes and circadian elements may provide the mechanism to produce the observed variety in circadian phase expression patterns (Yoo *et al.*, 2005; Akashi *et al.*, 2006).

The combination of elements that are regulated by anti-phasic factors, such as the E-box and DBP-binding site, can also result in robust expression rhythms through a 'feed-forward mechanism' (Ueda *et al.*, 2005; Akashi *et al.*, 2006). The 'circadian modules' in the *Pai-1* promoter examined here may therefore give rise to the observed robust rhythmic expression and delayed phase, particularly if BMAL1 and EPAS1 proteins are expressed in anti-phase, as suggested by their mRNA expression profiles (Chapter 3). Cloning these modules in isolation (as in (Ueda *et al.*, 2005), in one artificial construct and in their *in vivo* context and examining the differences in their temporal expression in synchronised cells would determine the contribution to *Pai-1* cycling by each module.

6.4.4 Context-specific regulation via the E-box element

As well as identifying a potential regulatory circadian module that may be present in other CCGs, the results presented allude to wider complexities surrounding circadian transcriptional regulation and the integration of other pathways. As discussed in Chapter 3, canonical E-box elements are largely represented throughout the genome and are bound by a range of bHLH family transcription factors that are involved in a wide array of physiological and developmental processes (Massari and Murre, 2000). In order to discriminate between bHLH factors mechanisms exist that modulate the affinity, strength of binding and subsequent activity of different binding proteins, such as tissue-specificity, phosphorylation of factors and methylation of the CpG site within the CACGTG motif (Corre and Galibert, 2005; Stormo and Zhao, 2007). From the results presented here one can speculate that the combination of different E-box elements and the competition or interaction of binding factors at these sites provides a mechanism for further differential regulation. In particular, co-operation between circadian and hypoxic factors may gate the response (and/or strength) of *Pai-1* expression in a

context-specific manner, such as in response to hypoxia, and at different times of day as the abundance of clock factors fluctuate. A similar mechanism of co-operation between circadian and hypoxic factors at a canonical E-box has been suggested for the SCN-expressed CCG *Vasopressin* (Ghorbel *et al.*, 2003). HIF-1α and CLOCK synergistically activated the rat *Vasopressin* promoter in an-E-box dependent manner. These two factors did not heterodimerise but recruited BMAL1 to the E-box, indicating a mechanism of cross-talk between circadian and hypoxic pathways. The *Vasopressin* promoter also contains two conserved non-canonical E-boxes that did not appear to be involved in the proposed cross-talk mechanism. The present study suggests that the canonical E-boxes in the *Pai-1* proximal promoter and distal module are important for circadian and hypoxia-induced regulation, while the non-canonical E-box in the proximal promoter may be involved in tissue-specific regulation.

The combined effects on circadian regulation of E-box and E-box-like elements (such as the HRE) observed here may represent a mechanism that is not unique to *Pai-1*. A recent study has suggested that a tandem repeat of E-box-like elements six or seven base pairs apart is the minimal required element for the generation of cell-autonomous transcriptional oscillations (Nakahata *et al.*, 2008b). The authors identified an element in the mouse *Pai-1* proximal promoter that matched the criteria involving the canonical E-box at -174/-179 and a mismatched E-box-like element CAAGTC six bases downstream. However, this region was not examined experimentally and the last base of the CAAGTC site is not conserved between mouse and rat so whether this motif is functional remains unknown. None-the-less, the combination of E-box and E-box-like elements, as identified in the *Pai-1* proximal promoter and distal module, may be necessary in imparting flexibility in regulation by bHLH factors.

As well as being dependent on binding site sequence the response of E-box elements varies depending on the genomic context and cellular environment. Both proximal and distal *Pai-1* upstream regulatory regions containing canonical E-boxes may be involved in the response of *Pai-1* to hypoxia and/or the clock but the response to common transcription factors varied. USF2 is a bHLH factor that has been shown to activate the rodent and human *Pai-1* proximal promoters *in vitro* by binding to the HRE and canonical E-box (Samoylenko *et al.*, 2001; Dimova and Kietzmann, 2006). In the present study USF2 over-expression was able to activate the proximal promoter, with greater activity being seen for the shorter -314 construct. In contrast, activity of the distal module was decreased by USF2. A differential response of the *Pai-1* promoter to USF2 over-expression has been observed before, with USF2 inhibiting rat *Pai-1* activity but activating human *Pai-1* through differential binding of the canonical E-boxes and HREs (Samoylenko *et al.*, 2001; Dimova and Kietzmann, 2006). This difference may result from differences in the genomic environment and cell type but a study using both rat and human *Pai-1* promoters in two different cell types found the effects of USF expression to be more dependent on the cell background than promoter context (Dimova and Kietzmann, 2006). A

different response of the mouse *Pai-1* distal module to USF2 over-expression was also seen here between the different cell types, NIH-3T3 and H9c2. The importance of USF factors in regulating *Pai-1* in these cell types is unclear since the dominant negative factor A-USF did not affect basal activity of the proximal or distal regulatory regions, however, the results demonstrate a further level of complexity generated by differences in genomic and cellular context.

Cell type-specific regulation of the mouse *Pai-1* proximal promoter may in part be mediated by the most distal E-box of the -707 construct, non-canonical E-box 1. In NIH-3T3 cells the basal activity of the longer -707 construct was the same as the shorter -314 construct whereas in H9c2 cells the longer construct had a higher activity. Therefore the region between -314 and -707, which includes E-box 1, may contain a binding site for a cardiac-specific factor. E-box 1 appears not to be involved in regulation of the *Pai-1* promoter by CLOCK and BMAL1, despite an E-box with the same sequence being identified in the mouse *Dbp* promoter that is bound by CLOCK and BMAL1 and governs cycling (Ripperger and Schibler, 2006; Kiyohara *et al.*, 2008). As well as binding circadian and hypoxic factors E-boxes can be targets for other bHLH factors, such as the cardiac-specific repressor Cardiovascular Helix-loop-helix Factor 1 (CHF1), which can inhibit EPAS1 binding (Chin *et al.*, 2000), and muscle-specific factors such as MyoD (which are expressed in H9c2 cells). Therefore, E-box 1 may be a site that can integrate circadian and tissue-specific responses, providing a 'fine-tuning' mechanism that allows adaptation of rhythmic circadian regulation to the environment.

6.4.5 A regulatory network involving clock-hypoxic factors-*Pai-1* may mediate cross-talk between diverse processes in the cardiovascular system

The results presented here and in previous studies demonstrate that *Pai-1* is regulated by hypoxia. Activation of the proximal promoter by EPAS1 over-expression was observed in both NIH-3T3 and H9c2 cells and this activity was augmented by co-transfection with BMAL1 in H9c2 cells but not NIH-3T3 cells, suggesting that the co-operation of circadian and hypoxia factors at this region is cell-type specific. HIF-1α and EPAS1 are likely to be important in heart development and function since deficiency of both genes causes severe cardiac and vascular effects. *Hif-1α*$^{-/-}$ mice die during gestation, mainly due to defective vascularisation, heart malformations and neural tube formation (Iyer *et al.*, 1998; Ryan *et al.*, 1998) and *Epas1* knock-out mice exhibit multiple-organ pathologies, including cardiac hypertrophy (Scortegagna *et al.*, 2003). EPAS1 deficiency also results in embryonic lethality caused by vascular defects (Peng *et al.*, 2000) and bradycardia (Tian *et al.*, 1998) and EPAS1 may play a role in the adaptation of cardiac myocytes during heart failure (Tanaka *et al.*, 2002). Therefore, the ability of the heart to respond timely to hypoxic stress is of likely importance.

PAI-1 is involved in diverse physiological and pathophysiological processes, many of which have been correlated with clock function and dysfunction, such as stress response, cardiovascular homeostasis, angiogenesis, diabetes, glucose metabolism, cell proliferation and cancer progression (Fu

et al., 2002; Hastings *et al.*, 2003; Rudic *et al.*, 2004; Oishi *et al.*, 2005b; Turek *et al.*, 2005; Curtis *et al.*, 2007). Many of these processes are also accompanied by hypoxia or oxidative stress (Dimova *et al.*, 2004) and hypoxia can perturb physiological circadian rhythms (Mortola, 2007). The proposed co-operative regulation of *Pai-1* by BMAL and EPAS1 may therefore mediate cross-talk between many diverse processes and provide a mechanism for both physiological dysfunction and circadian rhythm alteration following hypoxia.

6.4.6 *Bnp* – a putative target gene of the circadian clock

Bnp and *Anp* have also been identified as putative novel CCGs in previous chapters and contain canonical E-boxes conserved between mouse and rat in their promoters. Both genes are regulated by a number of mechanisms in the heart, including autocrine and paracrine processes, mechanical stress and extracellular matrix proteins and characterisation of the human and rodent *Bnp* and *Anp* promoters has revealed many basal and tissue-specific/inducible proximal and distal regulatory elements that confer responsiveness to a number of neurohumoural agonists (Houweling *et al.*, 2005; LaPointe, 2005; Ma *et al.*, 2005). *Bnp* and *Anp* are also up-regulated by hypoxia (Goetze *et al.*, 2004; Chun and Pratt, 2005) and the human *Bnp* promoter has an HRE that is activated by HIF-1α and is just nine base-pairs downstream of a non-canonical E-box that appears to contribute to HIF-1α mediated activation (Luo *et al.*, 2006). The co-operative regulatory mechanism outlined in this chapter may therefore be conserved between the putative CCGs *Pai-1* and *Bnp*.

Mouse *Bnp* promoter activity was examined in NIH-3T3 cells and preliminary results indicated regulation by CLOCK and BMAL1. High basal activity was also observed that may have masked activation by over-expressed factors in subsequent experiments. The use of a different cell line that expresses lower levels of *Bnp* or the investigation of the *Bnp* E-box in isolation may therefore yield more consistent results. BNP and ANP are involved in the cardiac hypertrophic response to stress (Rosenkranz *et al.*, 2003; Franco *et al.*, 2004) and disturbance of circadian rhythmicity in a mouse model prevented this physiological response (Martino *et al.*, 2007). Therefore, *Bnp* and *Anp* may be directly or indirectly regulated by the clock.

6.4.7 Conclusions and limitations

The results presented in this chapter demonstrate that both circadian and hypoxic factors can regulate the mouse *Pai-1* promoter via E-box and HRE elements *in vitro* and suggest previously undemonstrated competition or co-operation between the two mechanisms. However, the magnitude of activation observed in these *in vitro* transfections was often low and different doses of factors should have been used to confirm the response of the *Pai-1* promoter to each. Optimising the ratio of reporter to factor and examining different reporter constructs and mutants more systematically would also strengthen the findings of this chapter, as would further experiments with different techniques, such as electromobility shift assay (as discussed in section 7.2). A novel distal E-box-containing region was also identified that can be regulated by EPAS1 and BMAL and *may* mediate rhythmic expression (this also requires further investigation). This region may add to the circadian *Pai-1* regulation via the proximal promoter and provide additional sites for cross-talk between the circadian clock and hypoxic pathways, representing a conserved mechanism with humans. The E-boxes in the *Pai-1* promoter may also be targets for tissue-specific regulation. PAI-1 plays a key role in many physiological and pathophysiological processes, as do many other E-box containing genes. Therefore the integration of a variety of signals with circadian time-keeping via proximal and distal E-boxes in an array of genes may enable modulation of these signals and the target processes to the temporal environment.

7. General discussion

The discovery that circadian rhythms are generated by an intracellular molecular mechanism and that it exists in all cells and tissues allows the phenomenon of diurnal variations in cardiovascular physiology and pathologies to be investigated in a new way. In particular, the discovery of targets of the molecular clock in the heart will shed new light on the temporal functioning of this complex organ and the processes behind cardiac pathological events.

The circadian clock likely regulates expression of over 10% of the cardiac transcriptome by direct and indirect mechanisms but the targets of the cardiac clock are largely unknown. A four-stepped approach was used in this study to identify target genes of the cardiac clock and give direction to further study in this growing field. Findings were limited by the low number of samples used and lack of cross-validation using other techniques. However, the following findings emerged:

1. The expression of three cardiac genes, *Pai-1*, *Bnp* and *Ms1*, varies over the course of a day.
2. *Pai-1*, *Anp* and possibly *Bnp* are targets of the intracellular clock due to up-regulation by CLOCK and BMAL1 proteins in cells, and are responsive to glucocorticoid signalling *in vitro*.
3. While the heart clock is an autonomous oscillator, temporal gene expression in SCN-ablated mice demonstrated the dependence of this clock on synchronisation by the SCN *in vivo*.
4. Glucocorticoids such as DEX are synchronisers of gene expression *in vitro* and differentially affect gene expression *in vivo*. They are likely to be important synchronisers of the heart clock that may integrate response to various stimuli with circadian regulation, enabling adaptation to the environment.
5. The *Pai-1* promoter is regulated by circadian and hypoxic factors at proximal and distal E-box elements: the identified distal E-box may be sufficient to generate circadian activity of this novel region *in vitro* via glucocorticoid synchronisation.

7.1 Evidence supporting the importance of the cardiac clock

The present findings combined with previous observations of cardiac gene cycling (see Chapter 3) suggest that a varied array of cardiac genes are under the influence of the clock and that circadian regulation impacts many physiological and pathological processes in the rodent heart. Recent observations suggest that 10% or more of peripheral tissue transcriptomes may be rhythmically expressed. It has even been postulated that at least 50% of genes may be influenced by the circadian clock at the transcript level but due to biological variability between samples and the limitations of microarrays it may not have been possible to detect all cycling genes (Ptitsyn *et al.*, 2006). Therefore,

multiple approaches examining the temporal regulation of candidate genes (such as used in this study) are necessary to fully decipher the circadian transcriptome.

The observed diurnal variations in the onset of cardiovascular events, such as MI and stroke, have often been explained by alterations in external factors, such as sympathetic tone and levels of haematogenous factors (Muller *et al.*, 1989). The discovery of strong circadian rhythms in clock and cardiac gene expression within the whole heart and cardiomyocytes, demonstrated here and by others (Young *et al.*, 2001b; Young *et al.*, 2001c; Storch *et al.*, 2002; Durgan *et al.*, 2005), together with observed diurnal variations in cardiac function (Young *et al.*, 2001b), suggests that the responsiveness of the heart may also contribute. The circadian response of a system depends on the level of stimulus and the level of responsiveness, i.e. the ability to respond. Therefore, if either of these alters the ability of the heart to anticipate is compromised. The effect of changes in the level of stimuli, such as workload, light and nutrient intake, can be seen by the increased incidence of cardiovascular diseases among shift workers (see Chapter 1) and the pathological symptoms of many cardiovascular disorders, such as MI, are also caused by acute and unexpected stress, such as ischemia. The ability of the system to respond is dependent on the molecular composition of cardiac cells, therefore the cycling of clock genes and cardiac transcripts may directly influence the response of the heart to stress and its ability to adapt at different times of the day.

The variation in expression profile and thus responsiveness of the heart is underscored by the observation that in the mouse heart most rhythmic transcripts peak around CT4 (Storch *et al.*, 2002), which differs to the broader distribution seen in the liver. Therefore, the heart may be designed to adapt best to particular stimuli or conditions at a particular time-point. However, as shown in Chapters 3 and 4 common expression phases were observed for some genes but others exhibited different patterns. These differences reflect the delicate balance of factors needed to maintain an anticipating heart and the combination of and quantity of different circadian regulatory elements (Chapter 6) may provide this level of flexibility and allow adaptation to varied signals and pathways. Storch and colleagues (2002) also suggested a greater prevalence of low amplitude gene regulation in the heart compared to the liver, implying that cycling phase and fold change may be more important than amplitude in cardiac gene regulation.

The heart and liver clocks appear to differ in a number of ways and interestingly rhythmic expression of the clock genes *Clock* and *E4bp4* was not lost in hearts from SCN-ablated mice, in contrast to observation in the liver (Reddy *et al.*, 2007). The apparent ability of the heart clock to retain circadian expression of these genes in the absence of SCN-mediated synchronisation suggests that robust sustained circadian rhythms may be important in the heart. It also implies that the cardiac clock is regulated by a combination of external and intracellular/intra-tissue factors.

The suggestion that genes involved in multiple diverse processes are rhythmically expressed poses the question of what happens to cardiac physiology when the clock is disrupted? The documented circadian variations in cardiac function and occurrence of pathological cardiac events suggest that the heart is adapted to its temporal environment via its intrinsic clock. Pathological events may then derive from either; 1) the occurrence of stress at a time when the heart is less able to cope with it; or 2) disruption of clock gene expression in the heart, or desynchrony of the heart clock and SCN and/or other peripheral clocks. Both of these conditions provide a genetic explanation for the diurnal variation in CVD and the latter is also likely to exacerbate pathological conditions, suggesting that the clock may be involved in different stages of CVD progression.

The genes identified as CCGs in the current study implicate the processes of hypertrophy and remodelling as being important targets of the heart clock. This is suggested by the novel circadian regulation of the natriuretic peptide genes *Bnp* and *Anp*. The hypothesis that the significant temporal variation in *Bnp* transcript abundance observed in Chapter 3 is due to regulation by the circadian clock is supported by the observations in Chapters 4 and 5 that SCN-ablation abolished rhythmic *Bnp* expression (which was re-established by DEX injection) and over-expression of CLOCK and BMAL1 up-regulated *Bnp* expression in H9c2 cells. *Anp* was also strongly up-regulated by CLOCK and BMAL1 over-expression (Chapter 5). The importance of circadian regulation of these genes is supported by the recent observation that expression of *Bnp* and *Anp* and the normal hypertrophic response to aortic banding is attenuated in mice entrained to a non-physiological light-dark cycle (10:10 LD) (Martino *et al.*, 2007). The expression of clock genes was also altered in those mice, implying that a functioning clock is required for compensatory remodelling in the heart and disruption of the clock may exacerbate this disease state.

Cardiac hypertrophy is characterised by the growth of cardiac myocytes in response to a variety of neurohumoral and mechanical stimuli involving numerous pathways (Hunter and Chien, 1999; Olson and Schneider, 2003). It is primarily an adaptive response to increased demand placed on the heart following physiological stimuli such as exercise, or pathological stimuli such as chronic arterial hypertension or MI. The hypertrophic response to pathological stimuli can also become maladaptive, progressing to heart failure when stimuli are persistent. Therefore, the temporal-spatial regulation of genes involved in hypertrophy and remodelling is likely to be important in governing the daily response of the heart to variations in physiological and pathological stimuli and the ability of the heart to repair post-MI. The attenuation of circadian expression rhythms of cardiac CCGs by hypertrophy in the rat heart (Young *et al.*, 2001b) also suggests the importance of circadian regulation in the adaptation of the heart to stress and it may be the loss of this plasticity that contributes to the progression of hypertrophy to heart failure.

The temporal expression patterns of *Bnp* and *Ms1* discussed in Chapter 3 appeared to exhibit two peaks, although *post-hoc* analysis did not reveal these to be significant. The presence of two peaks would imply that temporal expression may be regulated by a combination of factors, including the clock. For example, the clock may be regulating expression at one time, e.g. during the circadian day (CT4) and other factors may be acting during the circadian night (CT20), overriding clock regulation, when rodents are active and there are greater demands on the heart. Alternatively, regulation via the multiple circadian regulatory elements found in the promoters of these genes (see Chapter 3) may result in such a pattern. Similar ultradian expression patterns have recently been observed in mouse aortic tissue for structural and remodelling genes including *tissue inhibitor of metalloproteinase 1* and *3* (*timp1*, *timp3*), *transgelin 1* (*sm22α*) and *calponin 1* (*cnn1*), so these rhythms may have important physiological functions (Chalmers *et al.*, 2008). However, only one peak in expression was observed in a synchronised vascular smooth muscle cell line used in the same study so it is likely that one of the peaks is driven by activity or neurohumoural signals as proposed above. If a similar mechanism exists in humans, it is tempting to speculate that such a mode of circadian regulation could have evolved to enable the heart to cope with both physiological demands during the day and increased incidence of MI at the end of the night, both of which could invoke hypertrophy and remodelling. The hypothesis that the peak at CT4 is a result of regulation by the clock is strengthened by findings from microarray studies that most of the rhythmically expressed transcripts in the heart peak around this time-point (Storch *et al.*, 2002). In the study by Storch and colleagues however, the *Bnp* transcript was found to peak at CT22 (around the time of *Bmal1*), which may reflect the second peak observed at CT20 in this study. Whether one or both of these peaks are driven by activity could be tested by studying expression in *Clock* mutant mice.

Adaptation of the heart to diurnal variations in workload may also be mediated by the circadian regulation of metabolic genes, such as *Ucp2/3*, *Pparα/γ* and *Glut4*. In the liver the majority of CCGs are believed to be involved in diverse essential metabolic pathways and processes (Akhtar *et al.*, 2002; Panda *et al.*, 2002), indicating the importance of circadian metabolic rhythms in this tissues. Circadian expression of *Ucp2/3*, *Pparα/γ* and *Glut4* was not observed in the current study but has been observed in the heart by others, as discussed in Chapter 3. The ability to adapt to different metabolic states is crucial for efficient cardiac function and protection, especially as variations in workload, oxygen demand and coronary blood flow result in the heart facing varying degrees of ischemia during the day, particularly in the morning (Quyyumi, 1992), that could result in cell death or MI. It is therefore logical that the heart has evolved a mechanism to adapt to diurnal variations in energy demand and substrate ability and the responsiveness of the heart to different energy sources exhibits clock-dependent diurnal variations. Factors such as PPARα, UCP2 and 3 and GLUT4 have also been observed to be dysregulated in the failing heart and under disease states such as diabetes, as fatty acid levels are increased (Murray *et al.*, 2004). Therefore, the circadian regulation of these factors is likely

to be important in enabling the heart to adapt to differences in fuel availability and pathological conditions.

The diurnal variations of plasma PAI-1 levels have been proposed to contribute to the increased morning risk of MI (see Chapter 1). The results presented here confirm that *Pai-1* is also rhythmically expressed in the heart (Chapter 3) and is a direct target of CLOCK and BMAL1/2 (Chapter 6). Plasma PAI-1 levels peak before dawn in humans and it is the inhibition of intravascular fibrinolysis that likely contributes to increased formation of blood clots and subsequent vascular occlusion and incidence of MI in the early morning. The role of PAI-1 in cardiac tissue is clearly different and the observation of strong circadian expression and direct regulation by the molecular clock in cell lines suggests that circadian expression of *Pai-1* in the heart is equally important. In cardiomyocytes and other tissues PAI-1 appears to predispose to fibrosis by inhibiting extracellular fibrin degradation, leading to collagen deposition (Pinsky *et al.*, 1998; Takeshita *et al.*, 2004). Cardiac fibrosis is involved in ventricular remodelling post-MI and is believed to be one of the factors that causes heart failure (Frohlich, 2001). Peak levels of cardiac PAI-1 in the morning may then be detrimental to the response of the heart following MI, just as peak plasma levels may contribute to increased incidence of MI. This raises the question of how such robust cycling of *Pai-1* could have evolved given these negative consequences and hints towards other unknown roles of PAI-1 that are advantageous in the early morning hours.

Evidence presented here indicates that *Pai-1* may be an integrator of various signalling pathways and circadian regulation, as summarised in Figure 7.1. In particular, the results presented in Chapter 6 demonstrate that circadian and hypoxic regulatory pathways may converge at two E-box-containing regions (one proximal and one distal) in the *Pai-1* promoter. The heart faces varying degrees of hypoxia over the course of a day and if the heart is unable to cope with severe hypoxia, ischemia, MI and heart failure can develop (Buja, 2005). Hypoxia is also an important regulator of vascular homeostasis and promotes thrombus formation and stabilisation via activation of PAI-1 (Kietzmann *et al.*, 1999). Up-regulation of PAI-1 by hypoxia is also believed to contribute to the development of atherosclerosis and enhanced *Pai-1* expression has been found in human atherosclerotic lesions (Schneiderman *et al.*, 1992; Lupu *et al.*, 1993; Robbie *et al.*, 1996). The ability of the clock to influence hypoxia-induced expression of *Pai-1* may therefore be important in protecting the heart from the effects of hypoxia at certain times of day, as well as influencing the role of PAI-1 in many other physiological and pathological processes (as discussed in Chapter 6). As hypoxia can regulate a number of processes and events in the cardiovascular system it will be of interest to see if other genes are regulated by a similar co-operative mechanism. It may be that E-box elements act as 'rheostat switches' to gate the response and/or strength of gene expression (and downstream processes) to stress such as hypoxia in a temporal and context-specific manner. The rodent *Pai-1* proximal canonical

E-box is also a target of the TGF-β signalling cascade (via USF binding and activation) (Kutz *et al.*, 2006) and mutation of the second canonical E-box in the human promoter inhibits TGF-β-dependent *Pai-1* activation (Hua *et al.*, 1998). TGF-β signalling can regulate many processes related to cell growth, migration and inflammation in response to a variety of signals and a similar mechanism of co-operative activation may allow integration of the clock with a variety of other pathways relevant that contribute to CVD, via *Pai-1*.

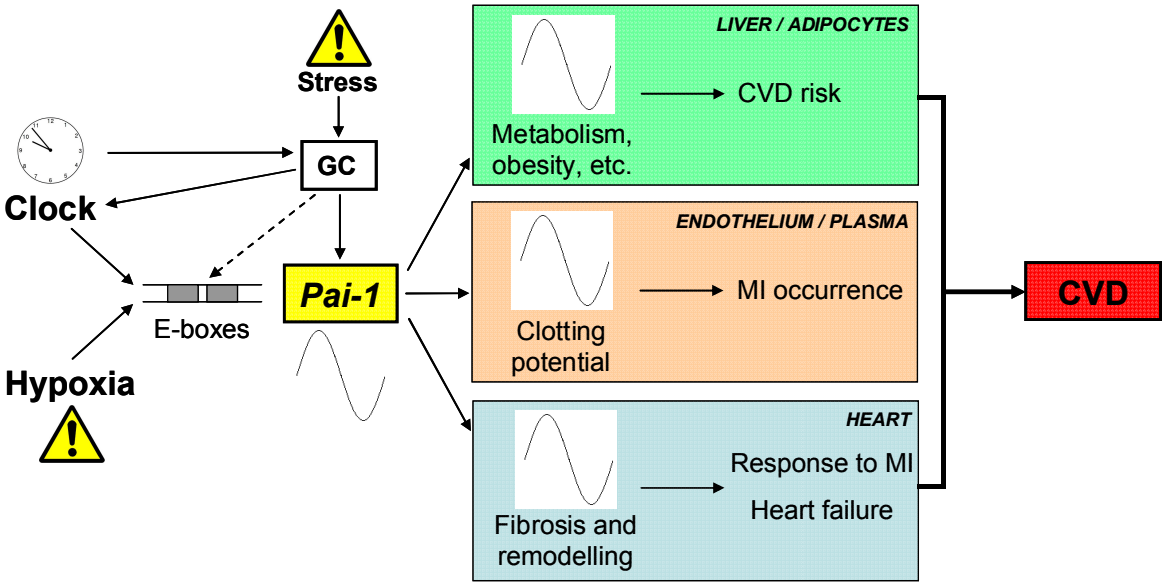


Figure 7.1. Proposed model of *Pai-1* as an integrator of responses in the CVS contributing to heart failure. Regulation of *Pai-1* expression is dependent on clock and hypoxic factors that may co-operate at E-box elements in the *Pai-1* promoter. Regulation by glucocorticoids (GC) may also mediate the integration of stress signals via clock/E-box-dependent and independent mechanisms. Rhythmic *Pai-1* expression can generate physiological oscillations in a tissue-specific manner, thus affecting the aetiology of CVD by modulating CVD risk factors (such as obesity), the occurrence of myocardial infarction (MI), and the response of the heart to MI. See text for further details.

Further evidence that transcriptional regulation of *Pai-1* may be an important integrator of processes is provided by the observed acute up-regulation of *Pai-1* expression in response to DEX (Chapter 5) and possible circadian expression of the *Pai-1* distal module following DEX treatment (Chapter 6). As mentioned in Chapter 1, glucocorticoids are rhythmically secreted, can regulate CVD risk factors and are believed to be involved in remodelling post-MI and atherosclerosis. Many of these processes overlap with those that PAI-1 has been implicated in (see Chapter 6) so the circadian regulation of both glucocorticoid secretion and *Pai-1* expression, and the regulation of *Pai-1* by glucocorticoids may combine to augment or fine-tune temporal control of cardiovascular physiology.

The loss of clock gene cycling in the heart of SCN-ablated mice demonstrates the importance of signals from the SCN in synchronising the cardiac clock (Chapter 4). The variety of responses of gene expression to DEX treatment suggests that glucocorticoids may play a role in signalling from the SCN clock but other factors are also required. The ability of glucocorticoids to regulate gene expression directly via GREs and indirectly via clock synchronisation may instead be important in integrating circadian synchrony with signals from peripheral tissues. The existence of independent peripheral clocks may allow flexibility to respond to tissue specific stimuli but as CVD and cardiovascular events are multi-component disorders contributed to by different tissues of the CVS, synchrony between these tissues may be important in the adaptation of the heart and CVS to the environment and prevention of CVD. Due to their wide range of targets, glucocorticoids are used therapeutically to treat a number of cardiovascular disorders, including MI outcome (Walker, 2007). As a result of their role in mediating internal synchrony they may also help to combat conditions that lead to loss of synchrony between peripheral oscillators and the SCN, such as shift work, and may therefore be important in preventing CVD as well as in treatment. It may also be necessary to consider the time of administering glucocorticoid treatments.

Over recent years the field of chronotherapeutics (delivering therapeutic treatments at optimal times in relation to circadian rhythms) has begun to develop and has already proved to be promising in increasing the efficacy of some drugs. For example, the rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase, is rhythmically expressed with peak levels during the night. HMG-CoA reductase is inhibited by the widely prescribed cholesterol-lowering drugs statins and the finding that statins are most effective when taken before bedtime has influenced the advice given to patients (Staels, 2006). The identification of cycling genes and the processes they are involved in may present new targets for chronotherapy and some of the genes examined in the current study are already therapeutic targets, such as *Pparα/γ* (Reilly and Lee, 2008). Recombinant human BNP (nesiritide) has also been shown to be an effective treatment of decompensated heart failure (Colucci *et al.*, 2000; Vasodilatation in the Management of Acute CHF study, 2002) and therefore may have potential chronotherpeutic uses that are beneficial in the treatment or prevention of MI. Understanding how the heart responds temporally to cardiovascular events through the circadian regulation of genes involved in processes such as hypertrophy and hypoxia may also enable the identification of optimal times for therapeutic cardiovascular interventions, when the heart is most able to cope with stress.

7.2 Further experiments

As discussed in previous chapters, further experiments and an increase in sample number are required to confirm and cross-validate the results presented in the thesis. The technique of transcript analysis used here enables the examination of expression of a wide range of genes and the direct output of the molecular clock and there are many cases where transcript cycling translates to protein expression.

However, the study of protein expression should also be considered as in the liver almost half of the cycling proteins identified are not cycling at the transcript level (Reddy *et al.*, 2006). Post-transcriptional and post-translational modifications also affect the activity and nuclear localisation of clock factors (see Chapter 1) so the analysis of phosphorylation state, etc. may provide further insight into the functioning of the cardiac clock.

Increasing the number of time-points to extend transcript analysis over two or three consecutive days would give a better picture of circadian expression rhythms and may exclude the possibility of isolated stress affecting the animals and gene expression. In Chapter 4 the use of three time-points with two of them 24 hours apart allowed the repetition of circadian rhythms to be seen and in general good comparison with the cycling seen with six time-points in Chapter 3 was observed. The use of fewer time-points has the obvious advantage of reduced animal numbers and cost, especially given the difficulty of SCN-ablation. However, only two points of the 24-hour circadian cycle were represented, which enabled the rhythmic expression of many clock and clock-related genes to be seen (as the majority peak around these times) but may not have been sufficient to observe the circadian expression of cardiac genes that peak at different time and cycle with weaker amplitudes.

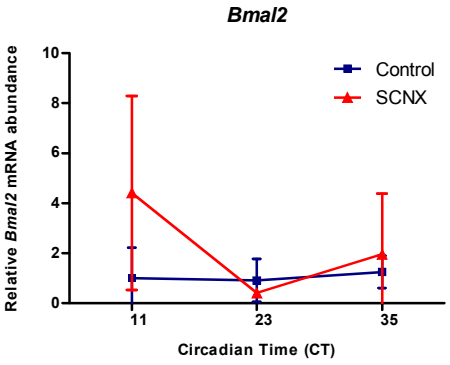
The demonstration that an E-box-containing distal module may have the potential to confer rhythmicity to the mouse *Pai-1* promoter *in vitro* is an interesting novel finding that requires further investigation and confirmation. Also, the effect of this region in the context of the whole promoter *in vivo*, which will be under the influence of a host of stimuli, remains unknown. Cloning of this long region was attempted but proved difficult and the creation of a "knock-in" bioluminescent reporter animal (like the *Per1 promoter:luciferase* rat) may demonstrate the importance of this region *in vivo*. The mutation of putative transcription factor binding sites in the *Pai-1* promoter combined with over-expression of transcription factors suggests a direct interaction between these sites and clock and hypoxic factors but can not be confirmed with this technique alone. The use of EMSA and *in vivo* techniques such as Chromatin Immunoprecipitation (ChIP) would allow the confirmation of known transcription factors binding to specific elements *in vivo*. Such techniques could also be used to give a profile of binding to a site over time if performed on samples from synchronised cells. Since the results presented here may have identified two circadian modules, whether these modules are present in the promoters of other identified (or unknown) CCGs should be investigated. Examining regions containing E-boxes and HREs cloned into reporter vectors over time in cells that are subject to simulated stress such as ischemia/reperfusion will also allude to the physiological importance of this proposed regulatory mechanism. The importance of EPAS1 in the regulation of *Pai-1* and other genes may also be confirmed by investigating transcript levels in *Epas1* knock-out mice.

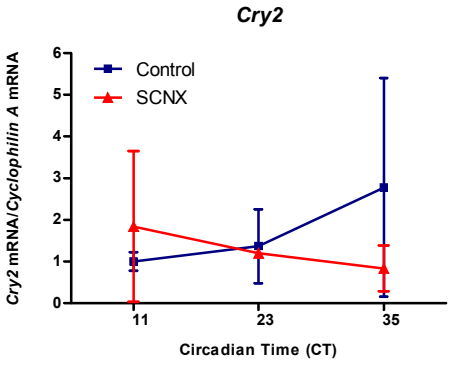
7.3 Concluding remarks and perspectives

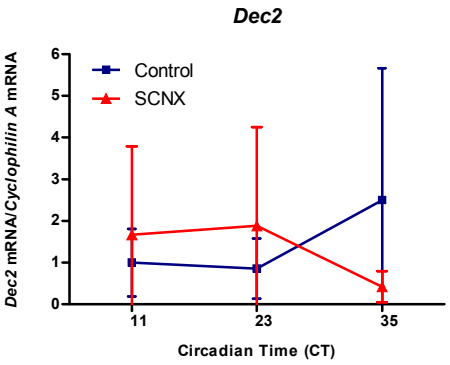
The primary aim of this study at the outset was to identify target genes of the circadian transcriptional-translational feedback loop in the heart to better understand the role of this recently identified peripheral clock and how it might contribute to the diurnal variations in cardiac function and pathological events. Due to the limited number of samples used and lack of cross-validation (as discussed in previous chapters) the findings of this study are limited. However they do contribute to existing findings that suggest the intrinsic cardiac clock may be involved in regulating many important processes in the heart through governing rhythmic gene expression, and thus enabling the heart to adapt to its temporal environment. Disturbances in cardiac circadian regulation may therefore contribute to cardiac pathologies. Circadian regulation of cardiac genes may also enable the integration of internal signals, such as glucocorticoid secretion and hypoxia, with adaptation to the temporal environment and *Pai-1* may play a key role in this mechanism.

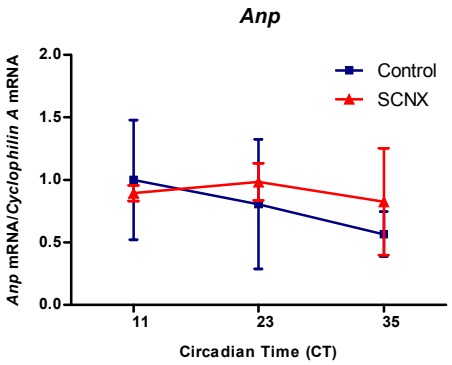
Since the start of this study the field investigating the role of peripheral clocks, including the heart, has rapidly expanded, highlighting the importance of a better understanding of peripheral clock function and clock targets. As this field continues to grow the development of chronotherapeutics is likely to become increasingly important and the results presented in this study may allude to pathways and genes that can be targeted in the temporal treatment and prevention of CVD through drug development or modification of behaviour. The consideration of circadian timing in experimentation is also likely to increase given the growing discovery of genes and processes that are influenced by the clock. The ubiquity and conservation of the circadian clock demonstrates that it is a crucial sensing mechanism in daily physiology. The growing network of interactions between the clock and other pathways presents many challenges in defining the precise role of the clock in physiology and pathology and the identification and further characterisation of clock-controlled genes and processes will highlight important mechanisms that can be therapeutically targeted.

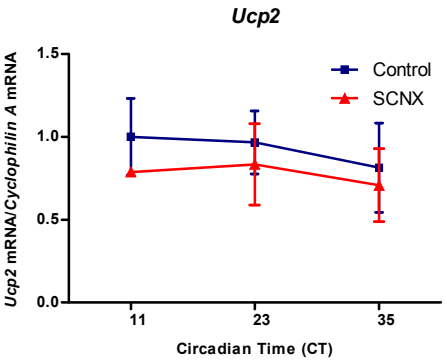
Appendix 1

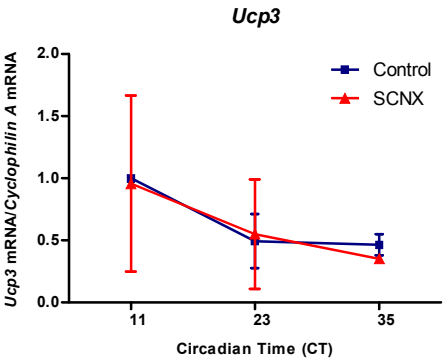

Bmal2
Relative Bmal2 mRNA abundance
Control
SCNX
Circadian Time (CT)


Cry2
Cry2 mRNA/Cyclophilin A mRNA
Control
SCNX
Circadian Time (CT)


Dec2
Dec2 mRNA/Cyclophilin A mRNA
Control
SCNX
Circadian Time (CT)


Anp
Anp mRNA/Cyclophilin A mRNA
Control
SCNX
Circadian Time (CT)


Ucp2
Ucp2 mRNA/Cyclophilin A mRNA
Control
SCNX
Circadian Time (CT)


Ucp3
Ucp3 mRNA/Cyclophilin A mRNA
Control
SCNX
Circadian Time (CT)

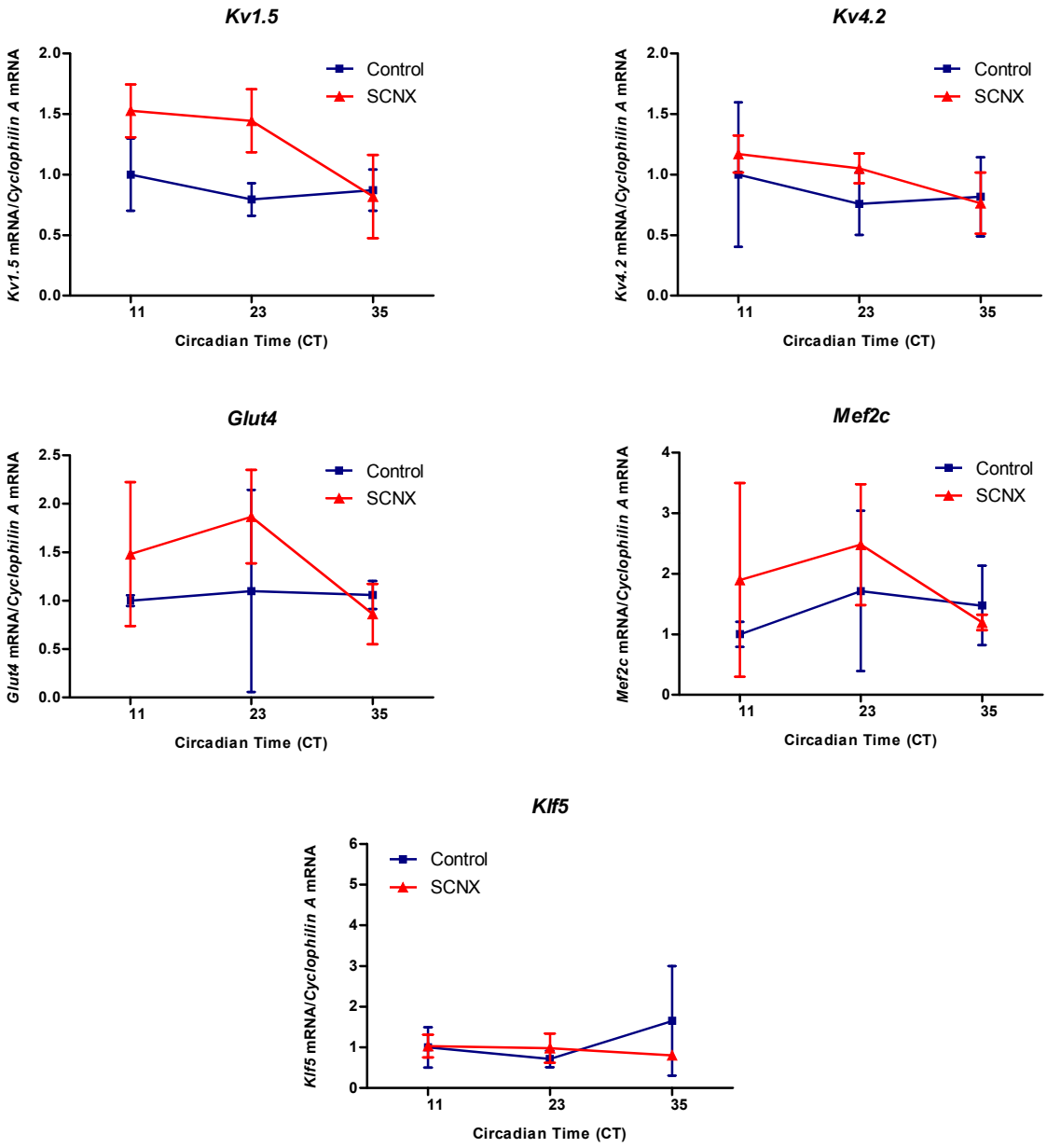
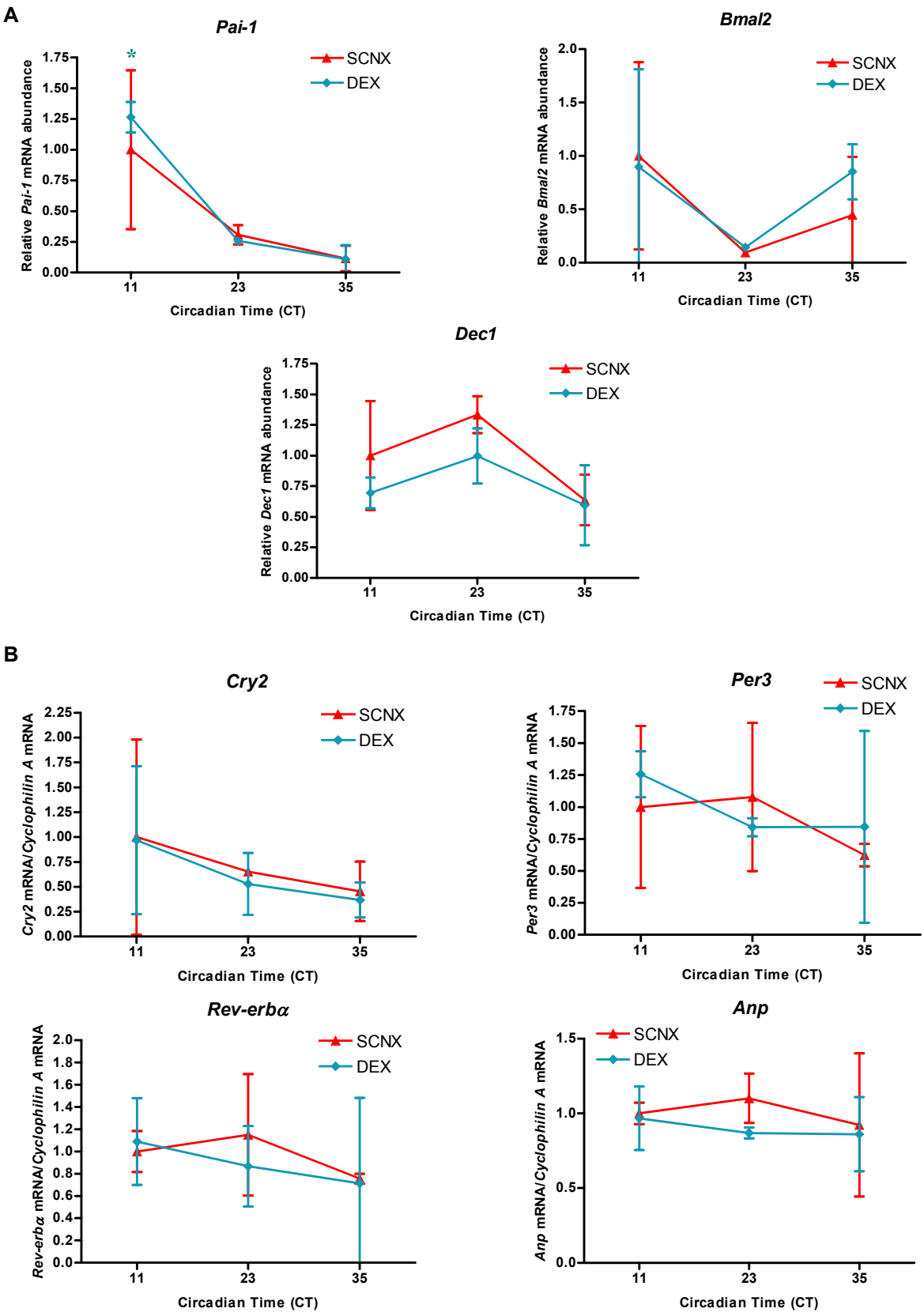


Figure 1. Temporal expression of genes in the hearts of SCN-ablated mice. Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene mouse *Cyclophilin A*. Values are the mean ±SD for two hearts per point (two-three observations) and are standardised to Control CT11 for each gene. Control = sham-operated mice, SCNX = SCN-ablated mice. Statistical analysis was performed by ANOVA, see Tables 4.1 and 4.2, Chapter 4.


A
Pai-1
SCNX
DEX
*
Relative Pai-1 mRNA abundance
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
11
23
35
Circadian Time (CT)
Bmal2
SCNX
DEX
Relative Bmal2 mRNA abundance
2.0
1.5
1.0
0.5
0.0
11
23
35
Circadian Time (CT)
Dec1
SCNX
DEX
Relative Dec1 mRNA abundance
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
11
23
35
Circadian Time (CT)
B
Cry2
SCNX
DEX
Cry2 mRNA/Cyclophilin A mRNA
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
11
23
35
Circadian Time (CT)
Per3
SCNX
DEX
Per3 mRNA/Cyclophilin A mRNA
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
11
23
35
Circadian Time (CT)
Rev-erbα
SCNX
DEX
Rev-erbα mRNA/Cyclophilin A mRNA
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
11
23
35
Circadian Time (CT)
Anp
SCNX
DEX
Anp mRNA/Cyclophilin A mRNA
1.5
1.0
0.5
0.0
11
23
35
Circadian Time (CT)

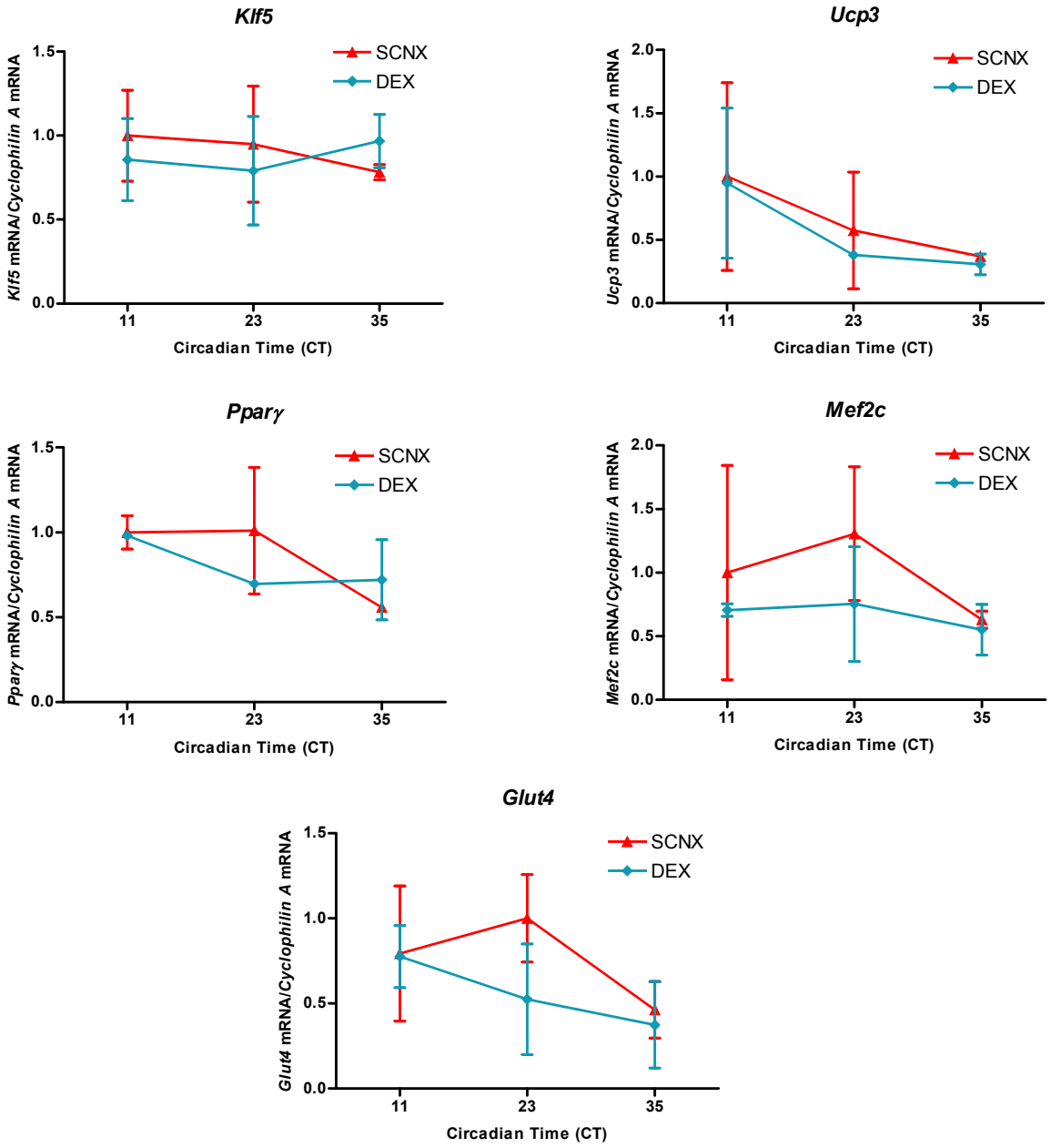


Figure 2. Effect of Dexamethasone injection on the temporal expression of genes in the hearts of SCN-ablated mice. Quantitative (A) and Semi-quantitative (B) RT-PCR expression values were normalised against the corresponding expression values of the housekeeping genes mouse TATA box binding protein (*mTbp*) and mouse *Cyclophilin A* respectively. Values are the mean ±SD for two hearts per point (two-three observations) and are standardised to SCNX CT11 for each gene. SCNX = SCN-ablated mice injected with a PBS control, DEX = Dexamethasone-injected SCN-ablated mice. Statistical analysis was performed by ANOVA, see Table 4.1, Chapter 4. * Indicates significant difference between adjacent time-points following *post-hoc* analysis with Newman-Keuls multiple comparison test, $P<0.05$.

Appendix 2

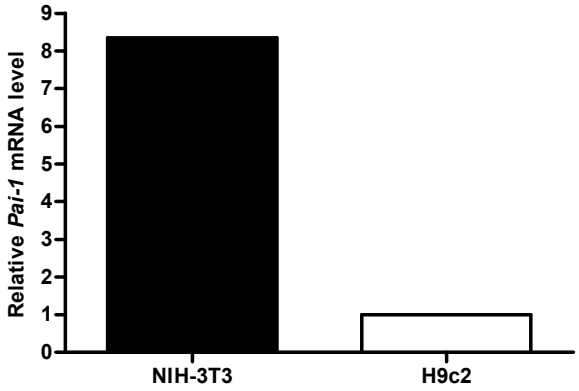


Figure 1. Relative *Pai-1* mRNA expression in NIH-3T3 and H9c2 cells. Semi-quantitative RT-PCR expression values were normalised against the corresponding expression values of the housekeeping gene *Cyclophilin A* and standardised to the expression level in H9c2 cells.

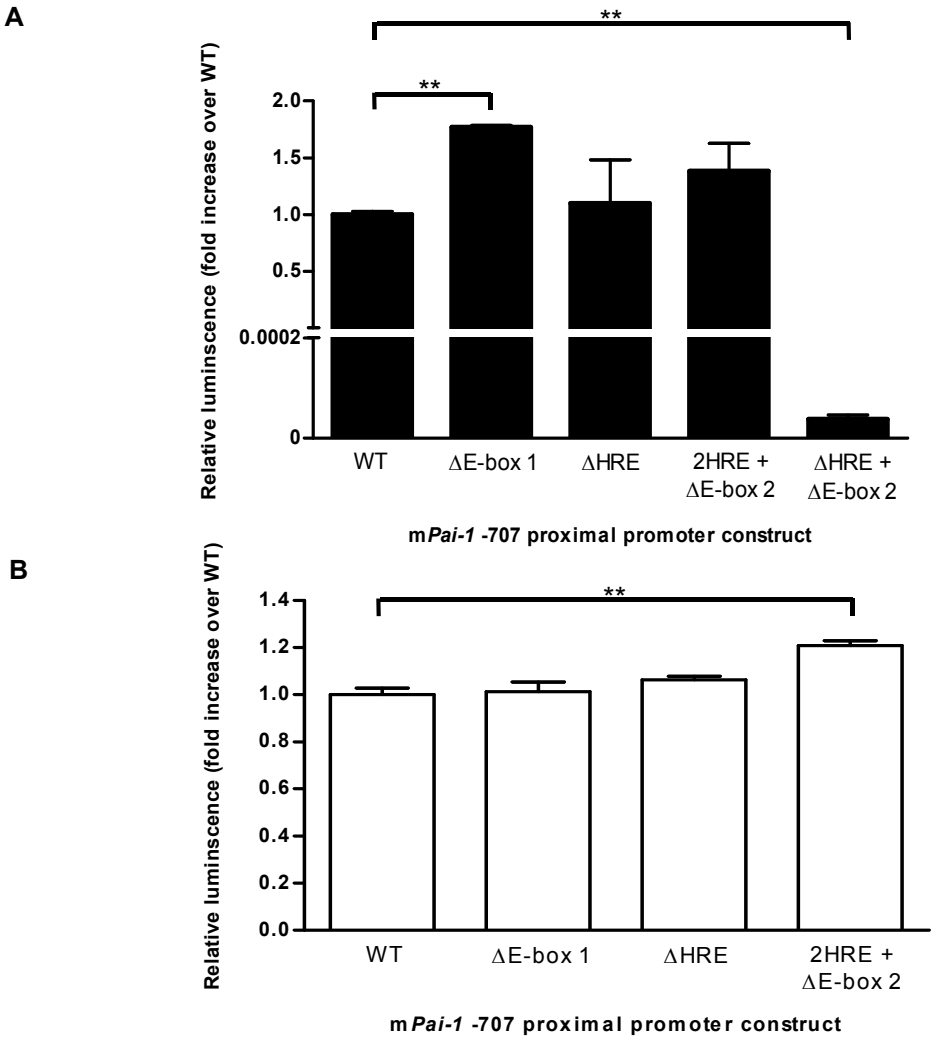


Figure 2. Mouse *Pai-1* proximal promoter basal activity. Basal activity of -707 mouse *Pai-1* proximal promoter and mutants in NIH-3T3 (B; black bars) and H9c2 (C; white bars) cells. Cells were transfected with 500ng of a pGL3-Basic reporter plasmid containing the wild-type, E-box 1-mutated (ΔE-box 1), HRE-mutated (ΔHRE), E-box 2-mutated (ΔE-box 2 + 2HRE) or HRE and E-box 2-mutated (ΔHRE + ΔE-box 2) -707 m*Pai-1* proximal promoter. ΔE-box 2 also contained an extra tandem repeat of the HRE site, later discovered to be a PCR-induced error. Values were standardised to WT activity and represent the mean ± SEM of three observations from one experiment, except for NIH-3T3 ΔHRE and ΔE-box 2 + 2HRE which represents the mean ± standard deviation of two independent experiments. (A) ANOVA $P<0.0001$, $F_{4,8}=47.68$, (B) ANOVA $P=0.0023$, $F_{3,8}=12.27$, ** $P<0.01$.

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